The acute effects of dioxidovanadium on blood glucose concentration and oxidative stress in the hippocampus of non-diabetic male Sprague Dawley rats and the chronic effects of dioxidovanadium on selected markers associated with hippocampal dysfunction in male Streptozotocin-induced diabetic rats.

By

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PREFACE

Diabetes mellitus is a metabolic disorder which is strongly associated with cognitive dysfunction. This metabolic disorder is characterised by chronic hyperglycaemia which promotes the development of oxidative stress, neuroinflammation, amyloid beta and hyperphosphorylated tau proteins in brain areas such as the hippocampus. Although exogenous insulin therapy efficiently manages diabetes, studies show that this treatment increases the risk of memory impairment four-fold due to side effects such as hypoglycaemia and insulin resistance. This has warranted the search for alternative treatment envisaged to circumvent undesirable effect associated with conventional therapies. In our laboratory, we have synthesised a vanadium complex, dioxidovanadium (V), by incorporating organic ligands which have been shown to improve potency and bioavailability whilst eliminating toxic accumulation. Dioxiodvanadium has been shown to successfully lower blood glucose concentration in a diabetic rat model without toxicity to the heart, skeletal muscle, liver, kidney and red blood cells. However, the effects of this vanadium complex on hippocampal function, particularly learning and memory are yet to be investigated. Therefore, this study aimed to investigate the effect of dioxidovanadium(V) on the hippocampus acutely and chronically as well as the effects on diabetes induced memory impairment in an STZ-induced diabetic animal model.

DECLARATION

I, Yalka Dayanand hereby declare that the dissertation entitled:

"The acute effects of dioxidovanadium on blood glucose concentration and oxidative stress in the hippocampus of non-diabetic male Sprague Dawley rats and the chronic effects of dioxidovanadium on selected markers associated with hippocampal dysfunction in male Streptozotocin-induced diabetic rats." is the result of my own investigation and research and that it has not been submitted in part or in full for any other degree or to any other university. Where use of the work of others was made, it is duly acknowledged in the text.

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PLAGARISM DECLARATION

School of Laboratory Medicine and Medical Sciences, College of Health Sciences

MASTER'S DEGREE IN MEDICAL SCIENCES 2022

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DEDICATION

This work is dedicated to my support system, God.

ACKNOWLEDGEMENTS

To God, the hope and support you bring me have helped me overcome some of the most challenging experiences in my life. None of this would be possible without you. You've taught me that not fear but perseverance, dedication, and hard work are always rewarded. To my family, thank you for making me the person I am today and supporting me throughout my education. To Preshen, thank you for being at my side at every step of this journey. Your companionship is one of my greatest gifts.

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ABBREVIATIONS

ATP - Adenosine triphosphate

AGE's - Advanced glycation end-products

AD - Alzheimer's disease

Aβ - Amyloid beta

APP - Amyloid precursor protein

BBB - Blood brain barrier

BRU - Biomedical resource unit

DM - Diabetes mellitus

DMSO - Dimethyl sulphide

Dioxidovanadium (V) - (cis-[VO2(obz)py] Hobz=2-hydroxyphenyl-1H-benzimidazole and py =pyridine])

GLUT - Glucose transporter

GPx1 - Glutathione peroxidase

GSK3β - Glycogen synthase kinase 3 beta

H_2O_2 - Hydrogen peroxide

pTau - Hyper-phsophorylated tau

IDE - Insulin degrading enzymes

IL-1 - Interleukin-1

IL-6 - Interleukin-6

MDA - Malonaldehyde

MAPK - Mitogen activated protein kinase

MWM - Morris water maze

ND - Non-diabetic

NADH - Nicotinamide adenine dinucleotide (NAD) + hydrogen (H)

