

UNIVERSITY OF KWAZULU-NATAL

**THE EFFECTS OF OXIDOVANADIUM COMPLEXES ON GLUCOSE
METABOLISM IN LIVER AND SKELETAL MUSCLE CELL LINES**

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2014

**THE EFFECTS OF OXIDOVANADIUM COMPLEXES ON GLUCOSE
METABOLISM IN LIVER AND SKELETAL MUSCLE CELL LINES**

BY

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Submitted in partial fulfilment of the requirements for the Master's degree of Medical Sciences in Human Physiology in the Discipline of Human Physiology, School of Laboratory Medicine and Medical Sciences, College of Health Sciences

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I DEDICATE ALL THIS WORK TO MY FAMILY

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Student Mr. N.H. Sibiya

Signature  Date 10/03/15

Supervisor Professor C.T. Musabayane

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LIST OF ABBREVIATIONS

α	Alpha
ADP	Adenine disphosphate
AGEs	Advanced glycosylation end products
AMPK	Adenine monophosphate protein kinase
ANOVA	One way analysis of variance
ATP	Adenosine triphosphate
AR	Aldose reductase
β	Beta
BCA	Binchoninic acid assay
Ca^{2+}	Calcium
cAMP	Cyclic monophosphate
Con	Control
DAG	Diacylglycerol
DM	Diabetes mellitus
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulphoxide
DN	Diabetic nephropathy
DNE	Diabetic neuropathy
DR	Diabetic retinopathy
ECM	Extracellular matrix
EMEM	Eagle's Minimal Essential Medium
ESR	Electron spin resonance
FCS	Foetal calf serum
Fpase	Fructose 1,6 bisphosphatase

G6pase	Glucose-6-phosphatase
GLUT	Glucose transporter
GFAT	Glutamine: fructose-6-phosphate amidotransferase
GS	Glycogen synthase
H	Hour
H ₂ O ₂	Hydrogen peroxide
HIF	Hypoxia-inducible factor
HLA	Human leukocyte antigen
Hpybz	2-pyridibenzimidazole
HRP	Horse radish peroxidase
IR	Insulin receptor
IRS-1	Insulin receptor substrate 1
IRS-2	Insulin receptor substrate 2
K ⁺	Potassium
KCl	Potassium chloride
KH ₂ PO ₄	Potassium dihydrogen phosphate
KOH	Potassium hydroxide
L	Litre
LDLs	Low density lipoproteins
M	Molar
MAPK	Mitogen activated protein kinase
Met	Metformin
MeOH	Methanol
mg	Milligrams
Min	Minutes
mL	Millilitre

mM	Millimolar
mmol	Millimol
μ	Micro
NaCl	Sodium chloride
NADPH	Nicotinamide adenine phosphate
Na ₂ EDTA	Disodium ethylenediaminetetraacetic acid
Na ₂ SO ₄	Sodium sulphate
nm	Nanometre
PBS	Phosphate buffered saline
PEP	Phosphoenolpyruvate
PEPCK	Phosphoenolpyruvate carboxykinase
PAI-1	Plasminogen activator inhibitor-1
PKC	Protein kinase C
PPR γ	Peroxisome proliferator activated receptor gamma
PVDF	Polyvinylidene difluoride
RIN	Rat insulinoma
ROS	Reactive oxygen species
RPMI	Rosal Park Memorial Institute
SA	South Africa
SDS	Sodium dodecyl sulphate
SGLT	Sodium dependent glucose transporter
TGF	Transforming growth factor
TTBS	Tris-tween buffered saline
TZDs	Thiozolidinediones
USA	United States of America
V	Oxidovanadium complex

v:v	Volume:volume
VEGF	Vascular endothelial growth factor
VO ₂	Dioxovanadium
VO (acac) ₂	Acetyl acetone oxovanadium
VOSO ₄	Sulphato oxovanadium

ABSTRACT

Introduction

Diabetes mellitus is a worldwide major concern associated with hyperglycaemia due to lack of insulin effects. Management of hyperglycaemia and diabetes associated complications relies on various strategies such as insulin injections and a host of oral anti-hyperglycaemic drugs. However, these therapies have limited efficacy, tolerability and significant side effects. Moreover, when glycaemic control is not achieved by a monotherapy, combination therapy is administered. Taken together, these challenges exert a major burden on health systems and facilities. Therefore, novel therapies with less undesirable effects and enhanced efficacy are under a constant evaluation. Recently, the anti-hyperglycaemic activity of vanadium complexes (IV) and (V) has received infinite attention for diabetes management. Accordingly, this study aimed to investigate the effects of novel oxidovanadium complexes *cis*-[VO₂(Hpybz)(pybz)] (1), (μ -O)[VO(Hpybz)(pybz).VO(Hpybz)(acac)] (2) and [VO(Hpbyz)₂SO₄].H₂O (3) on glucose metabolism *in vitro*. The first objective was to investigate the effects of oxidovanadium complexes on glucose utilisation and mechanisms involved in liver and muscle cell lines. The second objective was to investigate the effects of oxidovanadium complexes on insulin secretion in pancreatic cell line.

Materials and methods

Liver (Chang), muscle (C2C12) and pancreatic (RIN-5) cell lines were cultured using a well-established protocol in Eagle's Minimal Essential Medium, Dulbecco's Modified Eagle's Medium and Roswell Park Institute Medium 1640, respectively. Cell viability studies were carried in all cells. All doses of oxidovanadium complexes or standard drugs used in glucose utilisation and insulin secretion studies were extrapolated from preliminary studies. For glucose utilisation studies, separate preparations of muscle and liver cell lines incubated with media containing 29 and 19 mmol/L of glucose, respectively were treated with 12.5, 25 and 50 μ g/mL of oxidovanadium complexes (1, 2 and 3). Dimethyl sulphoxide (0.1%) and insulin (4 μ g/mL) or metformin (160 μ g/mL) treated cells served as a negative and positive control, respectively. Furthermore, to examine the effects of combined treatment, some cells were

treated with lowest dose of oxidovanadium complexes combined with either insulin or metformin. Media glucose concentrations were measured after 0, 12, 24 and 48 h. After 48 hours, liver and muscle cells were harvested for measurements of glycogen and expressions of GLUT4 and glycogen synthase. For insulin secretion studies, separate preparations of pancreatic cells incubated with media containing 18 mmol/L of glucose were treated with 12.5 µg/mL of oxidovanadium complexes (1, 2 and 3) and dimethyl sulphoxide (0.1%) treated cells served as a control. Media glucose and insulin concentrations were measured at 12, 24 and 48 hours.

Results

All 3 oxidovanadium complexes (12.5, 25 and 50 µg/mL) had no apparent effects on cell viability in liver and muscle cells. However, for pancreatic cell lines, only lowest dose was not toxic. By comparison to the control, administration of oxidovanadium complexes significantly increased ($p < 0.05$) glucose uptake after 48 h in both liver and muscle cell with a concomitant increase in glycogen concentrations. By comparison to the control, administration of oxidovanadium complex (2) significantly increased ($p < 0.05$) glucose uptake as indicated by a decrease in media glucose (11.5 ± 0.5 vs 7.8 ± 1.0 mmol/mL) with a concomitant increase in insulin secretion (191.1 ± 2.4 vs 255.0 ± 2.1 pmol/L) by the pancreatic cell. Administration of oxidovanadium complexes combined with either insulin or metformin showed no apparent additive effects on glucose utilization and glycogen synthesis. Furthermore, administration of oxidovanadium complex (2) significantly increased ($p < 0.05$) expressions of GLUT4 and glycogen synthase in muscle and liver cells, respectively.

Discussion

These results suggest that oxidovanadium complexes enhance glucose utilization and glycogen synthesis in both liver and muscle cells *in vitro*. The observed effects may be partly mediated via increased expression of GLUT4 and glycogen synthase. These observations extend the literature evidence; since various vanadium complexes have been shown to possess anti-hyperglycaemic effects. The increase in insulin secretion by pancreatic cells after oxidovanadium complex (2) administration cannot be explained by the present study. Taken together, these observations suggest that oxidovanadium complex (2) may have beneficial effects in the management of diabetes mellitus.

Conclusions

Oxidovanadium complexes enhance glucose metabolism in both liver and muscle cell lines. These results suggest that oxidovanadium complexes may exert a tight glycaemic control which is beneficial in diabetes management.

Recommendations

These observations highlight the necessity to further evaluate the effects of oxidovanadium complexes in experimental diabetic animal models, *in vivo*.

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- (b) **N. Sibiya**, M. Serumula, R. Myburg and C.TMusabayane. Antioxidant effects of oleanolic acid in the liver, heart and kidneys of streptozotocin-induced diabetic rats. Physiological Society of Southern Africa 2013, 16-19 September. 78
- (c) **N Sibiya**, M Serumula and C.T Musabayane. Evaluation of the effects of oxidovanadium 2-pyridylbenzimidazole (hpybz) on glucose metabolism in the liver and skeletal liver and skeletal muscle lines. Physiological Society 2014, 1 July. 79
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CHAPTER 1

INTRODUCTION/LITERATURE REVIEW

1.1 Background

Diabetes mellitus (DM) is a worldwide major concern associated with hyperglycaemia due to the lack of insulin effects. Insulin facilitates glucose uptake in the muscle and adipose tissue whilst promoting glycogen synthesis and inhibiting gluconeogenesis in the liver. These organs play a pivotal role in maintaining glucose homeostasis. Insufficiency of insulin or the hyporesponsiveness of the above-mentioned tissues to insulin results in hyperglycaemia which leads to the development of macro and microvascular complications. The management of hyperglycaemia and DM associated complications rely on various strategies such as insulin injections and a host of oral anti-hyperglycaemic drugs. However, these therapies have limited efficacy, limited tolerability and significant side effects. Therefore, novel therapies with less undesirable effects are under constant evaluation. Our laboratory is participating and contributing in a worldwide on-going research which seeks to develop novel anti-hyperglycaemic agents which include insulin formulations, bioactive plant compounds and anti-hyperglycaemic synthetic compounds. Novel approaches are not only meant to broaden treatment availability, but also to manage DM more efficiently with less undesirable effects. Recently, the anti-hyperglycaemic activity of vanadium complexes (IV) and (V) has received attention. Therefore, the focus of this study was directed to investigate the effects of oxidovanadium complexes on glucose metabolism in liver (Chang) and skeletal muscle (C2C12) lines.

1.2 Glucose homeostasis

Glucose serves as a main source of substrate for energy production in the form of adenosine triphosphate (ATP). Therefore, glucose has a pivotal role in various cellular processes for homeostasis maintenance. In the body, glucose homeostasis is maintained by three highly coordinated processes. These processes include glucose absorption by the small intestine, the production or release of glucose by the liver and the utilisation of this monosaccharide nearly by most tissues (Aronson and Rayfield, 2000). Some tissues such as brain are in a constant need of glucose supply, as a result hypoglycaemia can lead to seizures, loss of consciousness and irreversible neuronal cell damage. On the other hand, some tissues are vulnerable to hyperglycaemia; in these tissues, high glucose concentrations have deleterious effects referred to as glucotoxicity. Glucotoxicity leads to nephropathy, retinopathy, cardiac and peripheral vascular diseases (Novella *et al.*, 2001). Glucose acts as a main regulator of insulin production and secretion by beta (β)-pancreatic cells (Kaiser *et al.*, 2003). Thus prolonged excessive amounts of glucose have a negative impact in β -pancreatic cell function. Excessive glucose increases the release of insulin, which eventually causes a gradual depletion of insulin stores resulting in DM (Kaiser *et al.*, 2003). Therefore, there is an imperative necessity to maintain blood glucose within narrow limits. Glucose is a hydrophilic molecule, as result glucose cannot passively diffuse across the hydrophobic lipid bilayer membrane. Thus, there are various proteins located on the cell membranes to facilitate glucose transport into the cells.

1.3 Glucose transport

There are two types of glucose transporters which facilitate glucose transport into cells. Glucose transporters are classified into two; sodium dependent glucose transporters (SGLTs) and glucose transport facilitators (GLUTs). To date, known and well documented SGLTs include 1, 2, 4 and 6, and each SGLT has a distinct feature or function. They are integral proteins in the cell membranes mediating glucose transport, with high affinity for glucose (Wright *et al.*, 1994). This transport system mediates the transport for both glucose and sodium ions (Wright, 2001). The inward movement of sodium ions is favoured by concentration gradient of sodium; the membrane potential generated is coupled to glucose entry (Diez-Sampedro *et al.*, 2000). SGLT 1 is known to be a high affinity and low capacity sodium glucose transporter with sodium to glucose coupling ratio of 2:1. This co-transporter

is mainly found in intestines, heart and kidneys (Zhou *et al.*, 2003). On the other hand, SGLT 2 is known to have low affinity and high capacity, with sodium to glucose coupling ratio of 1:1 (Mackenzie *et al.*, 1994). SGLT 2 is ubiquitously expressed, however, there is high expression in the intestine and kidneys (Gerardi-Laffin *et al.*, 1993).

The human genome codes for 14 known GLUTs. Due to their amino acid sequence similarities and characteristics, GLUTs are divided into three subfamilies; Class 1 (1, 2, 3, 4 and 14), Class 2 (5, 7, 9, and 11) and Class 3 (6, 8, 10 and 12) (Joost and Thorens, 2001). Both GLUT1 and 2 are highly expressed in DM, which lead to high glucose absorption by small intestine and re-absorption in the kidneys (Kahn, 2002). Thus, inhibition of these transporters is beneficial in DM management. GLUT4 activity increases up to 20-folds when stimulated by insulin. In DM, GLUT4 expression is decreased and leads to inadequate glucose uptake by muscle and adipose tissue (Shepherd and Kahn, 1999). Various anti-hyperglycaemic agents have been shown to manipulate the glucose transport in muscle, adipose and liver to improve glucose homeostasis.

1.4 The role of skeletal muscle and liver in glucose homeostasis

As the main crucial detoxifying organ of the body, the liver plays a pivotal role in metabolic homeostasis and is a major site for synthesis, catabolism, storage and redistribution of carbohydrates, proteins, lipids as well as exogenous substances (Postic *et al.*, 2004). In the liver, insulin stimulates glycogen synthesis by increasing glycogen synthase expression and inhibition of glycogen breakdown. Additionally, insulin also stimulates glycolysis and inhibits gluconeogenesis in the hepatocytes thus lowering blood glucose (Aronson and Rayfield, 2000). In the absence of insulin, glycogenesis is impaired, on the other hand gluconeogenesis is enhanced and as a result more glucose is released by the liver to the circulatory system through a bidirectional GLUT2, and worsens hyperglycaemia. The rate of gluconeogenesis is controlled principally by the activities of unidirectional enzymes such as phosphoenolpyruvate carboxykinase (PEPCK), fructose-1,6-bisphosphatase (FBPase) and glucose-6-phosphatase (G6Pase) (Bechmann *et al.*, 2012). PEPCK catalyses one of the rate limiting steps of gluconeogenesis, the conversion of oxaloacetate to phosphoenolpyruvate (PEP), while G6Pase catalyses the final step of gluconeogenesis, the production of free glucose from glucose-6-phosphate (Postic *et al.*, 2004). Insulin inhibits gluconeogenesis by suppressing the expression of PEPCK and G6Pase (Bechmann *et al.*, 2012). In the liver,

vanadium complexes such as vanadyl sulphate have been reported to inhibit glycogenolysis by inhibiting G6Pase and glycogen phosphorylase (Shehzad, 2013). In addition, vanadium has also been found to improve glucokinase and pyruvate kinase which have positive effects on glycogenesis. However, the effects of vanadium on gluconeogenesis are unclear.

Unlike the liver, the muscle cannot regulate the release of glucose into circulation. However, the ability to rapidly increase glucose uptake is critical for maintenance of glucose homeostasis. In muscle, insulin stimulates amino acid uptake, protein synthesis, glucose uptake and incorporation of glucose into glycogen (Aronson and Rayfield, 2000). The muscle as well as adipose tissue expresses significant amounts of the GLUT4 which upon insulin stimulation, is translocated to the plasma membrane allowing glucose uptake (Bryant *et al.*, 2002). Vanadium complexes have been found to improve glucose homeostasis by promoting the activation of GLUT4 and hence promoting glucose uptake in skeletal muscle (Shehzad, 2013). In DM, the absence of insulin effects results in inadequate glucose uptake by muscle tissue which leads to hyperglycaemia. Studies have also reported that an increase in intramuscular fat content induces insulin resistance through generation of active fatty acid derivatives which can interfere with insulin signaling.

1.5 Insulin effects

Insulin is responsible for regulation of blood glucose homeostasis through promoting glucose utilisation by target tissues and storage in the form of glycogen in the liver. Insulin is a dimer made of alpha (α) and β chains which are joined together by disulphide bonds. This hormone is composed of 51 amino acids and is 5808 Da. Insulin is synthesized by β -cells of the pancreas in the form of pro-insulin which is further modified to insulin (Annandale *et al.*, 2004). In addition to carbohydrate metabolism, insulin also stimulates lipid, protein anabolism and growth (Ringborg *et al.*, 2010). Deficiency of insulin results in muscle wasting and lipid breakdown due to activation of hormone sensitive lipase and inhibition of lipoprotein lipase (Shen *et al.*, 2007). Insulin secretion is stimulated by high blood glucose and fatty acid. High blood glucose levels lead to depolarization of the β -cells due to activation of ATP-potassium (K^+) channels. Subsequent events include opening of calcium (Ca^{2+}) channels which lead to secretion of insulin. Low levels of blood glucose or starvation

inhibit secretion of insulin. In addition, there are various hormones such as glucagon and catecholamines which suppress insulin actions. Both glucagon and catecholamines promote hyperglycaemia and in DM high levels of these hormones worsens the condition. Bis-maltolato vanadium complex has been shown to decrease glucagon levels in the pancreatic cells of STZ-induced diabetic rats. Due to DM, to date, there are agents that have the ability to enhance insulin secretion such as sulphonylureas. On the other hand, some drugs such as insulin glargine have the ability to extend insulin half-life. Immunohistological staining studies have shown elevated insulin in pancreatic cells with a concomitant increase in plasma insulin levels after bis-maltolato vanadium and vanadate pyrrolodine-N-dithiocarbamate administration, *in vivo* (Conconi *et al.*, 2003)

1.6 Insulin mechanism

Insulin receptors (IR) are present particularly in adipose and muscle tissues. Insulin receptors have two components, α components which are found in the cell membrane surface and β components which are within the cell (Saltiel and Pessin, 2002). Insulin binds to α and activates β components. The activation of IR causes phosphorylation of two major downstream targets which are insulin receptor substrate 1 (IRS-1) and insulin receptor substrate 2 (IRS-2) (Cai and Dhe-Pagano, 2003). IRS-2 function is poorly understood, however, some studies have shown that IRS-2 is involved in lipid metabolism and mitogen activated pathway kinase (MAPK) signaling pathway. MAPK pathway signalling is important for DNA and protein synthesis which leads to cell proliferation. IRS-1, on the other hand is responsible for glucose metabolism. IRS-1 has various sites for tyrosine and serine/threonine phosphorylation which are significant in the activity of IRS-1. Reduced insulin-stimulated phosphorylation has been observed in type 2 DM. IRS-1 phosphorylation by tyrosine kinases activates phospho-inositol 3 kinase (PI3-K) which causes insertion of GLUT4 across the membrane in the muscle and adipose tissue (Engelman and Luo, 2006). GLUT4 then transport glucose into the cell (Annandale *et al.*, 2004). PI3-K also activates further pathways such as Akt. This pathway is involved in cell survival and also glycogen synthesis. Akt phosphorylates glycogen synthase 3-kinase (GS3-K) to activate glycogen kinase for glycogen synthesis in the muscle (Grimes and Jope, 2001; White, 2003). Defects in PI3-K have been reported also in type 2 DM, which lead to inadequate glucose uptake. Cells

using this transport system are said to be insulin dependent (Annandale *et al.*, 2004). Recently, tyrosine phosphatases have been found to inhibit insulin sensitivity, thus inhibition of phosphatases may present a novel approach in overcoming insulin resistance. Studies have shown that vanadium has the ability to inhibit phosphatases thus increasing insulin sensitivity (Bhattacharyya and Alan, 2001).