PUFA - Polyunsaturated fatty acids

AKT - Protein Kinase B

PKC - Protein Kinase C

PI3K - Phosphoinositide 3-kinase

ROS - Reactive oxygen species

STZ - Streptozoticin

TZDs - Thiazolidinediones

TNF-α - Tumour necrosis factor alpha

UKZN – University of KwaZulu- Natal

STUDY OUTLINE

This dissertation has been presented in manuscript format and consists of 4 chapters. Chapter 1: literature review, chapter 2: manuscript 1, chapter 3 manuscript 2 and chapter 4: synthesis, conclusions, study limitations and recommendations. The first chapter gives a brief background and illustrates the important literature pertinent to the justification of the study. Chapter 2 consists of the first research paper in manuscript format, that sought to investigate the acute effects of dioxidovanadium on blood glucose concentration and oxidative stress in the hippocampus of healthy male Sprague Dawley rats. This work is authored by Y. Dayanand under the supervision of Dr P.S Ngubane, Dr A. Kathi and Dr N. Sibiya and has been prepared for publication in the Journal of Neuroendocrinology according to the journal's guidelines for authors. Chapter 3 contains the second research experiment in manuscript format, that aimed to investigate the chronic effects of dioxidovanadium on selected markers associated with hippocampal dysfunction in male Streptozotocin-induced diabetic rats. The study was authored by Y. Dayanand under the supervision of Dr P.S Ngubane, Dr A. Kathi and Dr N. Sibiya and has been formatted in accordance to the journal guidelines for publication in the Canadian Journal of Diabetes. Chapter 4 consists of the synthesis that links the findings of both studies, highlighting their scientific relevance and the appendix that contains ethical clearance certificates and journal guidelines to authors.

ABSTRACT

Background

Diabetes mellitus is a disease associated with derangements in glucose metabolism and chronic hyperglycaemia. Chronic hyperglycaemia induces oxidative stress and inflammation that affect glucose sensitive hippocampal neurons resulting in generation of amyloid plaques and tau tangles. These are the primary markers used in the detection of neurodegenerative diseases such as Alzheimer's and dementia. Hence, there is a strong correlation between diabetes and memory impairment. Current therapeutic options such as bolus insulin have been successful in the management of the disease. Despite the efficacy of these therapies, they however have been shown to possess undesirable effects that exacerbate the secondary pathological effects of diabetes on the hippocampus thereby contributing to the detriment of cognitive tasks such as learning and memory. Therefore, there is a need to explore alternative treatments. Transition metals have been shown to possess therapeutic effects with vanadium possessing the greatest potency in lowering blood glucose concentrations. However, studies have demonstrated toxic accumulation of vanadium in the hippocampus which result in the generation of oxidative stress and neurodegeneration. In our laboratory, we have synthesised dioxidovanadium (V) complex by attaching organic ligands to reduce the toxicity and improve potency of the metal. This complex has been shown to efficiently reduce blood glucose and elicit cardio and reno-protective properties. Despite these advancements the effects of this complex on the hippocampus and learning and memory are yet to be established. Therefore, in this study the aim was to evaluate the effect of dioxidovanadium complex on selected learning and memory parameters.

Methodology

The effect of vanadium on the brain was studied acutely and chronically. In the acute study, animals were separated into 2 groups, non-diabetic control group and a non-diabetic animal group which was were treated with vanadium complex (40 $mg.kg^{-1}$ p.o). The treatment was administered at time 0. Subsequently an n=3 from each group was sacrificed at regular time intervals (1 hour, 2 hours, 6 hours, 24 hours, 5 days, 10 days) in each group. Blood glucose concentration was monitored before sacrificing and hippocampal tissue was harvested for malonaldehyde (MDA) analysis and glutathione peroxidase (GPx1) and tumour necrosis alpha (TNF-α). The second study was conducted over 5 weeks and consisted of an untreated non-diabetic control, a diabetic control, a positive insulin treated group (0.175 $mg.kg^{-1}$ s.c) and two dioxidovanadium (V) treated groups (40 $mg.kg^{-1}$ p.o), a non-diabetic and a diabetic group. Blood glucose was monitored weekly and the Morris water maze was conducted on the last week of the study. After 5 weeks the animals were sacrificed and hippocampal tissue was harvested for malonaldehyde (MDA) analysis, glutathione peroxidase (GPx1) tumour necrosis alpha (TNF-α), amyloid beta (Aβ) and hyperphosphorylated tau (pTau) ELISA's.

Results

Acutely**,** dioxidovandium (V) did not lower blood glucose significantly in comparison to the control group. Interestingly, MDA, GPx1 and (TNF-α) were also not significantly different from the control group over all time periods in the study. Chronically, the glucose concentration of the dioxidovandium (V) treated diabetic group was significantly lowered when compared to the untreated group which displayed significantly increased glucose concentration in comparison to the non-diabetic control. The non-diabetic dioxidovanadium (V) treated group did not show a significant difference in glycaemic level. Increased MDA concentration in the diabetic group was significantly lowered by dioxidovanadium(V) treatment. GPx1 concentration in the dioxidovanadium (V) treated group significantly improved in comparison to the diabetic untreated control. The non-diabetic dioxidovandium (V) treated group showed no significant change in MDA and Gpx1 after the 5-week period. There was no significant difference in TNF-α in dioxidovanadium (V) treated groups, diabetic and non-diabetic. The concentration of Amyloid β was significantly lower in the diabetic control when compared to the non-diabetic control. The dioxidovanadium (V) treated groups, both diabetic and non-diabetic did not have a significant difference in comparison to the diabetic control. pTau concentrations in all groups did not significantly differ. Latency times for the last day of training the Morris water maze followed the same trend. The probe test results, which measured spatial memory, for the diabetic untreated and dioxidovanadium (V) treated groups were significantly reduced in comparison to the non-diabetic control group. The non-diabetic untreated and non-diabetic dioxodivanadium (V) treated were not significantly different.

Conclusion

Dioxidovanadium (V) treatment in non-diabetic animals did not induce hypoglycaemia acutely however reduced blood glucose concentration in diabetic animals when administered chronically. Dioxidovanadium (V) did not induce oxidative stress and may protect against neurodegeneration by enhancing antioxidant status and therefore was considered as a pro-oxidant in the hippocampus.

CHAPTER 1: LITERATURE REVIEW

1. Background

The continuous metabolic cycle of glucose handling for maintaining glucose homeostasis is a crucial factor contributing to wellness (1). However, the inability to restore or maintain glucose homeostasis in the event of dysfunction in metabolic pathways results in the continuous accumulation of abnormal blood glucose concentration (2). This concludes with the development of diabetes mellitus (DM), a disorder characterised by chronic hyperglycaemia (2). Diabetes occurs as a consequence of insufficient insulin production or ineffective insulin action resulting in dysregulated glucose handling (2). Approximately 50% of deaths resulted from secondary diseases induced or aggravated by the uncontrolled hyperglycaemia during the year 2019 (3). These secondary diseases are a consequence of hyperglycaemic blood circulation throughout the body resulting in organ system exposure to abnormal glucose concentration (4).

Microvascular and macro vascular complications in patients suffering with diabetes that occur due to glucotoxicity in organs that are highly sensitive to changes in blood glucose, such as the brain (5). The brain is dependent on a constant controlled supply of glucose to carry out neuronal and non-neuronal functions. However, roughly just under 50% of patients living with diabetes are subjected to decreased cognitive abilities due to glucotoxicity (5). Research has shown imbalances in reactive oxygen species and antioxidants as well as increased neurodegenerative proteins in the hippocampus of diabetic individuals (1). The hippocampus promotes learning and consolidation of memories, thereby suggesting a strong correlation between DM and memory loss (6).

Exogenous insulin is used as the primary treatment for type 1 diabetic individuals however bolus insulin has been shown to cause a hypoglycaemic environment in the brain resulting in a lack of ATP production (7). To avoid this and other associated side effects, our research team is exploring alternative therapies, including the use of the medicinal properties of vanadium. Furthermore, vanadium complexes have been synthesized to eliminate the toxic accumulative traits of vanadium while benefiting from its anti-hyperglycaemic qualities (8). We have synthesized a vanadium complex by attaching pyridine and bemidazole ligands to vanadyl to improve the bioavailability and clearance of the metal thereby preventing toxic accumulation (9). Since naturally occurring vanadium salts are toxic to the hippocampus and subsequent cognitive decline, this study aims to investigate the effects of a vanadium complex, dioxidovanadium (V), on the hippocampus and learning and memory in an attempt to advocate for its use as a possible alternative treatment for diabetes (10).

2. Glucose metabolism and function in the brain

Glucose transport proteins are required for transendothelial transport of glucose from the blood vessels through the blood-brain barrier (BBB) and into brain cells facilitated by GLUT1 and GLUT3

transporters present on endothelial cells of glia and neurons, respectively (11, 12). GLUT3 has a higher affinity for glucose than GLUT1 (11, 13). This difference in affinity ensures neurons receive enough glucose supply for adequate ATP generation, which encourages the transmission of signals and neurotransmitter production under varying systemic glucose concentrations (14). Under physiological conditions, glucose is metabolised via glycolysis and through oxidative phosphorylation by undergoing the TCA cycle, and the electron transport chain to produce ATP to fuel cellular function (15). The initial step of glycolysis is a rate-limiting step that involves the phosphorylation of glucose via a polar hydrophobic enzyme called hexokinase to produce glucose-6-phosphate (G-6-P), thereby trapping glucose in the cell (15, 16). Apart from being an intermediate in glycolysis, G-6-P is a substrate for the pentose phosphate pathway (PPP), which allows for NADPH production, a cofactor required to produce the antioxidant glutathione(GPx) (17). Glycolysis concludes with the production of pyruvate catalysed by the enzyme pyruvate kinase (17).