Despite the ability of vanadium to interact with insulin signaling pathways, vanadium has also been found to interact with other novel pathways to ensure adequate glucose uptake (Fantus and Tsiani, 1998; Medhi and Srivastava, 2005). Studies have reported a cytosolic protein-kinase which was activated 3-fold after vanadium administration in the muscle cell line (Shisheva and Shechter, 1991). The biological effects of vanadium which include glucose oxidation and lipogenesis were attributed to activation of cytosolic protein-kinase (Shisheva and Shechter, 1991). In addition, vanadium has also been shown to activate a non-receptor protein in the cell membrane which activates PI3-K for glucose uptake and glucose metabolism (Shisheva and Shechter, 1991). Thus vanadium seems to utilise various pathways to ensure adequate glucose uptake, which may be of importance in the management of hyperglycaemia and DM associated complications.

1.7 Diabetes mellitus

Approximately 3.2 million deaths caused by DM have been reported worldwide and of which the trend is expected to increase in future exerts a major burden in the health systems and facilities (Fazeli *et al.*, 2013). In South Africa and other developing countries, reports indicate that the disease is amongst the leading cause of mortality and morbidity (Abegunde *et al.*, 2007; Mash *et al.*, 2012). DM is a chronic metabolic disorder characterized by hyperglycaemia which is associated with development of both type 1 and 2 DM. Hyperglycaemia may result as a consequence of insulin insufficiency or insulin resistance. The insufficiency of insulin may be caused by exhaustion or dysfunction of the β -cells in the pancreas. Hyperglycaemia may also result as a consequence of insulin resistance which may be caused by hyporesponsiveness of target tissues such as muscle and adipose to insulin. Insulin promotes glucose uptake in the muscle and adipose tissue and also glucose storage in the liver through glycogen synthesis, thus maintaining glucose homeostasis. Therefore, the lack of insulin effects results in impaired glucose homeostasis (Kristen, 2007). Sustained

hyperglycaemia results in the derangement of carbohydrate, lipids and protein metabolism which lead to DM pathological manifestations.

1.7.1 Classification of diabetes mellitus

The major types of DM are type 1 DM which is insulin dependent, type 2 DM which is non-insulin dependent and gestational DM (Egede and Ellis, 2010). To date, the effects of various vanadium complexes such as vanadyl sulphate and bis-maltolato vanadium have been studied in type 1 and 2 DM experimental models (Medhi and Srivastava, 2005). Type 1 DM accounts for about 10% of all cases of diabetes and is often referred to as juvenile diabetes as this process takes place at some point during childhood or adolescence (Anabela *et al.*, 2006). Type 1 DM results as a consequence of insulin deficiency which occurs due to the pancreatic β cells destruction through various mechanisms (Belle *et al.*, 2001). One of the mechanisms involves the destruction of insulin producing cells as a consequence of autoimmunity (Belle *et al.*, 2001). Studies suggest that chromosome 6 gene codes for human leukocyte antigen (HLA) which sensitizes the cells to destruction by autoimmunity (Belle *et al.*, 2001). The initial presentation of type 1 DM is characterized by sudden onset of hyperglycaemia, often with ketoacidosis (Charpentier *et al.*, 2003). Ketoacidosis may arise as a consequence of fatty acids utilisation as a substrate for energy production by various tissues. Without insulin to normalize blood glucose, the patient often manifests with polyuria, polydipsia, polyphagia and weight loss (Saltiel and Kahn, 2001). In high blood glucose conditions, there is saturation of glucose transporters in the kidney which leads to glucosuria and polyuria, and as a consequence dehydration is often observed (John, 2014). Weight loss in DM is attributed to both dehydration and catabolism of lipids and proteins. The absence of insulin has been shown to inhibit the satiety centre in the hypothalamus and thus such patients exhibit higher food consumption. In order to circumvent these manifestations, type 1 DM patients depend entirely on exogenous insulin. Therefore, any novel agent that mimics insulin actions is of therapeutic value in the management of type 1 DM. Previous studies have shown that inorganic vanadium complexes such as sodium orthovanadate exert anti-diabetic effects in animal models of type 1 DM (Berhan and Habtewolde, 2012). However, there is an on-going controversy with regards to vanadium therapy in management of type 1 DM since vanadium has been shown to be catabolic (Berhan and Habtewolde, 2012). Interestingly, bis-maltolato vanadium complexes have been shown to decrease food consumption in STZ- induced diabetic rats, which is beneficial in DM management (Reul *et al.*, 1999).

Type 2 DM which is also known as late onset DM results from various causes and accounts for about 90% of morbidity and mortality. Type 2 DM is considered as the most difficult type to manage efficiently due to various reasons. Over the years, establishing a proper type 2 diabetic model has been difficult, thus there are still some gaps in the knowledge we have regarding type 2 DM. At the beginning of the disease, insulin is usually sufficient to sustain glycaemic control. However, the target tissues such as muscle and adipose progressively lose sensitivity to insulin (Suzuki *et al.*, 2003). There are several factors that sensitize individuals to type 2 DM. Sedentary life style and unhealthy diets increase the risk of type 2 DM, hence healthy diets and physical activities are recommended to the patients (Tessier *et al.*, 2000). Obesity and high circulating levels of free fatty acids have been shown to trigger insulin resistance by interacting and causing defects in the insulin signaling pathways (Adilson *et al.*, 2008). Insulin resistance can occur either in the insulin receptor or downstream in the cascade (Samuel and Shulman, 2012). Disturbance in the insulin signalling pathway has deleterious effects since glucose uptake is impaired. Unlike type 1 DM which is managed by administering insulin, type 2 is usually managed by the use of various oral hypoglycaemic agents such as metformin which enhance insulin sensitivity in both muscle and adipose tissue. Interestingly, reports have indicated that vanadium has the ability to increase insulin sensitivity in target tissues (Medhi and Srivastava, 2005). Studies have shown that oral vanadyl sulphate increases hepatic and peripheral insulin sensitivity in patients with type 2 DM (Cohen *et al.*, 2001). Furthermore, type 2 DM is associated with obesity and catabolic effects of vanadium may be of significance in the management of type 2 DM.

1.7.2 Diabetes mellitus complications

DM complications are classified into macrovascular and microvascular complications. Both complications are associated with high morbidity and mortality. Macrovascular complications involve the aberrations of large vessels such as veins and arteries (Kota *et al.*, 2012). The complications include atherosclerosis, hypertension and coronary heart diseases (Kota *et al.*, 2012). Treatment with inorganic and organic vanadium compounds has been shown to exert a wide range of cardiovascular-protective effects (Bhuiyan and Fukunaga, 2009). The central pathological mechanism in macrovascular disease is the process of atherosclerosis which leads to narrowing of arterial walls throughout the body (Fowler, 2008). In the development of atherosclerosis, oxidative stress results in the entrapment of oxidative low density lipoproteins (LDLs). Oxidative LDLs then cause inflammation and attract immune cells

which further form macrophages and foam cells (Fowler, 2008). These together with high levels of circulating free acid and adhesion molecules block vessels by forming plaques. In a study published in 1999, researchers at the Albert Einstein College of Medicine in New York compared the effects of vanadyl sulphate in moderately obese diabetic and non-diabetic people (Boden *et al.*, 2000). The results showed that LDL cholesterol levels and oxidative status were reduced. Since vanadyl sulphate has been shown to reduce cholesterol levels, vanadyl sulphate together with other vanadium complexes may be beneficial in treating atherosclerosis and heart disease and may play an important role in reducing incidences of heart attacks (Bhuiyan and Fukunaga, 2009). Hypertension is also recognized as a macrovascular complication, sustained hypertension is associated with progression of both diabetic nephropathy and neuropathy.

Microvascular complications involve aberrations in the small vessels such as capillaries. The complications include diabetic retinopathy, neuropathy and nephropathy. Angiogenesis has been hypothesized as the gateway to retinopathy and nephropathy (Kota *et al.*, 2012). The effect of angiogenesis in neuropathy has not been well studied (Kota *et al.*, 2012). Vanadium complexes such as peroxovanadium have been found to exhibit anti-angiogenic properties in animal models (Doillon *et al.*, 2000). The inhibition of angiogenesis by these complexes can be used in the prevention and treatment of diabetic retinopathy and nephropathy (Doillon *et al.*, 2000). Therefore, this highlights the significance of vanadium complexes as an alternative method for the management of microvascular complications in DM.

Diabetic retinopathy (DR) is the leading cause of visual disability and blindness in people with diabetes (Cade, 2008). Diabetic retinopathy is characterized by retinal vessel micro aneurysms, haemorrhages and oedema (Fowler, 2008). One of the primary changes in diabetic retinopathy involves loss of pericytes in retinal capillaries which may lead to vascular failure and chronic hypoxia (Maritim *et al.*, 2002). Hypoxia is one of the major inducers of angiogenesis (Kota *et al.*, 2012). Hypoxic conditions lead to the upregulation of hypoxia- inducible factor (HIF) and vascular endothelial growth factor (VEGF) which then promote the rapid formation of neovessels, resulting in exacerbated angiogenesis (Sone *et al.*, 1997). The sudden establishment of angiogenic vessels leads to leakiness and malfunctioning of vascular system (Kota *et al.*, 2012). Vitreous hemorrhage is also observed in DR, due to leaking of newly formed blood vessels and jell-like substance which fills the centre of the eye thus impairing vision (Pang *et al.*, 2011). Retinal detachment is also observed in DR, due to

abnormal new blood vessels which promote scar tissue growth, the latter pulls the retina away, ultimately causing spot floating vision (McHugh *et al.*, 2011). Moreover, leaking and new growth of blood vessels interrupt the fluid flow in the eyes and this causes the pressure to accumulate, in severe cases this damages the optic nerve which leads to permanent blindness. The underlying mechanism of DR is hyperglycaemia and the ability of vanadium to achieve glycaemic control may have beneficial effects in alleviation of DR. Studies have reported that oral vanadyl sulphates alleviate cataracts in DM (Bosia *et al.*, 2000).

In clinical practice, diabetic neuropathy (DNE) is defined as signs and symptoms of peripheral nerve dysfunction in a diabetic patient where other causes of peripheral nerve dysfunction have been excluded. DNE has caused more hospitalization of DM patients than other complications, with half of the patients having some degree of the disease such as polyneuropathy and ormononeuropathy (Won *et al.*, 2012). The prevalence of DNE has been found to be 66% and 59% for type 1 and type 2 DM, respectively (Kastenbauer *et al.*, 2004). This complication is heterogeneous by symptoms and signs, risk factors, underlying mechanism and pathologic alterations. Mono and polyneuropathies, plexopathies and radiculopathies are observed in DNE (Dyck and Sinnreich, 2003). Autonomic neuropathy has been considered irreversible and life threatening since there is high risk of mortality. However, some studies have shown that cardiac denervation regresses with high glycaemic control (Stevens *et al.*, 1999). For both prevention and therapeutic purposes, intense glycaemic control has been recommended. Early detection of hyperlipidaemia and hypertension, cessation of smoking and alcohol consumption may help in prevention and delaying the progression of DNE (Vinik, 2010).

Diabetic nephropathy (DN) is the leading cause of renal failure in USA (Gordois *et al.*, 2004). DN is amongst the leading cause of mortality and morbidity for both type 1 and type 2 DM (Thomson *et al.*, 2009). Most patients experience the condition before they are diagnosed. However, the false assumptions may be made since proteinuria may be due to renal infection, exercise and haematuria (Thomson *et al.*, 2009). Clinical hallmarks of DN include increased albumin excretion and declined glomerular filtration rate, both of which are associated with high blood pressure (Mogensen, 2003). These functional changes in the kidney occur as a consequence of structural changes which include the thickening of glomerular basement membrane, mesangial cell expansion and loss of podocyte, extracellular matrix deposition, glomerulosclerosis and tubulointerstitial fibrosis. The ability of vanadium

complexes to lower blood glucose and also to act as antioxidants may be beneficial in both prevention and management of diabetic nephropathy.

1.7.3 Aetiology of DM complications

Sustained hyperglycaemia results in derangement of glucose metabolism, especially in those tissues which are insulin independent such as kidneys, retina and nerves. As described in the preceding paragraphs, these tissues are highly susceptible to hyperglycaemia due to the activation of four major pathways which act together in the establishment of DM pathogenesis. These metabolic pathways involve advanced glycation end products (AGEs), polyol, protein kinase C (PKC) and the hexosamine pathway as illustrated in figure 1. The anti-hyperglycaemic and antioxidant effects of vanadium may be beneficial in declining the activation of these pathways.

The formation of AGEs is among the irreversible changes particularly in the kidney that occur as a result of hyperglycaemia (Ahmed, 2005). At high blood glucose concentrations there is an induction of non-enzymatic reaction between glucose and free amino acids (Singh *et al.*, 2001). The interaction between glucose and amino acids results in Schiff-base formation, thereafter Amadori products. Subsequent rearrangements and reactions lead to formation of irreversible compounds known as AGEs (Singh *et al.*, 2001). AGEs have a varying range of chemical, cellular and tissue effects through changes in charge, solubility and conformation. Extracellular matrix proteins are highly susceptible to AGE modification. This results in structure and function alterations, for example, collagen glycation results in inter and intramolecular crosslinks which alters structure and increase resistance of collagen to proteolytic digestion. Moreover, AGEs accumulation has been found to increase the production of reactive oxygen species (ROS). The use of AGEs inhibitor (aminoguanidine) has been found to alleviate AGEs associated complications in DM (Kelly *et al.*, 2001). Moreover, an intense glycaemic regulation by various conventional treatments, especially at the early stage of DM, delays AGEs formation. In this regard, vanadium has been shown to possess both hypoglycaemic and antioxidants effects and, as a result vanadium may circumvent or delay AGEs formation.

The polyol pathway has been shown to be involved in diabetic complications, particularly in microvascular damage in non-insulin sensitive tissues such as the lenses, peripheral nerves and the glomerulus (Dunlop, 2000). The polyol pathway is an alternative pathway activated by high intracellular glucose through aldose reductase (AR). Glucose is converted to sorbitol which is a highly osmotic active molecule. The conversion of glucose to sorbitol is facilitated by AR in a nicotinamide adenine phosphate (NADPH) dependent reaction. NADPH is known to be the universal hydrogen donor and responsible for the re-generation of the biological active glutathione which is an important antioxidant. Since polyol pathway consumes large quantities of NADPH levels, this results in impaired glutathione re-synthesis which ultimately causes redox imbalances leading to oxidative stress (Lorenzi, 2007). On the other hand, sorbitol accumulation activates sorbitol dehydrogenase which converts sorbitol to fructose (Lal *et al.*, 1997). Excessive levels of fructose are associated with high production of ROS through non enzymatic glycoxidation of proteins and lipids (Roger *et al.*, 2003). Vanadium has been shown to inhibit AR activity in diabetic rat model *in vivo* (Lei *et al.*, 2014).

Protein Kinase C (PKC) is a group of enzymes of the cyclic adenine monophosphate (cAMP) dependent protein kinase (AGC) family. PKC plays a pivotal role in many cellular functions and affects many signal transduction pathways (Alexandra and Newton, 2000). There are multiple isoforms of PKC that function in a wide variety of biological systems. The well-documented form of PKC (cPKC) isoforms include PKC- α , - β 1, - β 2, and - γ . These isoforms are activated by diacylglycerol (DAG) and ROS such as hydrogen peroxide (H₂O₂) that is generated in the polyol pathway (Alexandra and Newton, 2000). In DM, many abnormal vascular and cellular processes including endothelial dysfunction, vascular permeability, basement membrane thickening, extracellular matrix (ECM) expansion and enzymatic activity alterations are attributed to multiple PKC isoforms (Koya and King, 1998). Since PKC pathway descends from hyperglycaemia and ROS, intense glycaemic control by vanadium may attenuate hyper activation of PKC.

Previous studies have suggested that the hexosamine pathway is implicated in diabetic nephropathy. Under normal conditions glucose enters the glycolytic pathway where most of glucose is metabolized to pyruvate through glycolysis. However, a small quantity of fructose 6-phosphate from glycolysis is converted to glucosamine-6-phosphate, a reaction catalyzed by glutamine: fructose-6-phosphate amidotransferase (GFAT). This results in the formation

of uridine diphosphate-N-acetylglucosamine. Uridine diphosphate-N-acetylglucosamine serves as a building block for glycosyl side chains of various proteins (Sayeski and Kudlow, 1996). In DM, the rate through which fructose-6-phosphate is formed is elevated, leading to increased levels of uridine diphosphate -N-acetylglucosamine which results in increased formation of O-glycosylated proteins (Haltiwanger *et al.*, 1998). O-linked glycosylation of proteins leads to the induction of plasminogen activator inhibitor-1 (PAI-1) (Howard *et al.*, 2002). In DM, PAI-1 results in the accumulation of extracellular membrane proteins in the glomerular mesangium (Goldberg, 2000; Lee *et al.*, 2005). To date, no studies have been undertaken to investigate the effects of vanadium on the hexosamine pathway. The ability of vanadium to restore glucose homeostasis may delay the activation of hexosamine pathway.

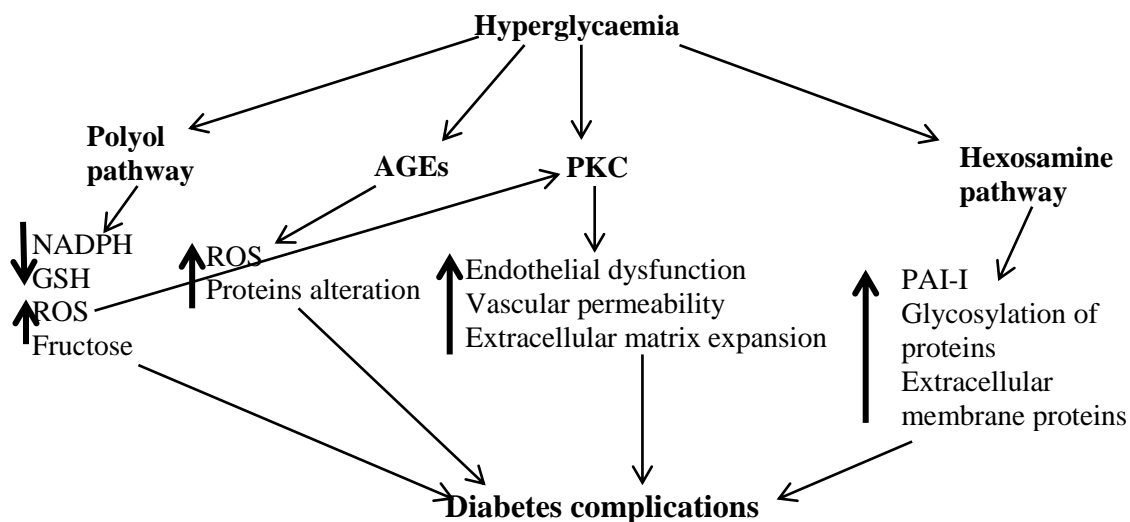


Figure 1: The diagram illustrates the pathways involved in the pathogenesis of diabetes mellitus. The activation of these pathways progressively alters cellular functions through protein modifications and impaired cell membrane integrity.

1.7.4 Conventional management of diabetes

To date, there are numerous therapeutic strategies used to manage diabetes since there are various pathogenic processes involved. Therapeutic agents used in DM treatment range from anti-hyperglycaemic agents, anti-hypertensive agents, anti-coagulant, anti-inflammatory and antioxidants. Thus management of DM and associated complications proves to be more complicated and challenging. The main causal link of DM is hyperglycaemia, therefore the primary intervention targets to maintain euglycaemia. Various anti-hyperglycaemic agents have been shown to maintain normal glycaemia and alleviate DM associated insults. In addition to exogenous subcutaneous insulin injections, there are four conventional classes of oral anti-hyperglycaemic drugs. These drugs act at different target organs to achieve their desirable effects. These classes of drugs include biguanides, sulphonylureas, α -glucosidase inhibitors and thiazolidinediones (TZDs) which can be administered as monotherapy. However, when the oral anti-diabetic monotherapy does not achieve glycaemic goal, the combination therapy is implemented. Despite the availability of these drugs, the evaluation of promising novel agents is continuous and vanadium based compounds are among these agents (Medhi and Srivastava, 2005).

Insulin administered for DM treatment is obtained from pork pancreas or is made chemically identical to human insulin by recombinant DNA technology or chemical modification of pork insulin (Retnakaran *et al.*, 2004). Insulin is available in rapid, short, intermediate and long acting types that may be injected separately or mixed (Retnakaran *et al.*, 2004). However, this form of insulin administration can often be embarrassing and painful (Retnakaran *et al.*, 2004). On average, the type 1 diabetics will administer between 5-6 injections per day. Importantly, continuous insulin injection progressively decrease receptor sensitivity and can result in insulin resistance (Uruska *et al.*, 2014). Even with these measures, the daily necessity for several insulin injections can be painful both physically and mentally. For these reasons, the development of a new class of pharmaceuticals for treatment of DM would be extremely desirable. Vanadium has been reported to mimic insulin actions both *in vivo* and *in vitro* (Medhi and Srivastava, 2005).