Pyruvate is subsequently converted to acetyl CoA via the pyruvate dehydrogenase complex and fed into the TCA cycle (17). Under conditions of the influx of glucose exceeding the rate of the TCA cycle or malate aspartate shuttle, excess pyruvate is shunted to the lactate dehydrogenase cycle to regenerate NAD+ (15). The metabolites produced from glycolysis and the TCA cycle are used to produce neurotransmitters (NT) to allow for neurons to transmit signals and communicate with each other to carry out brain function (15). Non-essential amino acids cannot pass the BBB and therefore must be synthesised in the brain (15). Metabolites such as α-ketoglutarate, as seen in figure 1, act as precursors to produce non-essential amino acids required to produce neurotransmitters such as glutamate, an excitatory NT required for memory and learning (18).

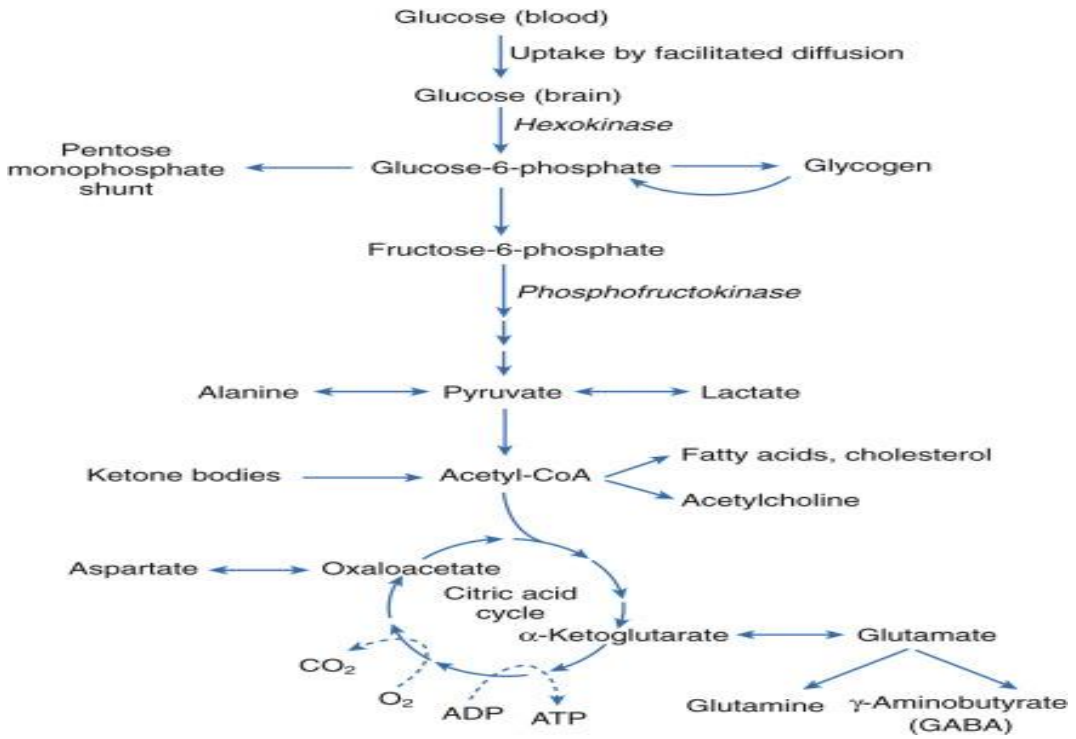

Figure 1: Diagram depicting metabolic processing of glucose by brain cells contributing to brain function (16).

The final metabolic pathway, the electron transport chain, promotes the formation of ATP via ATP synthase located on the mitochondrial membrane (15). ATP is used by neurons and glial cells to perform physiological functions such as the facilitation of learning and defining dedicated neuronal memory traces (19). During metabolic diseases such as diabetes, the brain receives an unregulated amount of glucose which exceeds the rate at which it is physiologically processed. This results in the shunting of glucose to pathological pathways. One of the brain areas greatly affected by the dysregulation of glucose metabolism is the hippocampus thereby leading to cell degeneration and loss of function (20).