Biguanides class includes drugs such as metformin, buformin and phenformin. To date, metformin is the only drug belonging to this class available for management of DM (Correia *et al.*, 2008). Metformin utilizes three mechanisms to maintain normal glucose levels. These

mechanisms include gluconeogenesis suppression in the liver, intestinal glucose absorption inhibition and peripheral glucose uptake stimulation in the presence of insulin (Ohiraa *et al.*, 2007). In addition, metformin has been reported to decrease glycohaemoglobin and improves both fibronolytic activity and lipid profile. Metformin therapy appears to be beneficial in type 2 DM patients since no weight gain has been reported in DM patients (Seufert *et al.*, 2004). Metformin administration is however, associated with diarrhoea and poor intestinal absorption of vitamin B complexes (Adams *et al.*, 1993). Buformin and phenformin were withdrawn since they were associated with lactic acidosis (Misbin *et al.*, 1998). Metformin-vanadium derivatives have, however, been evaluated and [VO (metformin)₂] H₂O has been shown to improve glycaemic control (Conconi *et al.*, 2003).

Sulphonylurea compounds continue to be the mainstay therapy for type 2 DM and have been available nearly half a century (Kar and Holt, 2008). Currently, glibenclamide is one of the sulphonylureas available for DM treatment. Glibenclamide achieves glycaemic goal through stimulation of insulin release by the β -pancreatic cells (Hu *et al.*, 2001). Sulphonylureas exert their effects by activation of ATP-dependent K⁺ channels, which allows depolarization of the cells, leading to insulin secretion (Fuhlendorf *et al.*, 2001). Despite their beneficial feature, sulphonylureas are associated with cardiovascular complications since they have been reported to impair myocardial blood flow and they also elicit proarrhythmic effects (Smits and Thien, 1999; Garratt *et al.*, 2000).

TZDs include anti-diabetic medications such as rosiglitazone, troglitazone and pioglitazone (Lebovits, 2002). The TZDs are insulin sensitizers which bind to peroxisome proliferator-activated receptor gamma (PPAR γ) followed by elevated GLUT4 expression and glucose uptake (Saltiel and Olefsky, 2000). TZDs have also been shown to inhibit gluconeogenesis in the liver (Edgerton *et al.*, 2009). Despite anti-hyperglycaemic effects, TZDs have been associated with weight gain in DM patients. Increased weight gain is attributed to reduction of leptin levels during TZDs treatment (Brakis *et al.*, 2002; Nissen and Wolski, 2007). This feature of TZDs has harmful implications and has been documented in both experimental diabetes and human studies.

Some anti-diabetic medications such as alpha-glucosidase inhibitors act primarily on the gastrointestinal tract, inhibiting starch hydrolysis and thus delaying glucose absorption (Kamawori *et al.*, 2009). The compounds like voglibose, miglitol and acarbose inhibit various

enzymes of the α -glucosidase group (sucrase, maltase, isomaltase and glucoamylase). The membrane-bound enzymes in the brush border of the small intestine hydrolyze oligosaccharides and disaccharides to glucose (Kamawori *et al.*, 2009). The hypoglycaemic potency of these drugs is less than that of biguanides and sulphonylureas (Moritoh *et al.*, 2009). The side effects of these drugs include mild pain, diarrhoea and flatulence (Chiasson *et al.*, 2002). Taking into consideration the burden exerted by DM in health care systems and also the inability of conventional therapies to manage diabetes efficiently, there is a necessity to further search for alternative strategies in the management of DM. One of these strategies involves evaluation of various vanadium compounds for diabetes management.

1.7.5 Alternative strategies for diabetes management

Literature has documented a variety of alternative strategies in the management of DM. Various medicinal plants (crude extracts and bioactive compounds) have been reported to have anti-diabetic properties (Musabayane *et al.*, 2005; Ugochukwu and Babady, 2008; Wang *et al.*, 2010). In addition to natural medicinal products, there is also a continuous search and evaluation of several synthetic compounds, including various classes of vanadium complexes (Berhan and Habtewolde, 2012). Vanadium is a trace element which exists in a variety of oxidation states (+1 to +6) (Rehder, 2003). In the body, vanadium exists in low quantities with highest concentrations found in the liver, kidney and bone. Reports indicate that insulin-sensitive tissues such as the liver and adipose metabolize vanadium more quickly compared to other tissues such as bone (Thompson *et al.*, 2009). Vanadium ions cannot easily diffuse through the lipid bilayer; hence vanadium is not easily absorbed in the small intestine. However, vanadium is a highly flexible mineral and can form co-ordination complexes with ligands which can allow greater permeability of vanadium. Since the 1980's, the hypoglycaemic effects of vanadium complexes in experimental diabetes have been reported together with the mechanisms relevant to both glucose and lipid metabolism (Medhi and Srivastava, 2005). Reports indicate that vanadium complexes (+4) and (+5) possess potent anti-diabetic insulin-like effects (Thompson *et al.*, 2009). Various vanadium complexes have been shown to promote glucose uptake, glucose oxidation and glycogen synthesis (Reul *et al.*, 1999).

Depending on the nature of ligand, vanadium complexes can be divided into inorganic and organic complexes. Inorganic vanadium such as vanadyl sulphates and sodium orthovanadate

complexes were among the first to be investigated in DM and were shown to have promising anti-diabetic effects (Reul *et al.*, 1999). Irrespective of their promising anti-diabetic properties, some inorganic vanadium complexes are associated with toxic effects such as diarrhoea, decreased fluid and food uptake, dehydration and reduced body weight gain (Reul *et al.*, 1999). Thus, there is urgency in continuously developing new non-toxic and more potent vanadium complexes analogues. Furthermore, vanadium also has the ability to utilise other non-insulin pathways to lower blood glucose (Medhi and Srivastava, 2005). There is also an interesting growing body of evidence indicating that this class of metallo-pharmaceuticals requires organic biological active ligand systems to provide stability and promote bioavailability of the metal complex. Consequently, finding an appropriate organic ligand system may enhance the potency and circumvent the downfalls of vanadium salts. To date, there are various organic vanadium complexes such as vanadyl acetylacetonate, bis-maltolato oxovanadium and vanadyl 3-ethylacetylacetonate which have been shown to exert anti-diabetic effects with minimal toxic effects as compared to inorganic vanadium complexes (Reul *et al.*, 1999). Therefore, a continuous search for an appropriate ligand system is significant.

2-pyridylbenzimidazole (Hpybz) is one of the well-established promising heterocyclic ligands which have been shown to also have an array of biological activities such as antimicrobial, antibacterial and anti-diabetic activities. Theoretically, an appropriate fusion of Hpybz with vanadium complex may have an additive effect in glucose lowering capacity thus improving the therapeutic value of vanadium. Since the causal link of DM and associated insults is hyperglycaemia, the ability of a novel agent to efficiently lower glucose or facilitate glucose uptake is of importance in the therapy of the disease.

1.7.6 Experimental diabetic models

There are various diabetic models used to study DM related pathology and also to investigate the effects of various therapeutic modalities in management of DM. Experimental diabetes studies can be conducted *in vivo* and *in vitro* using animals and cell lines, respectively. Although both do not perfectly mimic human diabetes, they are valuable tools for evaluating novel anti-diabetic agents. There are various *in vivo* diabetic models, which include surgical diabetes, chemical induced and genetically engineered diabetes models. Surgical models involve removal of pancreas and have limitations. Limitations of surgical models include,

high degree of technical expertise required, post-operative analgesia and antibiotics administration and pancreatic enzymes supplementation (Masiello, 2006). The majority of documented studies in the field of experimental diabetes between 1996 and 2006 employed chemical induced-diabetes models. Streptozotocin and alloxan are the most frequently utilized drugs to induce diabetes (Federiuk *et al.*, 2004). These two drugs selectively destroy β -pancreatic cells, which allow the study of various aspects of the disease (Mythili *et al.*, 2004). The diabetogenic actions of these drugs are exerted when administered intravenously, intraperitoneally and subcutaneously (Federiuk *et al.*, 2004). The dose of these agents necessary to induce diabetes depends on the animal species, route of administrations and also nutritional status. Various vanadium complexes have been investigated using chemically induced-diabetic models. However, biological tests in whole animal have been found to be expensive, time consuming and have a limited sensitivity to differentiate between structurally similar compounds. These limits drive researchers to prior conduct *in vitro* studies using cell lines models before using animal models.

Cell culture is a process where cells are grown under controlled conditions which can be easily manipulated and analysed (Li *et al.*, 2004). Cell culture studies are the precursor to *in vivo* animal studies and help to elucidate various mechanisms of various therapeutic compounds and also to determine whether significant cytotoxicity exists for a particular given compound (Li *et al.*, 2004). Therefore, cell culture is essential for biomedical research which include medicine, cell metabolism, genetic engineering and molecular biology (Dunham and Guthmiller, 2008). Cell lines have been widely utilised in the field of cell metabolism, particularly evaluating the effects of various anti-diabetic agents. An anti-diabetic agent can affect various components of glucose metabolism and homeostasis including insulin secretion, glucose absorption by the small intestine, glucose storage or release by the liver, glucose uptake by muscle and adipose tissue and glucose re-absorption by the kidney. Therefore, appropriate cell lines allow studying and evaluating the effects of anti-diabetic agents on various isolated aspect of glucose homeostasis.

There are various cell lines utilised as glucose utilisation models to determine the ability of a given compound in promoting and inhibiting glucose uptake. These models also allow researchers to monitor glucose utilisation and associated molecular events over time. Literature has documented various cell lines which have been used with adipose, muscle and liver lines being widely employed (Verma *et al.*, 2004). Adipose tissue and skeletal muscle

are the key contributors to insulin resistance and hyperglycaemia. With recent advances, pathways related to glucose metabolism and insulin resistance can be successfully studied in the cell lines of adipose tissue such as murine 3T3-L1 cells and the skeletal muscle cell lines such as L6 engineered and C2C12 (Karalee *et al.*, 2001; Yen *et al.*, 2014). The muscle cell line (C2C12) is an immortal and adherent cell line of mouse skeletal myoblasts and was originally derived from satellite cells of mouse's thigh muscle (Diel *et al.*, 2008). This cell line differentiates well into myocytes under appropriate cell culture conditions (Diel *et al.*, 2008). C2C12 cell line is an adherent cell line and grows as undifferentiated myoblasts in growth media. C2C12 cell line also expresses significant amounts of GLUT4 and glycogen synthase, therefore, C2C12 is a significant tool in studying the effects of a given compound on glucose uptake (Karalee *et al.*, 2001; Yen *et al.*, 2014). C2C12 cell line was chosen in the study to determine the effects of oxidovanadium compounds on glucose uptake, glycogen synthesis and GLUT4 expression.

There are various liver cell lines employed in biological science and pharmaceutical industry. These cell lines include hepatocyte derived human transformed cell line (HepG2) and Chang cell line. Chang liver cells are immortalized non tumour cells derived from normal liver tissue of a mouse. Chang cell line, like other cell lines, are termed "immortal", thus they can divide in an unlimited number of times in a laboratory cell culture plate as long as fundamental cell survival conditions are met (Strick-Marchand and Weiss, 2003). Chang cells are also adherent cells and grow as undifferentiated hepatoblasts in growth media (Strick-Marchand and Weiss, 2003). Furthermore, Chang cells are significant tools in assessing the effects of a given compound on glucose utilisation, glycogen synthesis as well as toxicity (Nadu, 2007; Ventera *et al.*, 2008). Therefore, the Chang cell line was chosen in this study to determine the effects of oxidovanadium complexes on glucose uptake, glycogen synthesis and glycogen synthase expression.

Recent advances provide opportunities to establish and improve various appropriate cell lines to facilitate studying mechanisms of both insulin secretion and cell dysfunction (Zhao *et al.*, 2005). To date, there are various insulin secreting cell lines, and widely used cell lines include rat insulinoma (RIN-5) (Poitout *et al.*, 1996). The RIN-5 cell line was derived from a radiation induced transplantable rat islet cell tumour. RIN-5 cell line mainly secretes insulin, small amounts of glucagon and somatostatin (Fernández-Millán *et al.*, 2014). The behaviour of these cells, however, do not perfectly resemble cell physiology of β pancreatic cells,

nonetheless, they are extremely significant tools for the evaluation of drugs on insulin secretion together with associated molecular events (Fernández-Millán *et al.*, 2014). Therefore, RIN-5 cell line was chosen in the study to determine the effects of oxidovanadium complexes on insulin secretion.

1.8 Justification of the present study

The literature evidence described in the preceding sections indicates that numerous vanadium compounds have potential in the management of diabetes. Of interest in this study, were the effects of oxidovanadium complexes on glucose metabolism in muscle and liver cell lines and insulin secretion by pancreatic cell lines. The outcome of the study may justify further evaluations of various potential vanadium complexes in the management of diabetes

1.9 Aims

The overall aim of the study was to investigate the effects of oxidovanadium complexes on glucose metabolism in the liver (Chang) and skeletal muscle (C2C12) cell lines. The other aim was to evaluate the effects of oxidovanadium complexes on insulin secretion in pancreatic (RIN-5) cell line.

1.10 Objectives

The aims of this study were achieved by:

- i. synthesis and characterization of novel oxidovanadium complexes
- i. analysis of cytotoxic effects of oxidovanadium complexes using a luminescent-based cell viability assay
- ii. determination of glucose utilisation in liver and muscle cells using a glucometer
- iii. analysis of glycogen synthesis in liver and anthrone-based glycogen assay
- iv. determination of insulin secretion using ELISA
- v. analysis of GLUT 4 and glycogen synthase expression using western blotting method

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals and drugs

Chemicals and drugs were sourced as follows:

dimethyl sulphoxide (DMSO), Tris-HCL, glycerol, Tris-base, glycine, sodium dodecyl sulphate (SDS), vanadyl sulphate, bovine serum albumin (BSA), phosphate buffered saline (PBS), anthrone, dioxovanadium (VO_2), acetyl acetone oxovanadium ($\text{VO}(\text{acac})_2$), sulphato oxovanadium (VOSO_4), Hpybz, ammonium vanadate, mercapthoethanol, bromophenol blue and Tween 20 (Sigma-Aldrich, St Louis, Missouri, USA);

Dulbecco's Modified Essential Medium (DMEM), Eagle's Minimum Essential Medium (EMEM), Roswell Park Memorial Institute Medium (RPMI) 1640, L-glutamine, penicillin/streptomycin (Pen/Strep), foetal calf serum (FCS) and trypsin (Highveld Biological, Johannesburg, South Africa);

methanol (MeOH), ethanol, sucrose, sodium chloride (NaCl), sodium sulphate (NaSO_4), potassium hydroxide (KOH), potassium chloride (KCl), dithiothreitol, glycerol (Merck chemicals, Johannesburg, South Africa);

insulin (NovoRapid Pen Refill, Novo Nordisk Pty Ltd, Sandton, South Africa) and

GLUT4, glycogen synthase, β actin and horse radish peroxidase (HRP) coupled antibodies (Abcam, Cambridge, United Kingdom).

All chemicals used were of analytical grade.

2.2 Methods

2.2.1 Synthesis of oxidovanadium complexes

The synthesis of oxidovanadium complexes (1, 2 and 3) was conducted in Dr I Booyens' laboratory. The oxidovanadium complexes, *cis*-[VO₂(Hpybz)(pybz)] (1), (μ -O)[VO(Hpybz)(pybz).VO(Hpybz)(acac)] (2) and [VO(Hpybz)₂SO₄].H₂O (3) were synthesized from the 2:1 molar ratio reactions of the ligand 2-pyridylbenzimidazole (Hpybz) with the respective metal precursors, ammonium vanadate, vanadyl (IV) acetylacetonate and vanadyl (IV) sulphate as reported by Booyens *et al* (2013) (Booyens *et al.*, 2013). The complexes were characterized by the following conductance measurements; UV/Vis, nuclear magnetic resonance (NMR), electron spin resonance (ESR) and infrared resonance (IR) spectroscopy as well as single crystal X-ray diffraction.

2.2.2 Experimental design

The study was divided into 3 series. First series investigated the effects of oxidovanadium complexes (1, 2 and 3) on cell viability in liver, muscle and pancreatic cell lines. Second series investigated the effects of oxidovanadium complexes (1, 2 and 3) on glucose uptake, glycogen synthesis and expressions of GLUT4 and glycogen synthase in liver and muscle cell lines. Lastly, third series investigated the effects of oxidovanadium complexes (1, 2 and 3) on insulin secretion in pancreatic cell line. An outline summarizing the experimental protocols is shown in Figure 1.

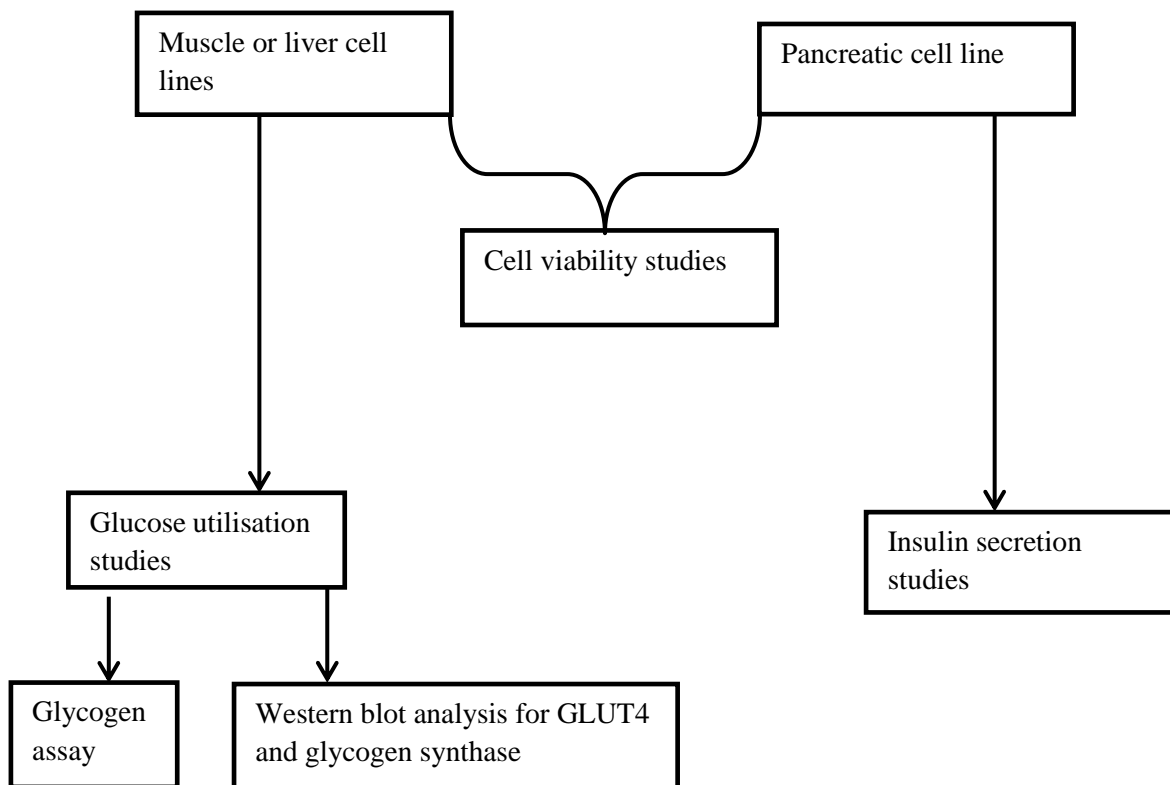


Figure 2: Summarized outline of protocols used in the study. The effects of oxidovanadium on cell viability were investigated in liver, muscle and pancreatic cell lines. Subsequently, the effects of oxidovanadium complexes on glucose utilisation were monitored in liver and muscle cell lines, followed by biochemical parameters analysis. The effects of oxidovanadium complexes on insulin secretion were investigated in pancreatic cell line.

2.2.3 Cell culture protocol

The cell lines used in the study were kindly donated by Dr Christo J.F. Muller from the Diabetes Discovery Platform at the South African Medical Research Council (MRC), Cape Town, South Africa. Cell culture studies were conducted using a well-established protocol (Czifra *et al.*, 2006). Briefly, DMEM, EMEM and RPMI for culturing muscle (C2C12), liver (Chang) and pancreatic (RIN-5) cell lines, respectively, were supplemented with FCS (10%), Pen/strep (1%) and L-glutamine (1%). Frozen muscle, pancreatic and liver cell lines were reconstituted in respective media and transferred into 25cm³ flasks which were incubated at 37 °C in the presence of CO₂ (5%) in a humidified (89%) incubator (Shel Lab, Cornelius, Oregon, USA). The cell lines were allowed to grow, attach and become confluent. The attached confluent cells were trypsinized with trypsin (0.25%) after washing three times with PBS. The cells were then sub-cultured in new flasks and incubated. Thereafter, the cell lines were plated in 24 and 96 well plates for experiments. To prepare doses required for each experiment, oxidovanadium complexes (1, 2 and 3) were freshly prepared in DMSO (0.1%) and subsequently diluted in fully supplemented respective cell culture media. Preliminary studies were undertaken to determine appropriate cell density, doses of drugs and time intervals for the study.

2.2.4 Cell viability studies

Cell viability studies were conducted in liver, muscle and pancreatic cell lines to examine the cytotoxic effects of oxidovanadium complexes. Cell viability was assayed using the Cell Titer-Glo Luminescent Cell Viability Assay Kit (Promega, Madison, Wisconsin, USA). The assay reagent produces luminescence in the presence of ATP from viable cells. The luminescence produced is proportional to the quantity of ATP present. The quantity of ATP is proportional to the amount of viable cells. The cell viability assay was conducted as follows; briefly, cells (10, 000 cells/mL) were seeded in 96-well plates and incubated in their respective media (200 µL) overnight. Thereafter the cells were treated with oxidovanadium complexes (12.5, 25 and 50 µg/mL). Cells which were treated with DMSO (0.1%) , served as controls. Each treatment was conducted in 6 separate wells (n=6). The combined treatment of oxidovanadium complexes (12.5µg/mL) with insulin (4µg/mL) or metformin

(160µg/mL) was also assessed on cell viability in liver and muscle cell lines. After 12, 24 and 48 h, plates were equilibrated at room temperature for 30 min. The assay reagent (100 µL) was added to each well, followed by shaking the plates for 2 min to induce cell lysis. After shaking, plates were incubated at room temperature for 10 min to stabilize the luminescence signal; thereafter the luminescence was read on the Promega microplate luminometer (Promega, Madison, Wisconsin, USA). For background correction, wells with medium only were used as blanks.. Data were expressed as percentage of viable cells (treatment value-blank/ control value-blank×100%). For each time point (12, 24 and 48 h), the cell viability percentage was calculated based on the respective control.

2.2.5 Glucose utilisation studies

The glucose utilisation experiments were conducted as previously described by Ventera *et al* (2008) with slight modifications (Ventera *et al.*, 2008). Separate 80% confluent liver and muscle cell lines (1.5×10^5) in 24 well plates were incubated at 37°C with EMEM and DMEM (1 mL) containing 19 and 29 mmol/L of glucose, respectively, in the presence of various concentrations (12.5, 25 and 50 µg/mL) of oxidovanadium compounds (1, 2 or 3). Cells incubated with DMSO (0.1%) and insulin (4 µg/mL) or metformin (160 µg/mL) acted as untreated and treated positive controls, respectively. Each treatment was conducted in 6 separate wells (n=6).. Media glucose concentrations were measured at 0, 12, 24 and 48 h with OneTouch select glucometer (Lifescan, Mosta, Malta, and United Kingdom).

To examine the effects of combined treatments, a dose of 12.5 µg/mL of oxidovanadium complexes (1, 2 or 3) was combined with either insulin (4 µg/mL) or metformin (160 µg/mL). This dose was the least effective in glucose utilisation and as a result this allowed observing whether combination with either insulin/metformin has positive additional effects on glucose utilisation. Media glucose concentrations were monitored as described above. After 48 h period, cells were trypsinised and harvested for measurements of glycogen and expressions of GLUT4 and glycogen synthase.

2.2.6 Glycogen assay

Glycogen analysis was performed in liver and muscle cells harvested at 48 h. Glycogen assay was performed using a well-established laboratory protocol (Musabayane *et al.*, 2005; Ngubane *et al.*, 2011; Khathi *et al.*, 2013). The harvested muscle and liver cells were heated with KOH (30%, 2 mL) at 100°C for 30 min. Thereafter, NaSO₄ (10%, 0.194 mL) was added to stop the reaction and allowed to cool. For glycogen precipitation, the cooled mixture (200 µL) was aspirated and mixed with ethanol (95%, 200 µL). The precipitated glycogen was pelleted, washed and resolubilized in H₂O (1 mL). Thereafter, anthrone (0.5g dissolved in 250ml of sulphuric acid, 4 mL) was added and boiled for 10 min. After cooling, the absorbance was read using the Spectrostar Nano spectrophotometer (BMG Labtech, Ortenburg, Baden-Württemberg, Germany) at 620 nm. The glycogen concentrations were calculated from the glycogen standard curve. The standards ranged from 200 to 1000 mg/L

2.2.7 Insulin secretion studies

Separate confluent pancreatic cells (1.5×10^5) in 24 well plates were incubated at 37°C with RPMI (1 mL) containing 18 mmol/L of glucose, in the presence of oxidovanadium compounds (1, 2 or 3) (12 µg/mL). Each treatment was conducted in 6 separate wells (n=6). Cells incubated with DMSO (0.1%) untreated served as negative control, glucose concentrations were monitored 0, 12, 24 and 48 h. The media samples were harvested at 12, 24 and 48 h for insulin measurements.

Media samples collected at 48 h period were assayed for insulin concentrations. Insulin was measured using ultra-sensitive rat insulin ELISA kit (DRG International, USA). The kit contained a 96 well plate coated with mouse monoclonal anti-insulin, calibrator, enzyme conjugate, enzyme conjugate buffer, wash buffer, substrate 3,3',5,5'-tetramethylbenzidine (TMB) and a stop solution. The principle of the assay is a solid phase two-site enzyme immunoassay based on the direct sandwich technique in which two monoclonal antibodies are directed towards separate antigenic determinants on the insulin molecule. During the incubation period insulin in the sample reacts with peroxidase-conjugated anti-insulin antibodies and anti-insulin antibodies bound to the microtitration well. The washing step removes unbound enzyme labelled antibody, leaving the bound conjugate which reacts with

TMB. This reaction is stopped by adding acid to give a colorimetric endpoint that is read spectrophotometrically.

Each determination was conducted in duplicate for both calibrators and samples. The insulin assay was performed as follows; calibrators (0, 3.40, 8.50, 25.50, 68.10 and 170.27 pmol/L) or samples (10 μ L) were added in each well of the plate coated with anti-insulin antibody. Thereafter, an enzyme conjugate solution (100 μ L) was added in each well, followed by incubation of the plate at room temperature on an orbital shaker for 2 hours. After incubation, the reaction volume was discarded followed by washing (5 times) using a wash buffer solution (350 μ L) per well. After washing, a substrate TMB (200 μ L) was added in each well and incubated for 15 min at room temperature. Thereafter, a stop solution (50 μ L) was added in each well, followed by shaking for 5 seconds. The absorbance was read using a Spectrostar Nano spectrophotometer (BMG Labtech, Ortenburg, Baden-Württemberg, Germany) at 450 nm. Sample insulin concentrations were extrapolated from the insulin standard curve. The lower and upper limits of detection were 1.39 and 960 pmol/L, respectively. The intra-assay analytical coefficient of variation ranged from 4.4 to 5.5% and the inter-assay coefficient variation from 4.7 to 8.9%.

2.2.8 Western blot analysis

The muscle and liver cells harvested at 48 h were examined for expression of GLUT4 and glycogen synthase, respectively. Cells (1×10^5 cells/mL) were homogenized using a glass rod on ice in isolation buffer (0.5 mM Na₂EDTA, 0.1 M KH₂PO₄, 0.1 mM dithiothreitol, 0.25 M sucrose) and then centrifuged at 400 x g for 10 min (4 °C). The supernatant was harvested and the protein content was quantified using the bicinchoninic acid assay (BCA) (Sigma-Aldrich, St Louis, Missouri, USA). Briefly, the standards or samples (250 μ L) were mixed with BCA working solution (200 μ l) in a 96 well plate. Thereafter, the plate was incubated at 37 C° for 30 minutes, after cooling the absorbance was read at 562 nm. The protein concentrations were extrapolated from a protein standard curve (0.2-1 mg/mL). For Western blot analysis, all protein samples were standardized to (1 mg/mL). The proteins were then denatured by boiling in laemmli sample buffer (0.5 M Tris-HCl, glycerol, 10% SDS, 2 mercaptoethanol, 1% bromophenol blue) for 5 min. The denatured proteins were loaded (20 μ L) on prepared resolving (10%) and stacking (4%) polyacrylamide gels along with

molecular weight marker (5 μ L). The gels were electrophoresed for 1 h at 150 V in electrode running buffer (Tris base, glycine, SDS), pH 8.3). After electrophoresis, the resolved proteins were electro-transferred to an equilibrated polyvinylidene difluoride (PVDF) membrane for 1 h in transfer buffer (192 mM glycine, 25 mM Tris, 10% methanol). Following transfer, the membranes were blocked with 5% non-fat dry milk in Tris-buffered saline with 0.1% Tween 20 (TTBS) (20 mM Tris, 150 mM NaCl, KCl, 0.05% Tween-20). The membranes were then immuno-probed with antibodies- GLUT 4 and glycogen synthase (1:1000 in 1% TTBS) for 1 h at room temperature. The PVDF membrane was then subjected to 5 washes (10 min each with gentle agitation) with TTBS. The membranes were then incubated in HRP-conjugated secondary antibody (rabbit anti-mouse 1:1000 in TTBS) for 1 h at room temperature. After washing, antigen-antibody complexes were detected by chemiluminescence using the Immune-star HRP substrate kit (Bio-Rad, Johannesburg, South Africa). Chemiluminescent signals were detected with the Chemi-doc XRS gel documentation system and analysed using the quantity one software (Bio-Rad, Johannesburg, South Africa). Band intensity analysis was conducted on the resultant bands. β -actin was used as reference protein and densitometry was expressed as band intensity of protein of interest over β - actin.

2.2.9 Statistical analysis

All data are expressed as means \pm standard error of means (SEM). Statistical analysis was performed using GraphPad Prism InStat Software (version 5.00, GraphPad Software, San Diego, California, USA). One-way analysis of variance (ANOVA) followed by Tukey-Kramer was used for analysis of differences between control and experimental groups. Values of $p < 0.05$ indicate statistical significance between the compared groups.

CHAPTER 3

RESULTS

3.1 Synthesis of oxidovanadium complexes

The complexes *cis*-[VO₂(Hpybz)(pybz)] (1), (μ -O)[VO(Hpybz)(pybz).VO(Hpybz)(acac)] (2) and [VO(Hpybz)₂SO₄].H₂O (3) were successfully synthesized and verified using the UV/Viz, NMR, IR and ESR spectroscopy and crystal X-ray diffraction. Complexes 1, 2 and 3 were yellow cubic crystals, green thin needles and green cubic crystals, respectively. Furthermore, all complexes exhibited good solubility in high boiling points solvents and less solubility in alcohol and chlorinated solvents.

3.2 Cell viability

3.2.1 The effects of oxidovanadium complexes

Figures 2 and 3 show the effects of oxidovanadium complexes on cell viability in liver cell and muscle cells. The administration of lower doses (12.5 and 25 μ g/mL) of oxidovanadium complex (1, 2 and 3) showed no toxic effects in comparison to the control at corresponding time intervals. However, the administration of high dose (50 μ g/mL) showed a decrease in cell viability in comparison to the control at 24 and 48 h incubation period in both liver and muscle cells (Figures 2 and 3). Figure 4 shows the percentage cell viability in pancreatic cells. There were no toxic effects in cells treated with low dose of oxidovanadium complexes (1, 2 and 3) in comparison to the control at corresponding time interval. However, there was a statistical significant decrease ($p < 0.05$) on cell viability in cells treated with high doses of oxidovanadium complexes (1, 2 and 3) (Figure 4).

3.2.2 The effects of oxidovanadium complexes combined with insulin

Figures 5 and 6 show the effects of combined oxidovanadium (1, 2 and 3) and insulin administration on cell viability in liver and muscle cells. The combined treatment showed no toxic effects in both liver and muscle cells in comparison to the control. Furthermore, non-

significant differences were observed between groups treated with combined treatment and oxidovanadium complexes or insulin alone after 12, 24 and 48 h (Figures 5 and 6)

3.2.3 The effects of oxidovanadium complexes combined with metformin

Figures 7 and 8 show the effect of administration of oxidovanadium (1, 2 and 3) combined with metformin on cell viability in liver and muscle cells. The combined treatment had no effects on cell viability in both liver and muscle cells in comparison to the control. Furthermore, non-significant differences were also observed between groups of combined treatment and oxidovanadium complexes or metformin after 12, 24 and 48 h (Figures 7 and 8).

Liver

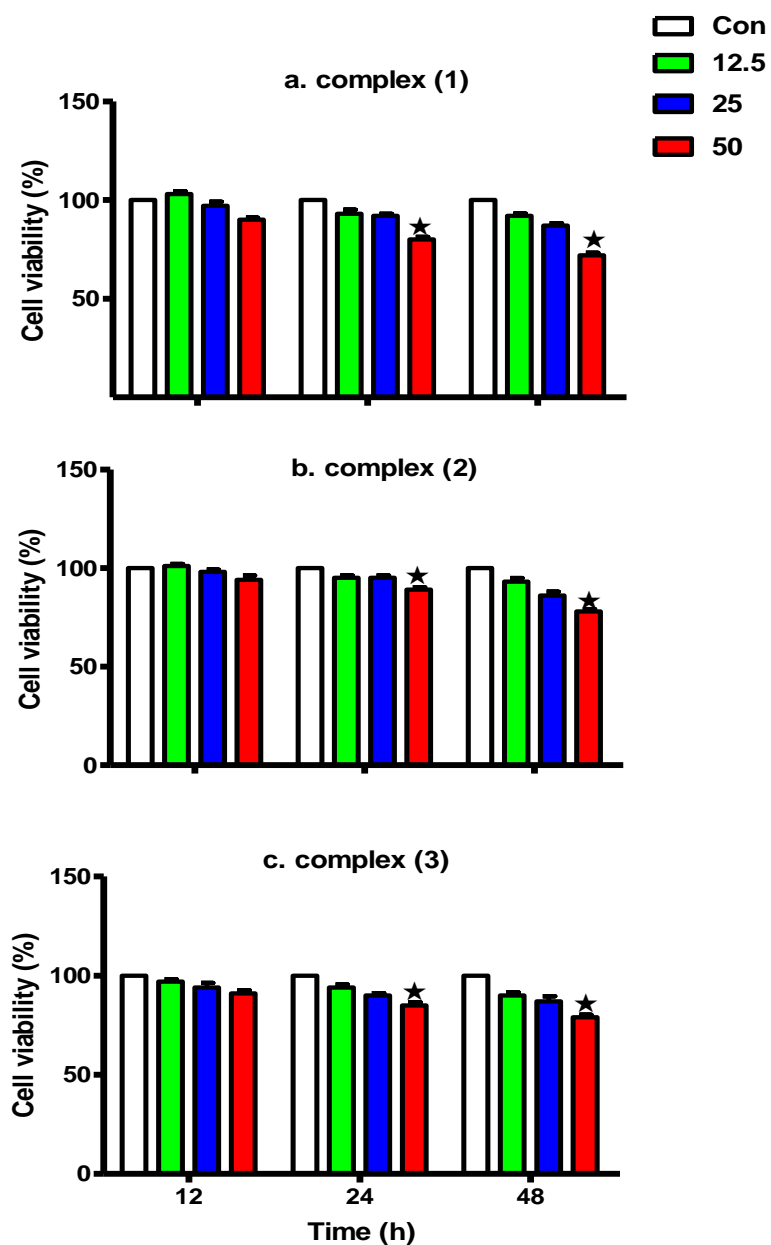


Figure 3: The effects of oxidovanadium complexes on cell viability in liver cells after 12, 24 and 48 h. Values are presented as means, and vertical bars indicate SEM (n=6 in each group). ★=p< 0.05 by comparison with control groups at each corresponding time.

Muscle

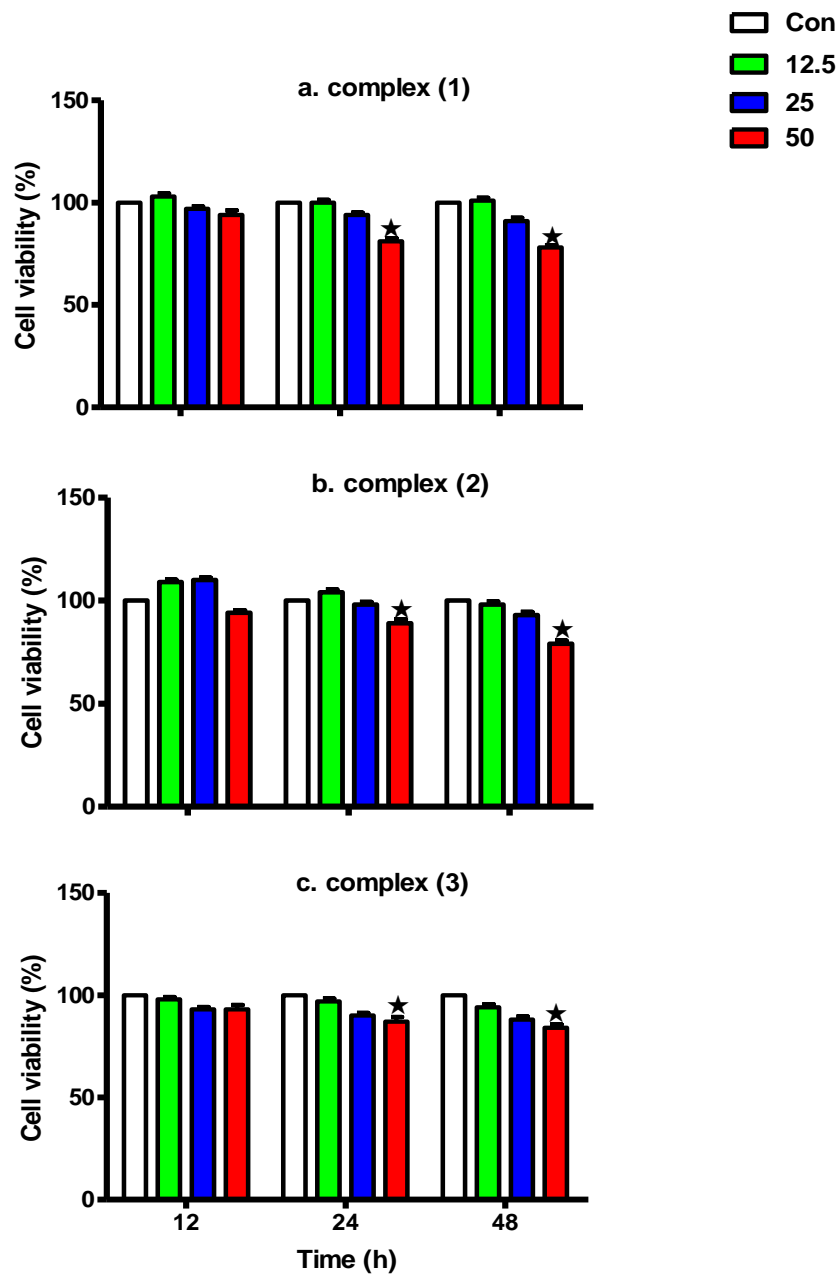


Figure 4: The effects of oxidovanadium complexes on cell viability in muscle cells after 12, 24 and 48 h. Values are presented as means, and vertical bars indicate SEM (n=6 in each group). ★=p<0.05 by comparison with control groups at each corresponding time.