3. The effects of diabetes on hippocampal metabolism

For an extended period, the brain was regarded as an insulin-independent organ; however, recent research has shown that receptors for insulin exist in multiple sites in the brain, such as the hippocampus, hypothalamus, cerebral cortex, and amygdala (21). In the absence of insulin, the body remains without a state of satiety, and food intake remains unregulated. Increased food intake then further contributes to the hyperglycaemic condition. Hyperglycaemia also disrupts proteins in tight junctions between micro-endothelial cells of the BBB, thus making the BBB porous, possibly allowing the entry of unwanted substances into the parenchyma of the brain (22). Large amounts of glucose enter neuronal and glial cells disrupting metabolic pathways. Increased formation of glucose-6-phosphate (G-6-P) in glycolysis downregulates hexokinase I (HKI) as it is regulated negatively against G-6-P; however, other isoforms of HK continue to convert absorbed glucose into G-6-P (20). The influx of

glucose exceeds the rate of glycolysis, and excess G-6-P is shunted to multiple destructive pathways, including the generation of reactive oxygen species (ROS), glycation product synthesis, accumulation of polyols, and neuroinflammation (20).

4. Pathological pathways of neurodegeneration in the hippocampus

4.1 Diabetes and the generation of oxidative stress in the hippocampus

During ischemia, as seen in diabetes, lactate production is favoured for the generation of ATP. This produces increased amounts of lactic acid and results in metabolic acidosis (23). There is subsequent degradation of cellular components such as the mitochondria and the production of free radicals (24). Another pathway that accepts G-6-P as a substrate is the polyol pathway. Excess glucose is reduced to sorbitol using the enzyme aldose reductase with NADPH as a cofactor in this pathway (24). The build-up of sorbitol induces oxidative stress in brain cells such as neurons. NADPH is also a cofactor required to generate the antioxidant glutathione; however, the polyol pathway consumes NADPH required for glutathione production (24). The production of oxidative stress as a result of accumulated sorbitol and diminished antioxidant status results in neurodegeneration (24, 25). Sorbitol production requires a reduction of NAD+ to NADH, resulting in an imbalance in the NAD+/NADH ratio.

The imbalance in the NAD+/NADH ratio results in decreased NAD+ availability, resulting in the inhibition of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (26). This causes the build-up of glyceraldehyde 3-phosphate (G3P), a substrate for α-glycerol phosphate, which is a precursor for the PKC binding domain diacylglycerol (26). This activates both PKC-α and PKC-β isoforms. The activation of these isoforms results in the shunting of glucose to the hexosamine pathway (26). Studies have shown that chitin-like polymer production introduces chitin scaffolds, which occurs due to the hexosamine pathway in the brain of patients affected by cognitive impairment diseases associated with patients living with diabetes (27). The mechanism by which these chitin-like scaffolds induce cognitive impairment is still unclear.

There is also a production of glycation products from the reaction of glucose with proteins and lipids that occurs at an accelerated rate during a hyperglycaemic state, referred to as advanced glycation products(AGEs) (28). These AGEs are elevated in the hippocampus along with inflammatory markers such as TNF-α (28, 29). Research has shown that increases in TNFα receptor, TNFR1, including increased binding affinity, is observed in patients with Alzheimer's disease (AD) (30). The expression of TNFα can be associated with abnormal amyloid β processing, causing synaptic loss and neuronal cell death and finally the development of dementia (30). Since oxidative stress is associated with diabetic complications such as memory impairment, a desirable trait of an anti-diabetic drug is to protect against the production of oxidative stress. By referring to figure 2, we can deduce that oxidative stress

is a result of an imbalance between reactive oxygen species (ROS) and antioxidants therefore a mechanism to prevent oxidative stress is by enhancing antioxidant mechanism. Later in this review, we will discuss how vanadium complexes have been shown to enhance the antioxidant system.