Pancreatic cells

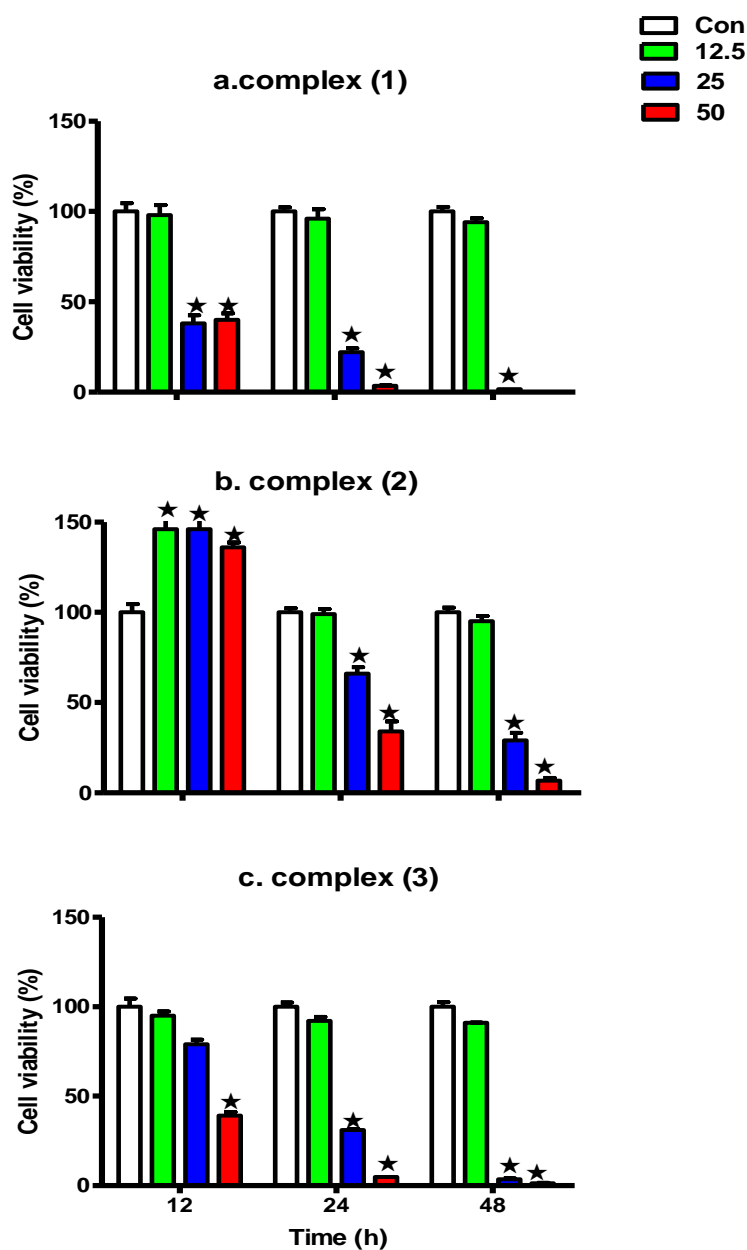


Figure 5: The effects of oxidovanadium complexes on cell viability in pancreatic cells after 12, 24 and 48 h. Values are presented as means, and vertical bars indicate SEM (n=6 in each group). ★=p<0.05 by comparison with control groups at each corresponding time.

Liver

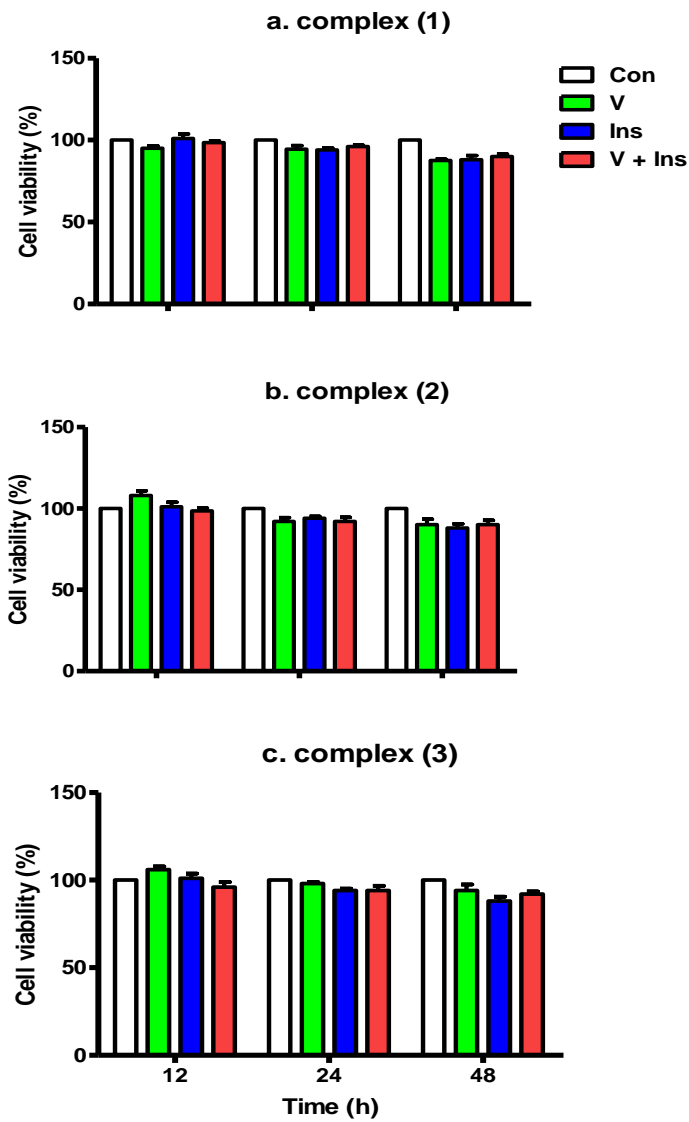


Figure 6: The effects of oxidovanadium complexes combined with insulin (V+Ins) on cell viability in liver cells after 12, 24 and 48 h. Values are presented as means, and vertical bars indicate SEM (n=6 in each group). ★ =p<0.05 by comparison with control groups at each corresponding time.

Muscle

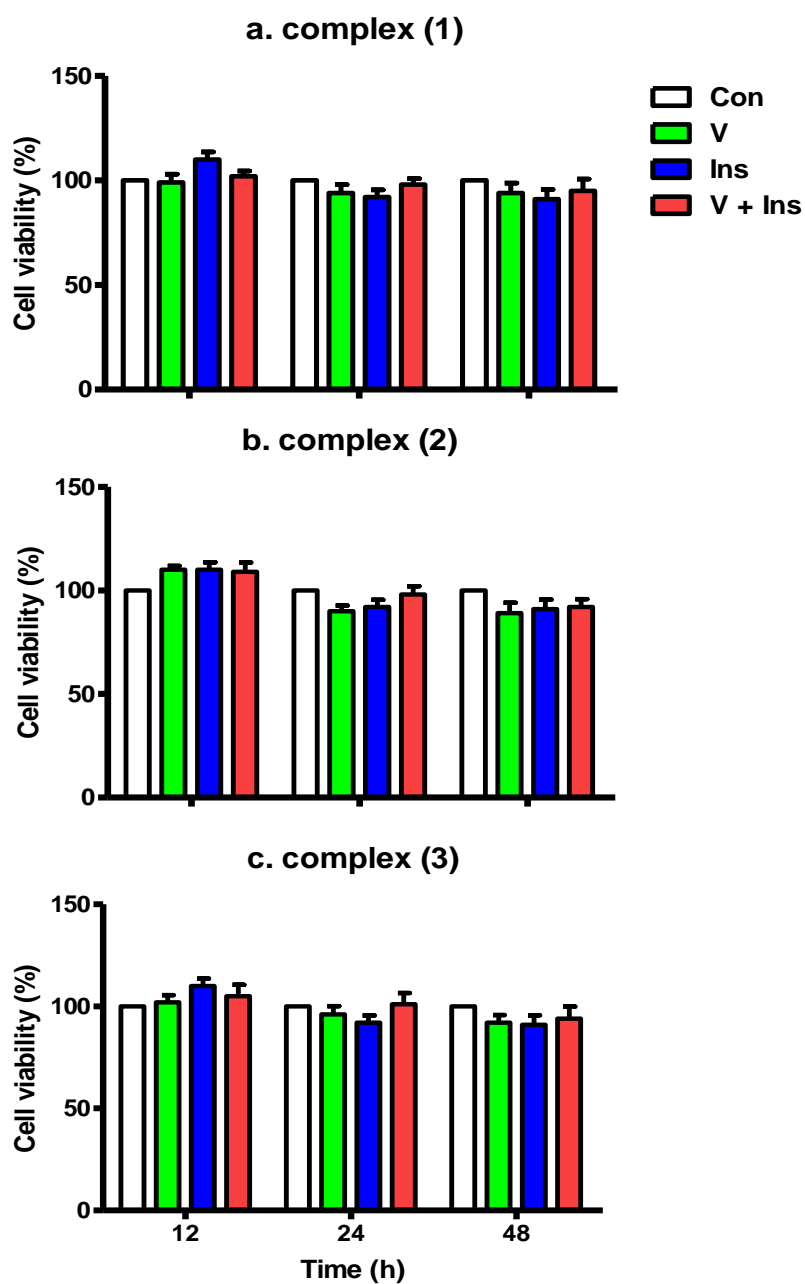


Figure 7: The effects of oxidovanadium complexes combined with insulin (V+Ins) on cell viability in muscle cells after 12, 24 and 48 h. Values are presented as means, and vertical bars indicate SEM (n=6 in each group). ★=p<0.05 by comparison with control groups at each corresponding time.

Liver

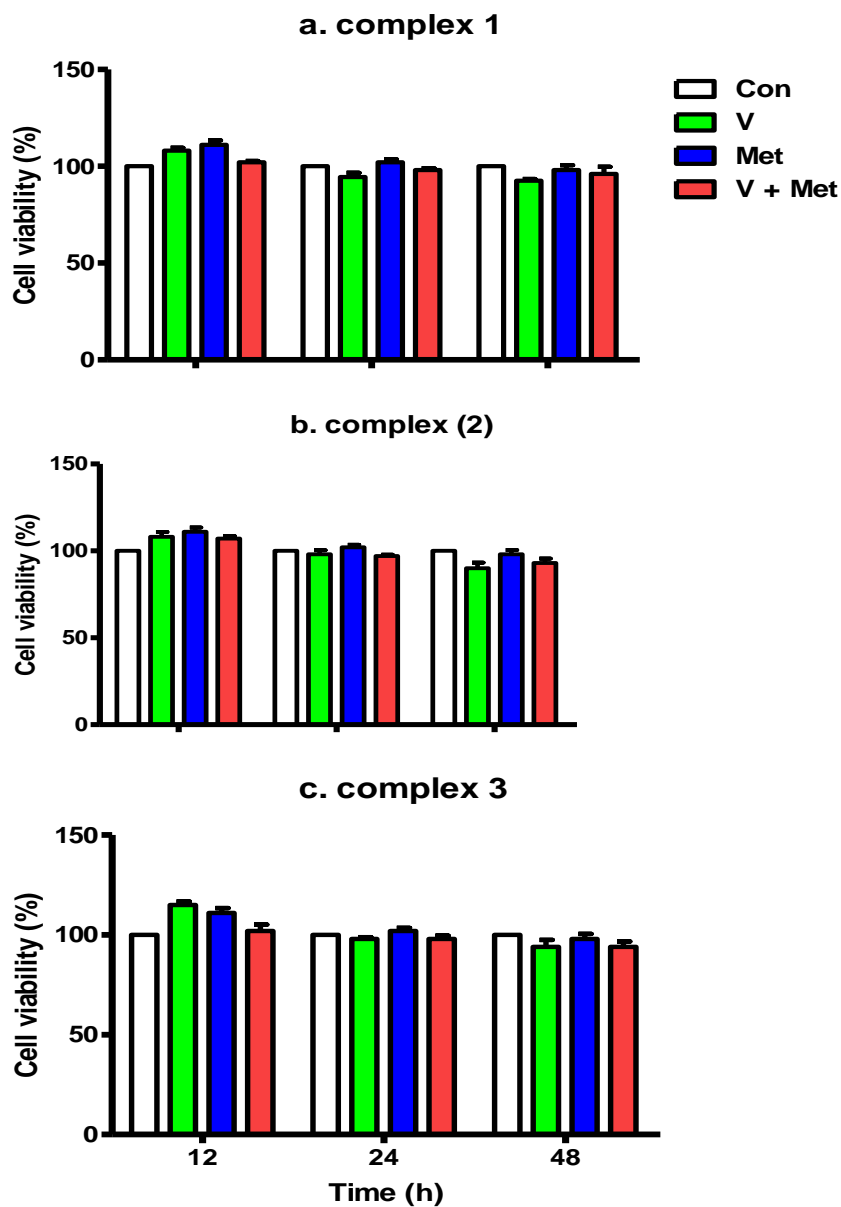


Figure 8: The effects of oxidovanadium complexes combined with metformin (V+Met) on cell viability in liver cells after 12, 24 and 48 h. Values are presented as means, and vertical bars indicate SEM (n=6 in each group). ★=p<0.05 by comparison with control groups at each corresponding time.

Muscle

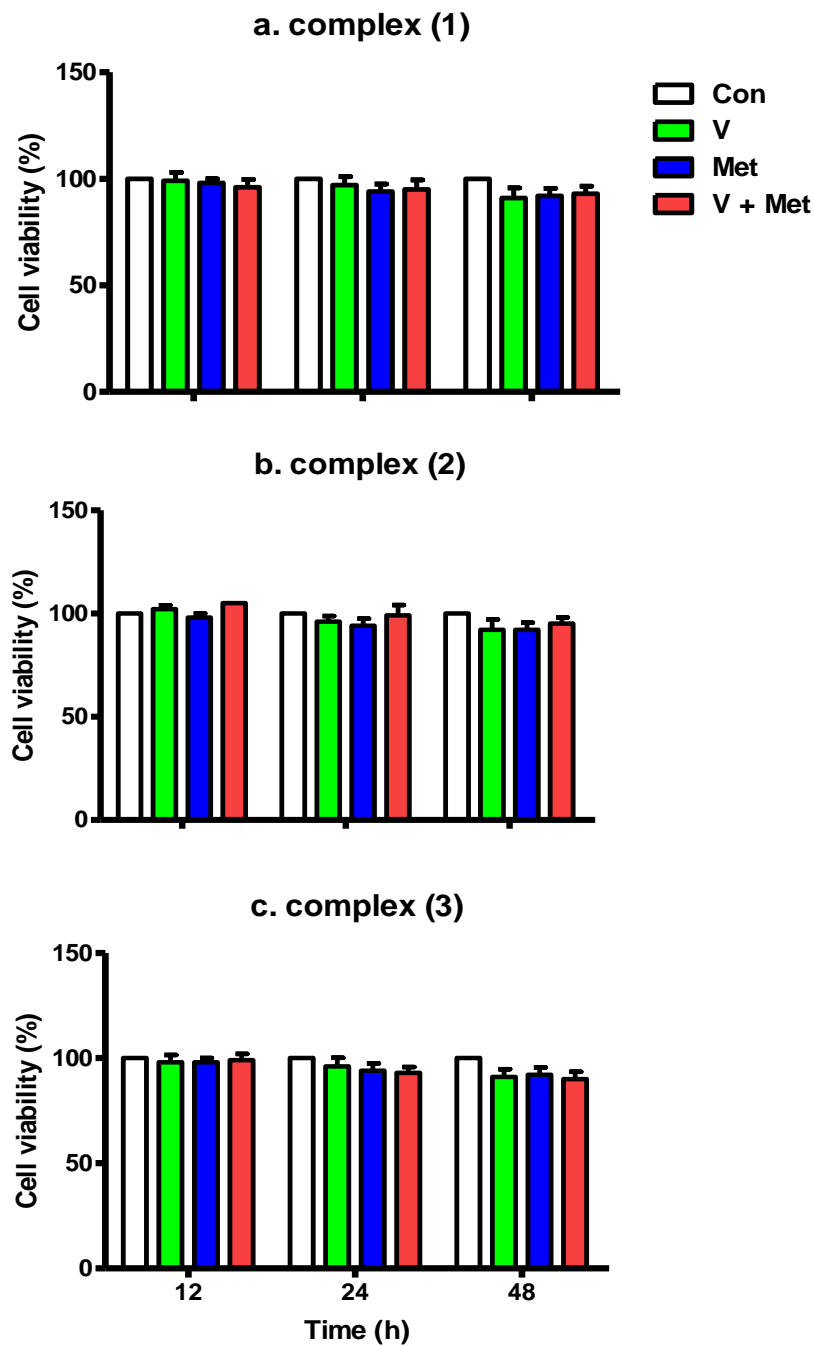


Figure 9: The effects of oxidovanadium complexes combined with metformin (V+Met) on cell viability in muscle cells after 12, 24 and 48 h. Values are presented as means, and vertical bars indicate SEM (n=6 in each group). ★=p<0.05 by comparison with control groups at each corresponding time.

3.3 Glucose utilisation

3.3.1 Effects of oxidovanadium complexes

Figures 9 and 10 show the effects of oxidovanadium complexes (1, 2 and 3) on glucose utilisation after 0, 12, 24 and 48 h in liver and muscle cells, respectively. The control groups for both muscle and liver cells showed a steady decline in glucose concentrations over 48 h experimental period. By comparison with the respective controls at corresponding time periods, the administration of the vanadium complexes (1, 2 and 3) significantly decreased ($p < 0.05$) media glucose concentrations with the lowest dose being the least effective in both liver and muscle cells after 12, 24 and 48 h incubation. Complex (2) showed to be the most effective oxidovanadium complex in reducing media glucose concentrations in both liver and muscle cells. The effects of oxidovanadium complexes (50 $\mu\text{g/mL}$) on glucose utilisation were comparable to insulin and more potent than metformin in both liver and muscle treated cells at all time points (Figures 9 and 10).

3.3.2 Effects of oxidovanadium complexes combined with insulin.

Figures 11 and 12 show the effects oxidovanadium complexes (1, 2 and 3) combined with insulin on glucose utilisation in both liver and muscle cells. By comparison with the respective controls at corresponding time periods, the administration of each combined treatment showed a statistical significant decline ($p < 0.05$) in media glucose concentrations after 24 and 48 h in both muscle and liver cells (Figure 11 and 12). Furthermore, combined treatment of oxidovanadium complex (1) with insulin showed an added effects since there was significantly reduced ($p < 0.05$) media glucose in comparison to oxidovanadium complex (1) or insulin alone, in muscle cells (Figure 12a).

3.3.3 The effects of oxidovanadium complexes combined with metformin

Figures 13 and 14 show the effects of oxidovanadium complexes (1, 2 and 3) combined with metformin on glucose utilisation in liver and muscle cells. By comparison with the respective controls at corresponding time intervals, the administration of each combined treatment showed a statistical significant decline ($p < 0.05$) in media glucose concentrations after 24 and 48 h in the muscle and liver cells. However, the administration of combined treatment

showed no added effects in glucose utilisation in both liver and muscle cells (Figures 12 and 13).

Liver

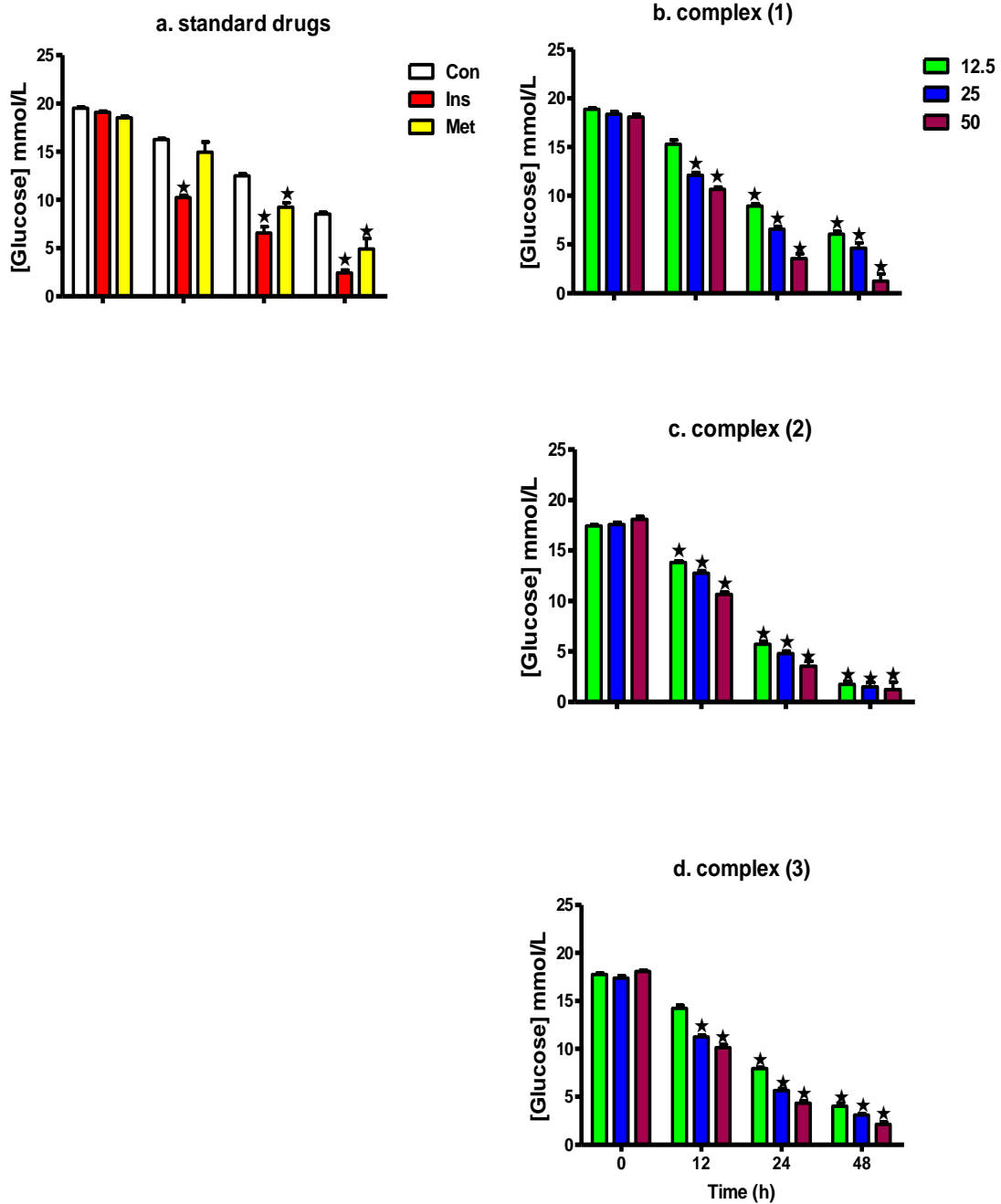


Figure 10: The effects of oxidovanadium complexes on glucose utilisation in liver cells after 0, 12, 24 and 48 h. Values are presented as means, and vertical bars indicate SEM (n=6 in each group). ★=p<0.05 by comparison with control groups at each corresponding time.