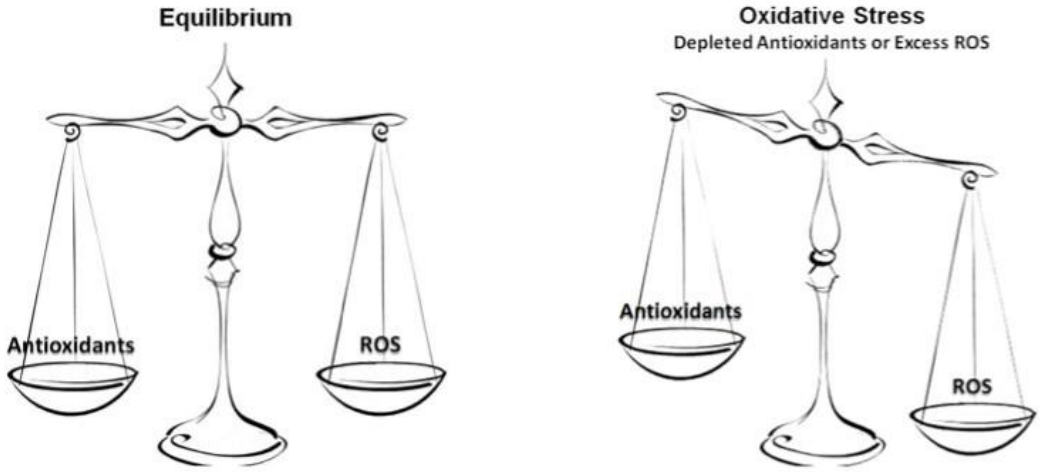

Figure 2: Diagram highlighting the difference between redox balance under physiological circumstances vs under oxidative stress.

4.2 The production of Amyloid Beta

Patients suffering from diabetes that experience memory loss or diagnosed with AD and dementia have been shown to present with increased levels of amyloid β in hippocampal neurons, causing neurodegeneration (31, 32). These are intracellular neuropeptides produced from the cleavage of amyloid precursor proteins (APP) in neurons by the enzyme beta secretase (33). Despite being nontoxic, these β-amyloid peptides aggregate into fibres and eventually form plaques due to their hydrophobic nature. β-amyloid has been shown to bind to RAGE and induce oxidative stress resulting in brain cell neuron and microglial degradation (33). There have been observations of 3 isoforms of β-amyloid, Aβ-40, Aβ-42, and Aβ-43. Aβ-42 and Aβ-43 are the isoforms considered neurotoxic. Insulin-degrading enzymes can degrade pro-β-amyloids; however, in an insulin-resistant setting caused by type 2 diabetes or insulin therapy, the activity of these enzymes is significantly reduced (31).

Mitochondria regulate energy production and intracellular Ca^{2+} however, there are increased levels of mitochondrial dysfunction in hyperglycaemia (27). Aβ-42 fibres contain amyloid channels that cause a rapid influx of Ca^{2+}, coupled with mitochondrial dysfunction, disrupting calcium homeostasis (34). Calcium homeostasis plays an essential role in neuronal synaptic plasticity, which is critical to memory formation (27). Therefore, β-amyloids expression disrupts calcium homeostasis, causing neuronal degradation and memory loss. Since vanadium has insulin-mimetic properties, further investigation of

vanadium complexes has revealed increased expression of insulin-degrading enzymes in the hippocampus, thereby reducing Aβ generation (35).

4.3 Hyper phosphorylation of Tau proteins

Another marker for memory loss and hippocampal neuron damage is the aggregation of pTau (36, 37). Mammalian TORC1 (mTOR) is responsible for regulating glucose metabolism by inhibiting glucose uptake and promoting glycolysis (36). In the hippocampus, ribosomal proteinS6 kinase (S6K) and mTOR signalling promote memory formation (33). Under physiological conditions, tau proteins are phosphorylated to maintain micro-stability in neurons. However, research has shown that in the presence of hyperglycaemia, there is an amplification of mTOR/S6K signalling resulting in the formation of hyper-phosphorylated tau (p-tau) and responsible for the activation of caspase 3, thereby inducing apoptosis in neurons (33, 36, 38). Locus coeruleus (LC) is a nucleus that projects neurons onto various brain regions, including the hippocampus and amygdala (39). Noradrenergic modulation of the hippocampus facilitates memory formation and emotional arousal (39). In cognitive disorders, these p-tau proteins are present in these LC neurons, thus promoting neuron degeneration. Neuron cell death induced by p-tau has strongly been implicated in AD, suggesting a strong correlation between hyperglycaemia and neurocognitive disorders such as AD(37). Many studies have displayed the ability of vanadium to promote the activation of phosphor-inositol 3 kinase (PI3K) and Akt as part of its anti-hyperglycaemic mechanism (40). These proteins favour cell survival and regulate the activity of mTOR (36). We speculate that this vanadium complex may prevent the aggregation of pTau by reducing its formation through regulated mTOR activity via increased PI3K/Akt activity.