Muscle

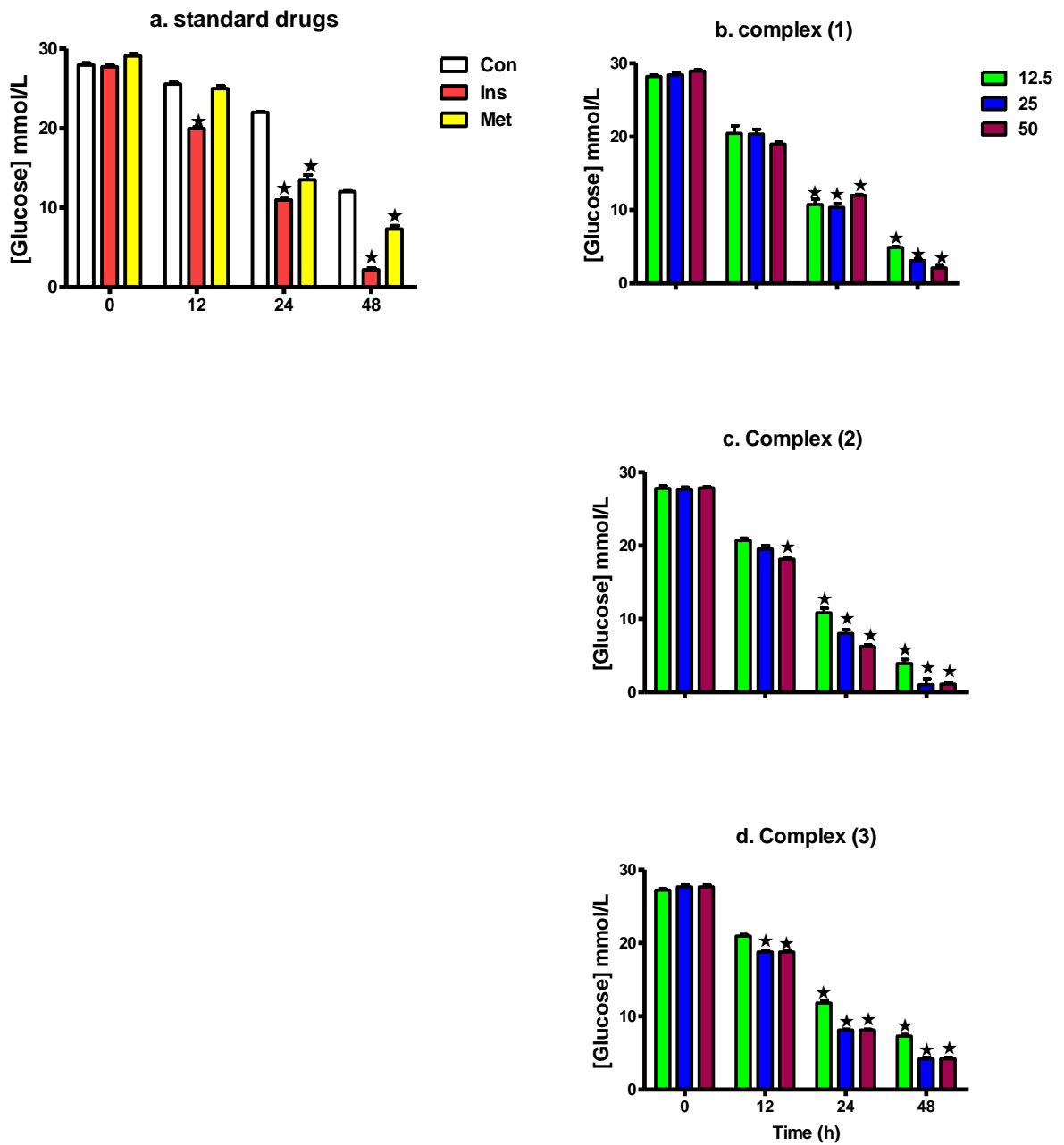


Figure 11: The effects of oxidovanadium complexes on glucose utilisation in muscle cells after 0, 12, 24 and 48 h. Values are presented as means, and vertical bars indicate SEM (n=6 in each group). ★=p<0.05 by comparison with control groups at each corresponding time.

Liver

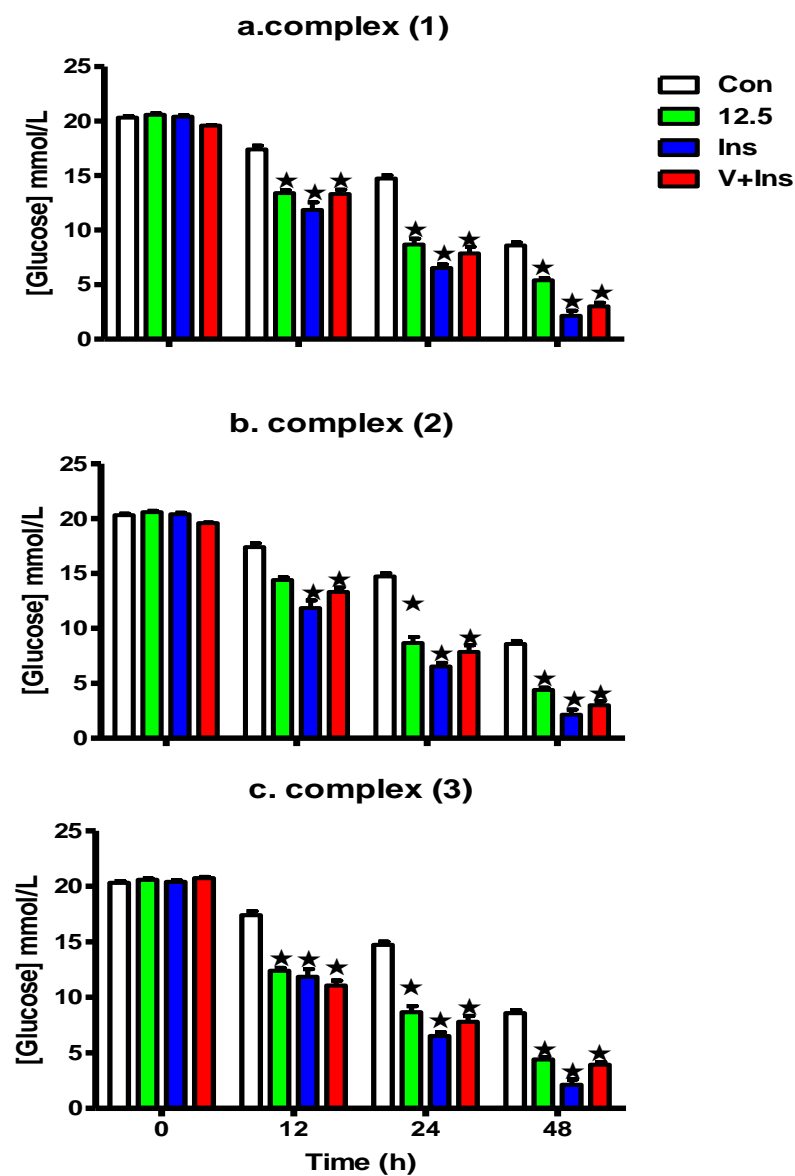


Figure 12: The effects of oxidovanadium complexes combined with insulin (V+Ins) on glucose utilisation in liver cells after 0, 12, 24 and 48 h. Values are presented as means and vertical bars indicate SEM (n=6 in each group). ★ =p<0.05 in comparison to the control group at corresponding time.

Muscle

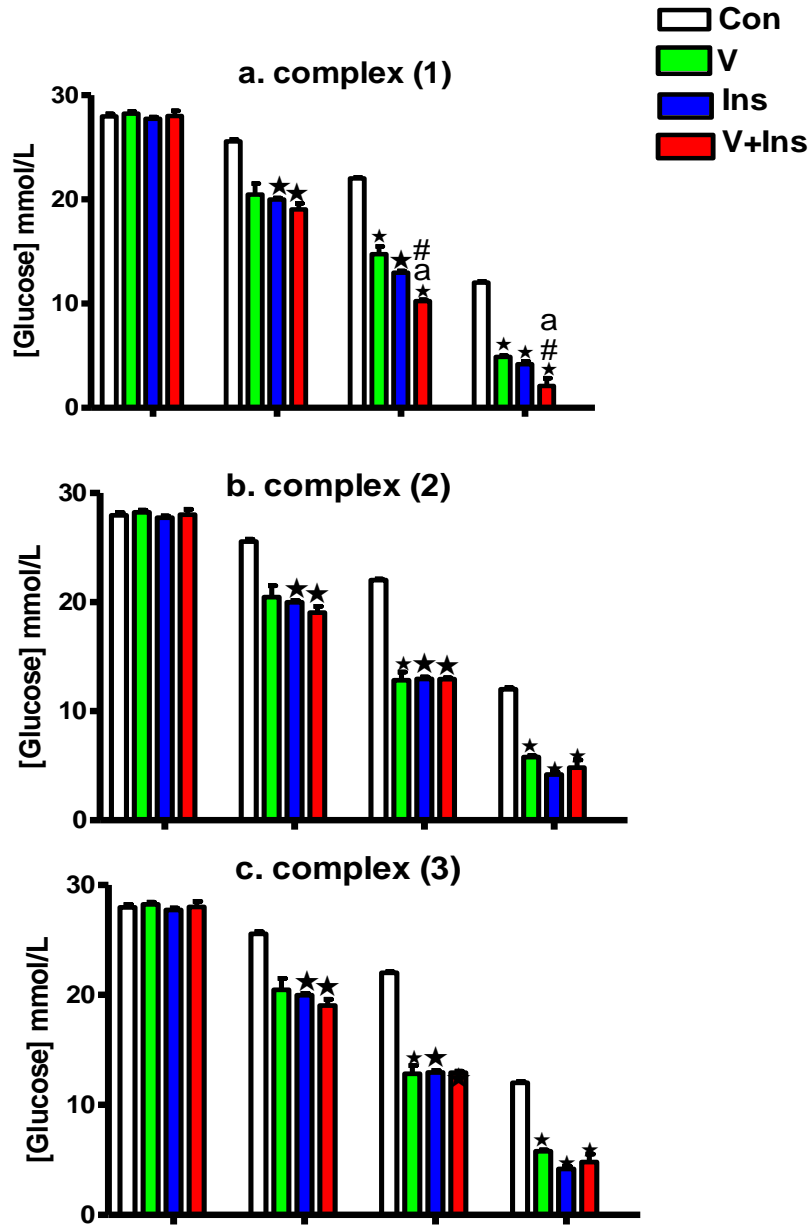


Figure 13: The effects of oxidovanadium complexes combined with insulin (V+Ins) on glucose utilisation in muscle cells after 0, 12, 24 and 48 h. Values are presented as means and vertical bars indicate SEM (n=6 in each group). ★=p<0.05 in comparison to the control group, #=p<0.05 in comparison to insulin treated group and a=p<0.05 in comparison to oxidovanadium complex (1) treated group in at each corresponding time.

Liver

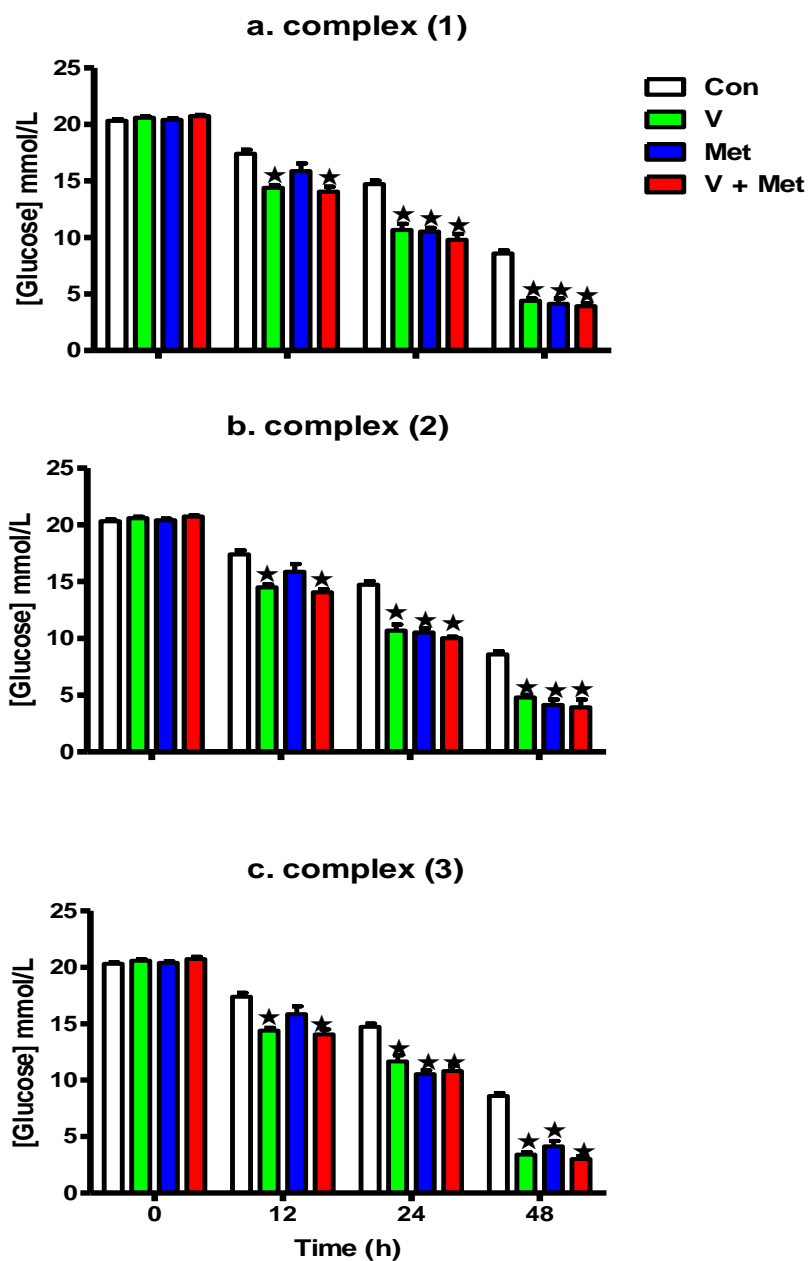


Figure 14: The effects of oxidovanadium complexes combined with metformin (V+Met) on glucose utilisation in liver cells after 0, 12, 24 and 48 h. Values are presented as means, and vertical bars indicate SEM (n=6 in each group). ★ =p<0.05 in comparison to the control group at corresponding time.

Muscle

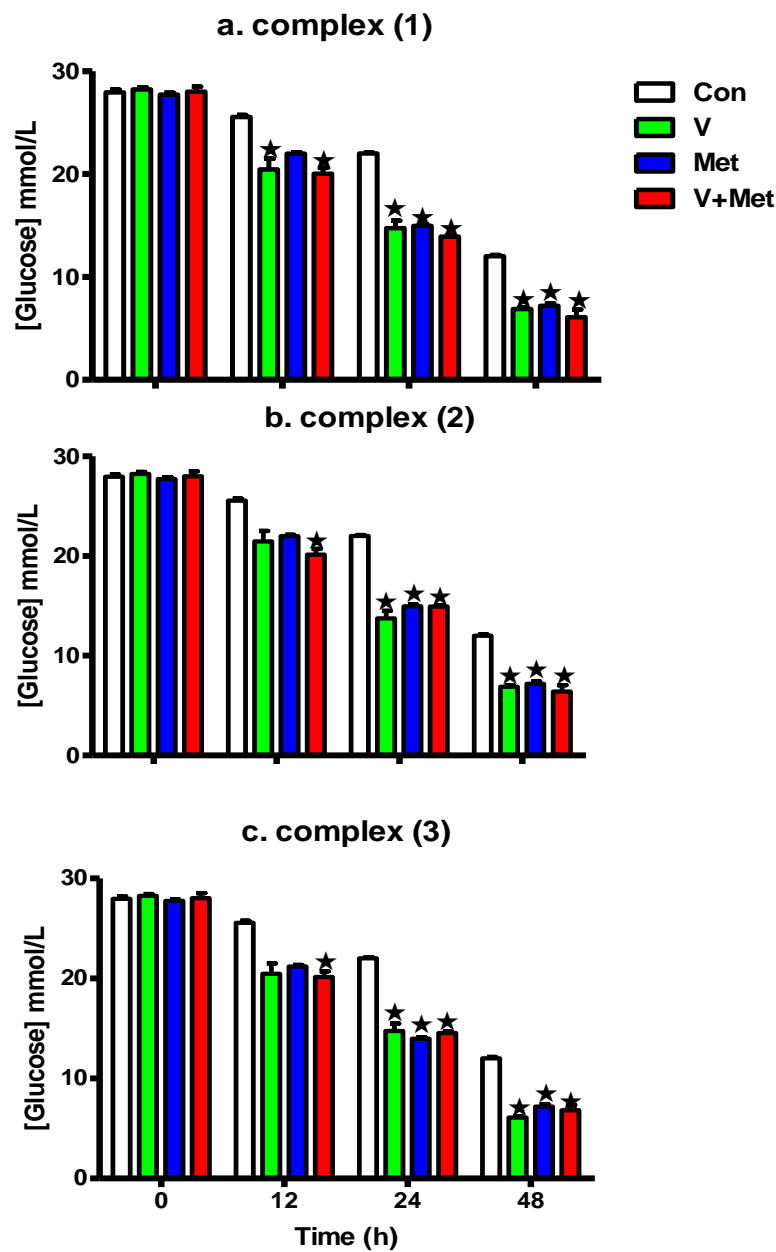


Figure 15: The effects of oxidovanadium complexes combined with metformin (V+Met) on glucose utilisation in muscle cells after 0, 12, 24 and 24 h. Values are presented as means, and vertical bars indicate SEM (n=6 in each group). ★ = p < 0.05 in comparison to the control.

3.4 The effects of oxidovanadium complexes on glycogen synthesis

Table 1 shows the effects of oxidovanadium complexes (50 µg/mL) (1, 2 and 3) and combined treatment administration on glycogen synthesis in both liver and muscle cells after 48 h. The administration of oxidovanadium complexes (1, 2 and 3) significantly increased ($p < 0.05$) glycogen concentrations in both liver and muscle cells in comparison to the control. Interestingly, the effects of oxidovanadium complex (2) were comparable to insulin treated groups in both liver and muscle cells. Furthermore, the administration of each combined treatment significantly increased ($p < 0.05$) glycogen concentrations in both liver and muscle cells in comparison to the control. Administration of oxidovanadium complex (1) combined with insulin showed a statistically significant increase ($p < 0.05$) in glycogen synthesis in comparison to either insulin or oxidovanadium complex (1) treated muscle cells (Table 1).

Table 1: The effects of oxidovanadium complexes and combined treatment on glycogen synthesis in liver and muscle cells after 48 h.

Groups	Muscle		Liver	
	Glucose (mmol/L)	Glycogen (mmol/cells)	Glucose (mmol/L)	Glycogen (mmol/cells)
Control	12.01±0.13	0.76±0.01	8.56±0.16*	0.64±0.07
Complex (1)	2.10±0.23*	1.40±0.02*	2.24±0.72*	0.86±0.02*
Complex (2)	1.06±0.25*	1.30±0.02*	1.25±0.24*	0.80±0.02*
Complex (3)	4.20±0.21*	1.20±0.02*	2.12±0.24*	1.00±0.01*
Insulin	2.20±0.23*	1.78±0.02*	2.42±0.30*	1.00±0.00*
Metformin	6.80±0.43*	1.17±0.00*	4.90±1.09*	0.92±0.03*
Complex (1)+Insulin	1.04±0.13*#a	1.91±0.02*#a	3.02±0.30*	0.90±0.04*
Complex (2)+Insulin	3.20±0.31*	1.30±0.05*	3.02±0.28*	0.86±0.01*
Complex (3)+ Insulin	3.50±0.21*	1.20±0.02*	3.18±0.52*	0.84±0.01*
Complex (1)+Metformin	6.10±0.23*	1.20±0.03*	3.90±0.29*	0.81±0.06*
Complex (2)+Metformin	6.14±0.43*	1.10±0.04*	3.50±0.33*	0.84±0.01*
Complex (3)+Metformin	6.50±0.42*	1.20±0.06*	3.40±0.22*	0.85±0.05*

*= p<0.05 in comparison to the control

#=p<0.05 in comparison to oxidovanadium complex (1)

a=p<0.05 in comparison to insulin

3.5 Effects of oxidovanadium complexes on insulin secretion

Table 2 shows the effects of oxidovanadium complexes (1, 2 and 3) (12.5 µg/mL) on insulin secretion in pancreatic cells after 12, 24 and 48 h. The administration of oxidovanadium complexes (1 and 3) had no effects on media glucose concentrations and insulin secretion in comparison to the control after 12, 24 and 48 h. However, administration of oxidovanadium complex (2) significantly decreased ($p < 0.05$) media glucose concentrations with concomitant increase in insulin secretion after 24 and 48 h (Table 2).

Table 2: The effects of oxidovanadium complexes on insulin secretion in pancreatic cells after 12, 24 and 48 h.

Groups	Glucose (mmol/L)	Insulin (pmol/L)
12 h		
Control	15.0±0.6	101.0±5.2
Complex (1)	14.5±0.5	105.0±4.3
Complex (2)	13.6±0.6	110.0±3.2*
Complex (3)	14.5±1.0	98.0±4.1
24 h		
Control	13.2±0.8	131.0±6.1
Complex (1)	13.9±0.2	129.0±3.9
Complex (2)	10.2±0.7*	143.0±3.2*
Complex (3)	13.1±0.5	138.0±4.5
48 h		
Control	11.5±0.5	191.0±2.4
Complex (1)	11.5±0.2	191.0±2.4
Complex (2)	7.8±1.00*	255.0±2.1*
Complex (3)	11.5±0.3	191.0±3.1

*=p<0.05 in comparison to the control

3.6 Western blots analysis

The effects of highest dose (50 µg/mL) of oxidovanadium complex (2) were examined on glycogen synthase and GLUT4 in liver and muscle cells, respectively after 48 h.

3.6.1 Glycogen synthase

Figure 15 shows the effects of oxidovanadium complex (2) on glycogen synthase (GS) expression in liver cells. Administration of oxidovanadium complex (2) significantly increased ($p < 0.05$) GS expression as indicated by increased densitometry in comparison to the control group (figure 15).

3.6.2 GLUT4

Figure 16 shows the effects of oxidovanadium complex (2) on GLUT4 expression in muscle cells. Administration of oxidovanadium complex (2) significantly increased ($p < 0.05$) the expression of GLUT4 as indicated by increased densitometry in comparison to the control group (figure 16).

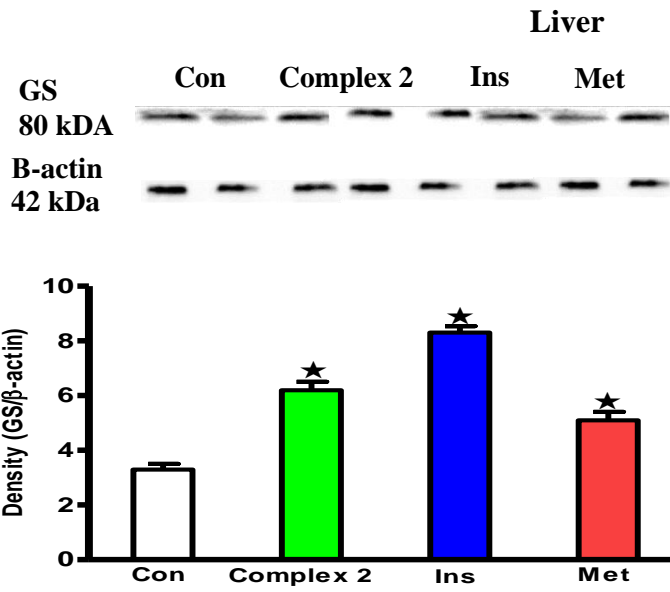


Figure 16: The effects of oxidovanadium complex (2) on GS expression in liver cells. Density of GS is expressed over β -actin. Values are presented as means, and vertical bars indicate SEM (n=3 in each group). ★=p<0.05 in comparison to the control.

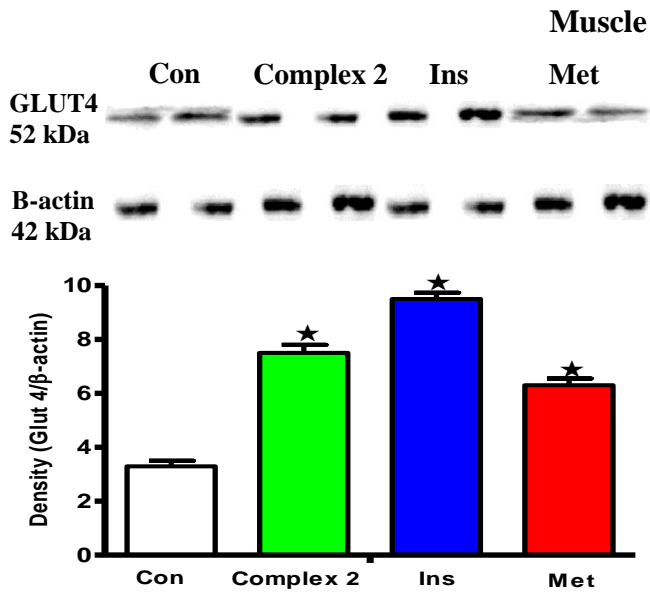


Figure 17: The effects of oxidovanadium complex (2) on GLUT4 expression in muscle cells. Density of GLUT4 is expressed over β -actin. Values are presented as means, and vertical bars indicate SEM (n=3 in each group). ★=p<0.05 in comparison to the control.

CHAPTER 4

DISCUSSION

The aim of the study was to synthesise and characterise the novel oxidovanadium complexes, and to investigate the effects of novel oxidovanadium complexes on glucose utilisation in liver and skeletal muscle cells and insulin secretion in pancreatic cells. The oxidovanadium complexes were synthesized and further verified using physical and chemical characteristics. The observations in cell viability studies suggested that lower doses of oxidovanadium had no cytotoxic effects in both liver and muscle cells. However, for the pancreatic cells, only lowest dose had no cytotoxic effects. Oxidovanadium complexes increased glucose uptake in both liver and muscle cell lines mediated perhaps via enhanced glycogen synthesis. The observations of the current study therefore extend literature reports where various vanadium complexes have been shown to possess anti-hyperglycaemic properties (Cohen *et al.*, 2001; Willsky *et al.*, 2011; Shehzad, 2013). Of the complexes studied only oxidovanadium complex (2) increased insulin secretion in pancreatic cell line suggesting that further studies are required on the effects of oxidovanadium complexes on insulin secretion. The ability of oxidovanadium complex (2) to promote both glucose disposal and insulin secretion suggests that this compound may ensure tight glycaemia *in vivo*. These observations may also be of clinical relevance considering the morbidity together with the burden exerted by diabetes in health systems and facilities.

Cell viability studies were undertaken to determine whether oxidovanadium complexes possess any cytotoxic effects. The effects of oxidovanadium complexes were investigated on cell viability in liver, muscle and pancreatic cell lines. There were no apparent cytotoxic effects after lower doses of oxidovanadium complexes administration in both liver and muscle cell lines. However, the dose of 50µg/mL administration showed a decline in cell viability, indicating cytotoxic effects, which may be due to oxidative stress elicited by oxidovanadium complexes. The increase in cell viability seen at 12 h may be due to proliferative effects of oxidovanadium complexes; however, we may not exclude other possibilities. Furthermore, there were no cytotoxic effects observed when the lowest dose of oxidovanadium was combined with either insulin or metformin. However, further studies are needed to determine whether insulin or metformin may correct the negative effects elicited by high doses in both liver and muscle cells. Administration of high doses of oxidovanadium

complexes, however, had cytotoxic effects to the pancreatic cell line as evidenced by a sharp decline in cell viability. Some vanadium complexes such vanadyl sulphates, sodium orthovanadate and vanadyl organic complexes have been reported to promote free radicals formation in biological systems (Zhao *et al.*, 2009). On the other hand, pancreas islets have been reported to have little amounts of antioxidants to neutralize free radicals formation (Kubisch *et al.*, 1997). Therefore, the reduced cell viability in pancreatic cells after oxidovanadium complexes administration may be attributed to oxidative stress. Oxidative stress has been shown to promote lipid peroxidation and DNA damage which is known to promote cell death mechanisms such as apoptosis (James *et al.*, 2002). However, we may not exclude other mechanisms and thus the mechanisms through which complexes exert toxicity in pancreatic cells remain unclear. Due to significant toxicity elicited by higher doses in pancreatic cells, only lowest dose was used to study the effects of oxidovanadium complexes on insulin secretion.

Anti-hyperglycaemic effects of various vanadium complexes have been investigated using chemically induced-diabetic models. However, biological tests in whole animal have been found to be expensive, time consuming and have a limited sensitivity to differentiate between structurally similar compounds. In this study, the yield of oxidovanadium complexes was very low and thus their effects on glucose utilisation and insulin secretion were only limited to *in vitro* studies. Muscle and liver tissues play a significant role in glucose homeostasis. Furthermore, glucose metabolism in these tissues is also controlled by insulin and various drugs have been shown to influence glucose metabolism in these tissues. Moreover, literature evidence indicates that muscle and liver cells are extensively employed in glucose metabolism research. Yen *et al* has demonstrated that the cinnamon extract promotes glucose uptake in muscle (C2C12) cells (Yen *et al.*, 2014). Resveratrol has also been shown to promote glucose uptake through adenosine monophosphate protein kinase (AMPK) pathway in C2C12 cells (Chang *et al.*, 2009). On the other hand, Nadu has shown that sex steroids increase glucose uptake in liver (Chang) cells (Nadu, 2007). Therefore, the liver (Chang) and muscle (C2C12) cells are significant tools in investigating the effects of a particular compound on glucose metabolism. For these reasons, liver (Chang) and muscle (C2C12) were chosen to investigate the effects of oxidovanadium complexes on glucose metabolism. In this study, the doses of drugs, glucose concentrations and time intervals were determined from a series of preliminary studies. For glucose utilisation experiments, a decline in media

glucose indicated an increase in glucose uptake by the cells. The poor glucose uptake observed in the control muscle cells was due to the lack of insulin. The glucose uptake observed in the muscle cells may be attributed to the basal glucose uptake, mainly facilitated by GLUT1 which has been reported to be present in almost all tissues (Ciaraldi *et al.*, 2005). In the liver control cells, the steady decline in media glucose may be attributed to the presence of a bidirectional GLUT2 which facilitates glucose uptake into the liver cells (Weinstein *et al.*, 1994). Insulin administration in both liver and muscle cells increased glucose uptake as evidenced by a sharp decline in media glucose concentrations. Insulin facilitates glucose uptake in the muscle and adipose through GLUT4 translocation and enhanced glycogen synthesis (Withers *et al.*, 1998). In the liver cells glucose uptake is independent of insulin and there are no drugs which have been found to directly influence glucose uptake (Postic *et al.*, 1993). However, insulin promote glucose uptake indirectly via increasing glycogen synthesis. Administration of oxidovanadium complexes significantly improved glucose uptake in both liver and muscle cells suggesting insulin mimetic effects. Several vanadium based compounds have been demonstrated to promote glucose uptake *in vitro*. A study by Mendes *et al* has also shown that vanadium complexes such vanadate 2-pyrideneformamide thiosemimacarbozones enhance glucose uptake in adipocytes (Pariante *et al.*, 1999). Furthermore, insulin or oxidovanadium complex (2) increased GLUT4 expression in muscle cells which may explain an increase in glucose uptake observed in the muscle cells. These observations are in agreement with the literature reports since some studies have shown elevated GLUT4 expression after vanadium administration (Li and McNeill, 2001; Mohammad *et al.*, 2002). In the absence of insulin, GLUT4 is trapped within the cysotol vesicles and GLUT4 translocation is influenced by insulin and other diverse signals (Nia *et al.*, 2002; Sano *et al.*, 2008). Upregulation of GLUT4 expression is critical for glucose uptake and a decreased GLUT4 expression has been reported in diabetes (Gaster *et al.*, 2001). A novel compound that has the ability to increase GLUT4 expression in muscle or adipose tissue is regarded as beneficial in the management of diabetes. The mechanisms through which our oxidovanadium complexes promote glucose uptake and GLUT4 expression cannot be explained by the present study. We speculate that our oxidovanadium complexes may facilitate glucose uptake partly through AMPK pathway activation. AMPK is a phylogenetically conserved serine/threonine kinase which functions independent of insulin. Activation of AMPK has been reported to increase glucose uptake through increased expression of GLUT4, hexokinase and enhanced glycogen synthesis via Akt pathway (Sakamoto and Holman, 2008). Furthermore, some anti-diabetic agents such as biguanides

and TZDs have been reported to target AMPK (Shaw *et al.*, 2005). In this study, metformin also had positive effects in the absence of insulin in both liver and muscle cells. Apart from AMPK, some anti-hyperglycaemic drugs interact with PPAR γ to promote glucose disposal in the muscle and adipose. The ability of oxidovanadium complexes to influence glucose uptake in both muscle and liver cells may be of a beneficial feature in improving glucose homeostasis. Glucose homeostasis improvement is essential and is regarded as the primary intervention in the management of diabetes to delay the onset or the progression of diabetes complications. In addition to insulin, conventional anti-hyperglycaemic drugs such as metformin and rosiglitazone achieve their effects through enhancing glucose disposal in liver and muscle (Lebovits, 2002; Ohiraa *et al.*, 2007).

After glucose has entered the liver or muscle cell, glucose is metabolized by various metabolic pathways. Upon entry to the cells, glucose is converted to glucose-6-phosphate which is then metabolised via glycolysis, citric acid cycle and oxidative phosphorylation to form ATP required for cell metabolic processes (Guoa *et al.*, 2012). The citric acid cycle intermediates are shunted towards lipid synthesis which is under insulin control in the liver, muscle and adipose (Inzucchi *et al.*, 2002). Furthermore, glucose is stored as glycogen in the liver and muscle, a metabolic process which is also influenced by insulin signaling (Aronson and Rayfield, 2000). In this study, we also investigated the effects of oxidovanadium complexes on glycogen synthesis as one of the mechanisms involved in glucose disposal. Both muscle and control liver cells showed poor glycogen synthesis perhaps due to lack of insulin. Furthermore, these observations correlated with reduced expression of glycogen synthase in control liver cells. Insulin is known to promote glycogen synthesis in both liver and muscle cells (Vollenweider, 2003). An increase in glycogen synthesis observed after oxidovanadium complexes administration may also explain or support the increase in glucose uptake observed in both liver and muscle cells. The increase in glycogen synthesis may be partly mediated via glycogen synthase expression which was also found to be elevated in liver cells. Our observations on glycogen levels slightly deviate from the literature perhaps due to different assays employed. Furthermore studies have reported correlation between glycogen synthesis and glycogen synthase expression *in vitro* which we also observed in this study (Mamedova and Shneyvays, 2003; Vigoda *et al.*, 2003). We speculate that the effects of oxidovanadium complexes on glycogen synthesis were partly mediated via activation of AMPK and Akt downstream effectors. Physiologically, GS expression is modulated by insulin or AMPK through PI3-K activation which further activates GS3-K to enhance GS

expression for glycogen synthesis (White, 2003). For these reasons, we speculate that our oxidovanadium complexes may act upstream PI3-K. Some vanadium complexes have been shown to enhance the fore mentioned metabolic process such as glycogen synthesis both *in vitro* and *in vivo* (Reul *et al.*, 1999). Vanadium complexes such bisperoxovanadate have been demonstrated to increase glycogen synthesis in fibroblast cells (Schmid *et al.*, 2004). Studies by Tolman also demonstrated that vanadium based compounds increase glucose uptake with concomitant increase in glycogen synthesis in the liver (Tolman *et al.*, 2000). A study by Furnsim *et al* has also demonstrated an increase in glycogen synthesis in rat isolated muscle after vanadium complex administration (Furnsim *et al.*, 2001). Since the causal link of DM and associated complications is hyperglycaemia, the ability of a novel agent to efficiently lower blood glucose concentrations or facilitate glucose disposal is of importance in diabetes therapy.

Furthermore, in this study we observed that the overall potency in glucose uptake by oxidovanadium complexes (high dose) was comparable to insulin. These observations together with enhanced glycogen synthesis and expressions of GLUT4 and GS may suggest that our oxidovanadium complexes mimic insulin's actions and may utilize the same or similar mechanisms as insulin to facilitate glucose disposal. The mechanisms through which vanadium enhance glucose disposal are not well-understood. Some mechanisms that have been reported involve activation of non-receptor cytosolic protein and inhibition of phosphotyrosine kinase phosphatases in the muscle. Inhibition of phosphotyrosine kinase phosphatases by vanadium complexes was found to enhance glucose uptake together with glycogen synthesis (Schmid *et al.*, 2004). Furthermore, inhibition of this enzyme by bismaltolato oxovanadium was found to increase the tyrosine kinase activity, leading to enhanced insulin sensitivity in muscle tissue (Bhattacharyya and Alan, 2001). For these reasons, further studies are required to elucidate the mechanisms through which our novel oxidovanadium complexes exert their effects on glucose metabolism in both liver and muscle cells.

Structurally different vanadium complexes have been demonstrated to have anti-hyperglycaemic and beneficial effects *in vivo*. Studies have shown that oral vanadyl sulphate administration increased hepatic and peripheral insulin sensitivity in patients with type 2 DM

(Cohen *et al.*, 2001). Studies by Subramanian *et al* in STZ-induced diabetic rats showed that a non-toxic vanadium-3-hydroxyflavone complex has anti-diabetic properties associated with improved lipid profile (Subramanian *et al.*, 2014). Willsky *et al* has demonstrated that vanadium dipicolinate administration normalizes hyperglycaemia and hyperlipidaemia in obese rats (Willsky *et al.*, 2011). Crans also investigated the effects of vanadium (IV) and (V) compounds and found that they had a long term insulin mimetic effects *in vivo* (Crans, 2000). For these reasons, we envisage that our novel oxidovanadium complexes may have positive effects in improving glucose homeostasis in diabetes.

To date, the potency of vanadium complexes combined with anti-hyperglycaemic agents remains controversial. Studies have documented that vanadium complexes combined with insulin or TZDs had no additive blood glucose lowering effects (Storr *et al.*, 2003). We also went a further step and investigated the effects of oxidovanadium complexes combined with insulin or metformin on glucose utilisation and glycogen synthesis in both liver and muscle cells. Our results are also controversial since only oxidovanadium complex (1) combined with insulin showed enhanced potency in both glucose utilisation and glycogen synthesis in the muscle cells. The enhanced potency may be possibly due to enhanced insulin sensitivity in the muscle after a combined treatment administration. Some vanadium complexes have been shown to influence insulin sensitivity in peripheral tissues (Srivastava and Mehdi, 2005). We speculate that our oxidovanadium complex (1) may sensitize insulin receptors to insulin. However, there is also a possibility that the synergistic effects observed may be due to more than one mechanism involved in glucose uptake after combined treatment administration. Furthermore, combining oxidovanadium complexes with metformin did not show positive synergistic effects. Metformin is known to have permissive effects to insulin through sensitizing insulin receptors. These observations may suggest that oxidovanadium complexes act downstream insulin receptor, possibly through interacting with tyrosine kinase, IRS-1 or PI-3K directly. Woo *et al* also studied the blood glucose lowering effects of vanadyl-biguanide compounds on STZ-induced diabetic rats and no apparent synergistic positive effects were observed (Woo *et al.*, 1999).