5. Implications of insulin therapy on the brain function

Insulin receptors are located on the soma of hippocampal neurons and have been associated with the long-term potentiation of signals. Insulin binding to its receptors results in the activation of the insulin receptor substrate followed by phosphorylation and activation of phospho-inositol-3 kinase (PI3K) and subsequently AKT. Phosphorylation of AKT regulates multiple signalling pathways that facilitate improved neuronal function in the hippocampus, such as upregulation of the AMPK pathway resulting in the activation of PGCα and PINK1, which are proteins responsible for improved mitochondria activity and decrease β oxidation and ROS formation in hippocampal neurons. As mentioned above insulin is used as the primary treatment for type 1 diabetic individuals (41). Although insulin has been shown to improve cognitive function when produced in the body or administered bolus, insulin has been shown to cause insulin resistance and acute episodes of hypoglycaemia in the brain due to the concentration given in 8 times the amount of insulin produced by the body, ultimately resulting in detrimental effects in neurons (41).

GLUT1 transporters on the BBB attempt to accommodate insulin-induced hypoglycaemia by increasing the affinity for glucose however are unable to establish normal glycaemic levels (42). Monocarboxylic transporters (MCT) on BBB capillaries upregulate the intake of ketone bodies and pyruvate to feed into the TCA cycle to continue ATP production (43). Exclusive to glial cells in the formation of acetyl CoA derived from acetate. The enzyme acetyl CoA synthetase catalyses this with a resultant prolonged TCA cycle. However, intermediates of glycolysis and the TCA cycle are precursors in the production of neurotransmitters (43). Therefore, decreased glucose metabolism prevents neurotransmitter production. Eventually, MCT substrates are depleted, and the TCA cycle is terminated (7). The production of ATP via oxidative phosphorylation is prohibited, and decreased neurotransmitter and ATP production result in brain cellular destruction, thereby contributing to memory loss and learning defects (43). Vanadium possesses insulin-mimetic effects. Therefore, scientists are exploring various vanadium-based complexes to discover if these effects can be manipulated to benefit patients living with diabetes without the toxic effects attributed to bolus insulin treatment and organic vanadium salts.

6. Alternative treatment for diabetes

There are various alternative treatment strategies proposed for diabetes. Synthetic treatments such as biguanides and insulin control hyperglycaemia, however not without adverse effects. (44)Medicinal plants, stem cell therapy, and metallotherapy have been some of the most extensively researched alternative methods for diabetes treatment, and the scientific results with regards to improving wellness in a diabetic individual are becoming more promising with time (45).

6.1 Transition metals and metal-based complexes

Studies have shown that transition metals such as zinc, magnesium, and vanadium have been shown to possess medicinal effects (45). The oxidation state and ligands attached to these metals contribute to their properties (35). The oxidation state directs which metabolic pathways the metal will be integrated to and the ligands control the reactivity and the type of interactions the metal makes (46). These are determining factors, differentiating between toxic and beneficial responses after the compound is administered. Without the addition of ligands, the metals possess a very unstable nature. The coordination geometry of these various ligands bound to a single metal atom provides stability as they donate a pair of electrons to the partially filled d shells of the metal, which stabilize its oxidation state and improve the potency of metallodrug (46).

6.2 Vanadium

Among them, vanadium has been the most potent in reducing blood glucose (47). Vanadium is a Group-V trace element with oxidation states of -1 to 5+ and are abundant in the environment (47). Under physiological conditions, vanadium is more commonly available in 2 forms, vanadyl and vanadate, which are interchangable with each other under the appropriate conditions (48). According to a study

done by Maanvizhi et al., the antidiabetic properties of vanadium salts were discovered in the early 1980s where the salt was ingested with drinking water and resulted in the reversal of diabetic symptoms in diabetic rats (49). Vanadate is stored as vanadyl intracellularly but converted to vanadate under oxygenated conditions because of its rich redox chemistry (48). This quality contributes to its insulin-mimetic effects as vanadate has been shown to phosphorylate glucose metabolizing enzymes and facilitate glucose uptake into cells (48). Vanadium is present in every organ in minute amounts and contributes to physiological function, however vanadium tends to accumulate in brain, bone, heart and kidney if administered (10). This results in toxicity experienced in various organs such as heart, kidney and brain (10).