The ability of a compound to promote insulin secretion is beneficial in the management of type 2 diabetes. In this study, we also investigated the effects of oxidovanadium complexes

on insulin secretion in pancreatic (RIN-5) cells. The RIN-5 cells have been reported to secrete substantial amounts of insulin, in addition to small amounts of glucagon and somatostatin (Poitout *et al.*, 1996; Fernández-Millán *et al.*, 2014). Furthermore, literature indicates that RIN-5 cells are among the widely used cell lines in studying insulin secretion (Polina *et al.*, 2010; Gorelick *et al.*, 2011). Studies by Gorelick *et al* and Polina *et al* have demonstrated that administration of plant extracts increase insulin secretion by RIN-5 cells (Polina *et al.*, 2010; Gorelick *et al.*, 2011). In this study, RIN-5 cells were chosen to investigate the effects of oxidovanadium complexes on insulin secretion. In control cells, we observed presence of insulin which was secreted into the media, probably due to presence of glucose. The β -cells are electrically excitable and transition in the membrane potential coupled to plasma glucose concentration variations stimulate or inhibit insulin secretion (Ashcroft and Rorsman, 1989). *In vitro*, in response to glucose challenge, from about 1-20 mM, insulin secretion increase about 15-folds (Henquin, 2000). Therefore, glucose concentrations play a well-understood significant role on insulin secretion by the β -cells. The entry of glucose in the β -cells through GLUT2 leads to changes in the ATP/ADP ratio with subsequent activation of ATP dependent K^+ channels and opening of Ca^{2+} channels (Donald *et al.*, 2013). Following successive events, insulin is released by Ca^{2+} dependant exocytosis through secretory granules which fuse with the cell membrane (Ammala *et al.*, 1993). Studies have documented that vanadium complexes such as bis-maltolato vanadium and vanadate pyrrolodine-N-dithiocarbamate increase insulin secretion in experimental animals (Conconi *et al.*, 2003). In spite of these observations, there have been no consensus mechanisms given. Our observations on insulin secretion in the pancreatic cells remain controversial since only oxidovanadium complex (2) increased insulin secretion. The increase in insulin secretion correlated with an increase in glucose uptake by the pancreatic cells. However, the mechanisms through which these observations occurred may not be explained in this study. We speculate that the increase in insulin secretion may be due to involvement of ATP dependent K^+ and Ca^{2+} channels since they play a crucial role on insulin secretion. Therapeutic modalities that affect insulin secretion such as sulphonylurea have been shown to activate ATP dependent K^+ channels (Fuhlendorf *et al.*, 2001). Furthermore, Pariente *et al* has shown that vanadate compounds promote Ca^{2+} mobilization and Ca^{2+} influx in pancreatic acinar cells (Pariente *et al.*, 1999). Apart from well-known mechanisms, our oxidovanadium complex may also interact with SNARE proteins which have been reported to be involved in insulin secretion. Some studies have shown that interfering with SNARE proteins influence insulin secretion. SNARE phosphorylation has been shown to play a significant role in

modulating the secretory capacity of β -cells (Seino and Shibasaki, 2005). Furthermore, studies have also reported that overexpression of SNARE leads to cAMP dependant exocytosis of insulin from secretory granules (Barnard *et al.*, 1997). For these reasons, further studies are required to investigate the exact mechanisms through which our oxidovanadium complex (2) enhances insulin secretion.

CHAPTER 5

CONCLUSIONS

5.0 Conclusions

Oxidovanadium complexes (1, 2 and 3) enhance glucose metabolism in both liver and muscle cell lines *in vitro*. Furthermore, the effects of oxidovanadium complexes on insulin secretion remain controversial in pancreatic cells. The ability of oxidovanadium complexes to independently promote glucose metabolism in the liver and muscle may be beneficial in the management of both type 1 and type 2 diabetes. The overall observations warrant further investigations in a rat model to determine whether complex 2 might exert a tight glycaemic control *in vivo*.

5.1 Limitations

In the current study we did not investigate the blood glucose lowering effects of oxidovanadium complexes and effects on insulin secretion *in vivo*.

5.2 Recommendations for future studies

The observations of the current study highlight the significance to further evaluate the effects of oxidovanadium complexes on glucose metabolism in experimental diabetic animal models, *in vivo*.

CHAPTER 6

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CHAPTER 7

APPENDICES

Appendix I: Conference presentations

(a) Poster presentation titled “The effects of *Syzygium aromaticum*-derived-oleanolic acid (OA) on reactive oxygen species in the heart, liver and kidneys of STZ-induced diabetic rats” was delivered in the Society for Endocrinology BES 2013 (21 March 2013)

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THE EFFECTS OF SYZYGIUM AROMATICUM-DERIVED OLEANOLIC ACID (OA) ON REACTIVE OXYGEN SPECIES IN THE HEART, LIVER AND THE KIDNEY OF STZ-INDUCED DIABETIC RATS

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INTRODUCTION

The onset of diabetic complications is attributed to sustained hyperglycaemia which triggers the generation of free radicals and oxidative-related micro- and macrovascular damages¹. Recent studies report that intense glycaemic control by the subcutaneous administration of insulin cannot completely restore the balance between reactive oxygen species (ROS) and antioxidants².

Preliminary studies in our laboratory indicate that transdermally delivered *Syzygium aromaticum*-derived oleanolic acid (OA) has the ability to lower blood glucose in experimental diabetes mellitus due to the sustained release of the diterpene. Research has indicated that some plant derived bioactive compounds such as flavonoids and tannins have antioxidant properties³. However, no work has been done to determine the effects of OA on oxidative stress.

OBJECTIVES

The aim of the study was to evaluate the effects of *Syzygium aromaticum*-derived OA on some biomarkers of oxidative stress in the heart, liver and kidney of the STZ induced diabetic rats.

Effects of OA oxidative stress

The acute effects of OA on malondialdehyde (MDA), an indicator of lipid peroxidation and the antioxidant enzyme, glutathione (GSH) concentrations were evaluated in the heart, liver and kidney of STZ-induced diabetic rats following a glucose load after an 18-hour fast. Rats administered pectin-free OA or transdermally delivered insulin acted as untreated and treated positive controls, respectively. Heart, liver and kidney samples of liver and gastrocnemius muscle (1–1.5 g) were harvested after 6-h in separate groups of non-diabetic and STZ-induced diabetic rats for the determination of MDA and GSH.

RESULTS AND DISCUSSION

Effects of OA on oxidative stress

By comparison with non-diabetic rats, STZ-induced diabetic rats exhibited oxidative stress and impaired antioxidant defense mechanisms in the heart, liver and kidney tissues of as assessed by elevated MDA levels and reduced GSH concentration (Figures 1 and 2). Free radicals are extremely reactive and produce damage and modify cell functions. Under normal circumstances, there are antioxidant enzymes and macromolecules that remove and protect the cells from the damaging effects of free radicals.

OA like insulin significantly reduced the levels of MDA in the hearts and livers of STZ-induced diabetic rats (Figure 1).

OA significantly increased GSH concentration in the heart of STZ-induced diabetic rats although levels in the kidney and liver were not altered (Figure 2).

Interestingly, insulin treatment did not alter the GSH concentrations in the heart, liver and kidney tissues.

MATERIALS AND METHODS

Extraction of OA

- OA was extracted from *Syzygium aromaticum* [(Linnaeus) Merril & Perry] (Myrtaceae) clove flower buds using a previously validated protocol⁴.
- Cloves (500 g) were sequentially extracted twice at 24h interval with dichloromethane, and ethyl acetate (EA) yielding ethyl acetate solutions (EAS) containing MA/OA and methyl carosolate.
- OA was isolated using silica gel column and recrystallized from methanol/chloroform and its structure confirmed by ¹H and ¹³C-NMR compared with literature data⁵.

Animals

Male Sprague–Dawley rats (250–300 body weight) bred and maintained at Biomedical Research Unit (BRU), University of KwaZulu-Natal were used in this study.

Induction of diabetes mellitus

Diabetes mellitus was induced in rats with a single intraperitoneal injection of streptozotocin (STZ; 60mg/kg) dissolved in freshly prepared 0.1M citrate buffer (pH 4.3).

CONCLUSION

These results suggest that OA is a potential drug for diabetes mellitus that would not only lower blood glucose, but also can avert complications that arise due to oxidative stress.

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Figure 1 The effect of transdermal administration of pectin-free OA on MDA levels in the heart, liver and kidney of STZ-induced diabetic rats.



Figure 2 The effect of transdermal administration of pectin-free OA on GSH levels in the heart, liver and kidney of STZ-induced diabetic rats.

(b) Oral presentation titled “**Antioxidant effects of oleanolic acid in livers, hearts and kidneys of streptozotocin-induced diabetic rats**” was delivered in Physiological Society of Southern Africa (PSSA) Conference (18 September 2013)

ANTIOXIDANT EFFECTS OF OLEANOLIC ACID IN LIVERS, HEARTS AND KIDNEYS OF STREPTOZOTOCIN-INDUCED DIABETIC RATS

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Hyperglycaemia-induced oxidative stress triggers the development and progression of microvascular and macrovascular complications in diabetes. However, some plant extracts containing quercetin, flavonoids and triterpenes decrease blood glucose concentrations and oxidative stress in experimental diabetes. Hence we postulated that the triterpene oleanolic acid (OA) which possesses anti-hyperglycaemic properties can prevent oxidative stress to consequently avert complications in diabetes. This study was, therefore, designed to investigate the effects of *Syzygium aromaticum*-derived OA on oxidative stress in the hepatic, renal and cardiac tissues of streptozotocin (STZ)-induced diabetic rats. Pectin hydrogel OA patches were applied on shaved back of the neck of STZ-induced diabetic rats, twice after every third day, for five weeks. Pectin free OA patch and insulin subcutaneously administered rats served as untreated and treated positive controls while non-diabetic untreated rats served as negative controls. After five weeks, animals were sacrificed followed by measurement of malondialdehyde (MDA), superoxide dismutase (SOD) and glutathione peroxidase (GPx) in the hepatic, cardiac and renal tissues. Topical OA administration significantly reduced MDA levels in the hepatic, renal and cardiac tissues of STZ-induced diabetic rats with concomitant increase of the concentration of antioxidant enzymes, SOD and GPx. These results suggest that OA ameliorates oxidative stress in the liver, heart and kidneys and this may consequently avert macrovascular and microvascular complications of DM. It can thus be concluded that OA is a promising modality for DM management, since it possesses both anti-hyperglycaemic and antioxidant effects.

(c) Poster presentation titled “Evaluation of the effects of oxidovanadium 2-pyridylbenzimidazole (Hpybz) compounds on glucose metabolism in liver and skeletal muscle cell lines” was delivered in the Society of Endocrinology (1 July 2014)

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EFFECTS OF VANADIUM COMPLEXES ON GLUCOSE METABOLISM IN THE LIVER AND SKELETAL MUSCLE CELL LINES

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INTRODUCTION

The various medications available to treat diabetes mellitus include insulin injections and a host of synthetic hypoglycaemic drugs. However, these therapeutic approaches are associated with inconveniences such as multiple of daily insulin injections, cardiovascular disorders and weight gain. On-going research seeks to develop anti-hyperglycaemic novel agents with less undesirable effects. More recently, the anti-diabetic activity of vanadium compounds has received attention particularly inorganic vanadium salts, sodium orthovanadate and vanadyl sulphates which have been shown to lower blood glucose and increase glycogen synthesis in experimental diabetes [1]. Accordingly this study, investigated the effects of novel oxidovanadium formulations on glucose utilization in liver and muscle cell lines.

OBJECTIVES

The objectives of this study were to evaluate the effects of oxidovanadium compounds in the liver (Chang) and muscle (C2C12) cell lines on:

- i. glucose utilization in the liver
- ii. glycogen synthesis
- iii. cell viability

MATERIALS AND METHODS

Synthesis of oxidovanadium compounds
 The oxidovanadium complexes, cis-[VO2(Hpybz)(pybz)] (1), (μ-O)[VO(Hpybz)(pybz)VO(Hpybz)(acac)] (2) and [VO(Hpybz)2SO4].H2O (3) were synthesized from the 2:1 molar ratio reactions of the ligand, 2-pyridylbenzimidazole (Hpybz) with the respective metal precursors, ammonium vanadate, vanadyl(IV) acetylacetonate and vanadyl(IV) sulphate as reported by Booysen [2]. The octahedral vanadium (IV) complex, [VO(Hpybz)2SO4] (3).H2O was characterized by means of conductance measurements, UV/Vis, ESR and IR spectroscopy as well as single crystal X-ray diffraction. Each of the oxidovanadium complexes was evaluated for effects on glucose uptake in C2C12 skeletal muscle and Chang cell lines.

EXPERIMENTAL PROTOCOL

Separate confluent liver and muscle cell lines in 24 well plates were incubated at 37°C with media containing 19 and 29mmol/L of glucose, respectively in the presence of oxidovanadium compounds (1, 2 or 3) at various concentrations (12.5, 25 and 50µg/ml). Blood glucose concentrations and cell viability were determined after 12h, 24h and 48h whilst glycogen was measured after 48h. Cells incubated with DMSO (0.1%), or insulin (0.004mg/ml) acted as untreated and treated positive controls, respectively.

STATISTICAL ANALYSIS

All data are expressed as means ± S.E.M. Statistical comparisons between the control means and experimental groups were performed with GraphPad InStat Software (version 5.00, GraphPad Software, San Diego, California, USA), using one-way analysis of variance (ANOVA), followed by Tukey-Kramer multiple comparison test. A value of p < 0.05 was considered significant.

RESULTS

- Figures 1 and 2 show that all treatments stimulated glucose uptake in liver and muscle cell lines at all time points in a dose-dependent manner.
- The complexes also increased glycogen synthesis although no dose-dependent effects were observed (Table 1).
- There was no toxicity in all cell lines after exposure to vanadium complexes (Figures 3 and 4).
- The effects were comparable to cells treated with insulin.

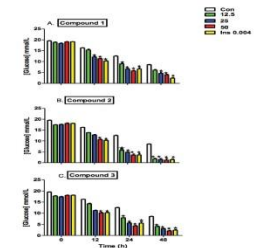
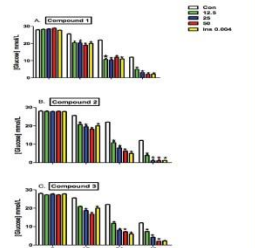
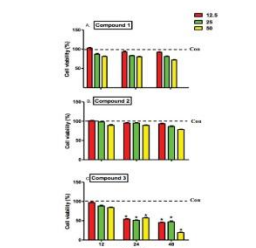
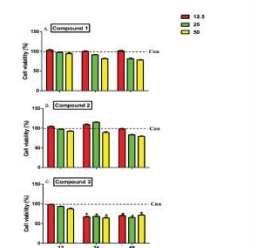



Table 1: The effects of oxidovanadium (1, 2 and 3) on glycogen concentrations in the liver and muscle cell lines. *p < 0.05 by comparison with control value.

Groups	Glycogen concentrations (µmol / cells)	
	Muscle (C2C12) cells (µmol / 1.5 × 10 ⁶ cells)	Liver (Chang) cells (µmol / 1.5 × 10 ⁶ cells)
Control	960±15	645±15
Compound 1	1410±15*	865±15*
Compound 2	1300±15*	860±18*
Compound 3	1165±18*	960±14*
Insulin	1780±18*	983±22*

DISCUSSION

The present observations indicate that oxidovanadium 2-pyridylbenzimidazole (Hpybz) compounds improve glucose utilization in skeletal muscle and liver without evoking toxicity. Some vanadium complexes such as vanadyl sulphates are associated toxic effects. For this reason, oxidovanadium 2-pyridylbenzimidazole (Hpybz) compounds complexes may have beneficial effects in diabetes treatment and can thus provide novel approaches in diabetes management.

CONCLUSION

The results suggest that the novel vanadium Hpybz compounds possess hypoglycaemic properties and hence can provide novel approaches in diabetes management.

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(d) Oral presentation titled “**The effects of vanadium complexes on insulin secretion in pancreatic cell lines**” was delivered in the College of Health Sciences Research Symposium (11-12 September 2014).

THE EFFECTS OF VANADIUM COMPLEXES ON INSULIN SECRETION IN PANCREATIC CELL LINES

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Vanadium complexes possess anti-hyperglycaemic properties but their mechanisms of action remain unclear. Accordingly, the study was designed to investigate the effects of vanadium complex 2-pyridylbenzamidazole acetylacetonate oxovanadium Hpybzvo(acac)_2 on insulin secretion and furthermore investigate glucose utilization *in vitro*. The study was carried out using a well-established cell culture protocol. Separate preparations of pancreatic and skeletal muscle cell lines were incubated with Roswell Park Memorial Institute and Dulbecco Minimal Essential Media containing 18 and 29 mmol/L of glucose respectively in the presence of hpyzvo(acac)_2 (12, 25 and 50 $\mu\text{g/mL}$). Dimethyl sulphoxide (0.1%) treated cells served as a control. Glucose concentrations and cell viability were monitored at specific time intervals (12, 24 and 48 hours). Thereafter, muscle cells were harvested for glycogen analysis and media samples of the pancreatic cell lines were collected for insulin measurements. The highest dose was more effective in eliciting glucose utilization in the skeletal muscle. This was associated with concomitant increase in glycogen synthesis. This can be seen with the glucose concentrations in comparison with the control (12.00 ± 0.05 vs 1.00 ± 0.05 mmol/L) with concomitant increase in glycogen synthesis (0.76 ± 0.02 vs 1.35 ± 0.02 mmol/L). Furthermore, hpyzvo(acac)_2 (12.5 $\mu\text{g/mL}$) significantly ($p < 0.05$) increased insulin secretion in comparison with the control (0.19 ± 0.01 vs 0.23 ± 0.01 nmol/L). This vanadium compound showed no toxic effects in both muscle and pancreatic cell lines. These results suggest that the anti-hyperglycaemic effects of hpyzvo(acac)_2 may be exerted via enhanced glucose uptake in the skeletal muscle and insulin secretion in the pancreas. Therefore, hpyzvo(acac)_2 may be beneficial in the management of hyperglycaemia.

(e) Oral presentation titled “**The effects of vanadium complexes combined with hypoglycaemic agents on glucose utilisation in the liver and muscle cell lines**” was delivered in the College of Health Sciences Research Symposium (11-12 September 2014).

THE EFFECTS OF VANADIUM COMPLEXES COMBINED WITH HYPOGLYCAEMIC AGENTS ON GLUCOSE UTILISATION IN THE LIVER AND MUSCLE CELL LINES

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Studies in our laboratory have shown that 2-pyridylbenzimidazole dioxovanadium complex (hpybz (VO) 2) enhance glucose metabolism in the liver and skeletal muscle cell lines. However, the effects of hpybz (VO)2 with other hypoglycaemic agents is unknown. Accordingly, this study investigated the effects of 2 hpybz (VO) 2 complex in combination with metformin and insulin on glucose utilization in liver and muscle cell lines. The hpybz (VO) 2 (12.50 µg/mL) was combined with either insulin (4 µg/mL) or metformin (160 µg/mL). Glucose concentrations were monitored at 0, 12, 24 and 48 hours. Thereafter, cells were harvested for glycogen analysis. By comparison to the control, hpybz (VO) 2 combined with insulin significantly decreased media glucose in both liver (13.60 ± 0.26 vs 4.90 ± 1.15 mmol/L) and muscle cell lines (16.33 ± 0.52 vs 5.00 ± 0.73 mmol/L) with concomitant increase in glycogen synthesis in both liver (1.70 ± 0.02 vs 0.76 ± 0.02 mmol/L) and muscle cell lines (1.93 ± 0.02 vs 0.76 ± 0.02 mmol/L). Interestingly, an insulin combined treatment showed a significant additive effect on glucose utilization and glycogen synthesis when compared to hpybz (VO) 2 or insulin alone, in both liver and muscle cell lines. These results suggest that a combination administration of hpybz (VO) 2 with insulin is effective in glucose utilisation compared to a single drug administration in liver and muscle cell lines, *in vitro*. Therefore, administration of hpybz (VO) 2 in combination with insulin may be beneficiary in the management of diabetes mellitus.