6.2.1Vanadium salts and the brain

Vanadium can freely pass through the BBB into the brain parenchyma, which consists of the neurons and glial cells (10). It is actively transported via transferrin and divalent metal ion transporter (DMT1) (10, 50). Vanadium prefers delivery to the olfactory bulb, hippocampus, and cerebellum (50, 51). Studies have shown that exposure to increased accumulation of vanadium correlates with increased DMT1 and transferrin. Once accumulated in the brain, it contributes to the formation of ROS by depleting the antioxidant glutathione. Acute exposure to vanadium results in microglial activation in the hippocampus and cerebellar area and promotes the microglia inflammatory pathway by which cytokines are released (51). This is an attempt at functional recovery and axon regeneration. However, prolonged exposure results in CA-1 pyramidal neuron degeneration and dendritic spine loss (51, 52). These neurons are responsible for spatial memory. Vanadium has also been shown to play a role in demyelination, disruption of the BBB, and behavioural and locomotor defects by promoting lipid peroxidation, apoptosis, and DNA cleavage (10).

6.3 Vanadium complexes

Vanadyl has been shown to possess less toxic effects as opposed to vanadate (37). Complexes synthesised from vanadyl and maltol (3-hydroxy-2-methyl-4-pyrone) and kojic acid (3-hydroxy-2-hydroxymethyl-4-pyrone) ligands were shown to mimic insulin with minimal toxic effects. However the Bis(maltolato)oxovanadium(IV) was the first vanadium complex that showed enhanced antidiabetic effects when compared to vanadium salts (53). The chelation of ligands to the metal ion provides increased bioavailability and removal of the drug after its use, thereby avoiding toxic accumulation (53). Unlike vanadium salts, vanadium complexes have been shown to reduce oxidative stress by increasing the concentration of antioxidants such as GSH (54). Scientists have not been able to study all interactions made by vanadium complexes therefore, the exact mechanism used to treat diabetes is still under investigation.

7. Justification of the study

Vanadyl has been shown to possess insulin mimetic effects by inhibiting tyrosine phosphatase. This results in prolonged activation of insulin signalling proteins and, it is ineffectively cleared from organs. It is however associated with toxic accumulation. We have therefore synthesized vanadium complex dioxidovanadium (V) [$VO(Hpybz)_2SO_4.H_2O$] by the 2:1 molar ratio reactions of the heterocyclic ligand 2-pyridylbenzimidazole (Hpybz) with vanadyl (IV) sulphate. The organic ligand functioned as a chelator to promote the clearance of vanadyl from the body(44).

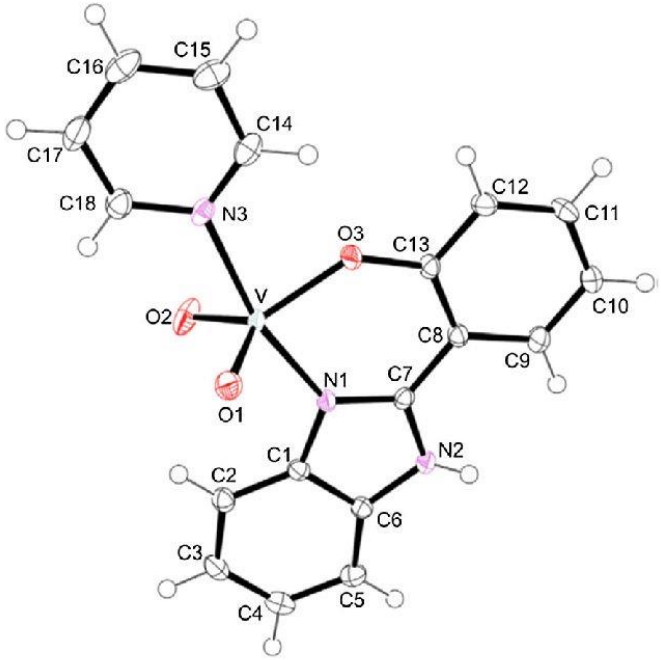

Figure 3: The molecular structure of dioxidovanadium (48)

Pyridylbenzimidazole (Hpybz) is also one of the well-established promising heterocyclic ligands which have also been shown to have an array of biological activities such as antimicrobial, antibacterial, and anti-diabetic activities (46). Currently there are no studies which document the effects of novel dioxidovanadium complex on brain function. However, since the complex has successfully been shown to alleviate hyperglycaemia and hyperglycaemia associated complications in the liver, heart muscle and red blood cell, with limited traces of toxicity in our laboratory, we speculate that treatment with this complex might be beneficial in alleviating hyperglycaemia-induced brain dysfunction such as memory impairment thereby favouring investigation of dioxidovanadium in the hippocampus (9, 55, 56).However, organic vanadium salts have been considered toxic to the hippocampus without disease, it is essential to understand the effects of the complex on non-diabetic rats. Following the successful results of the first study, we considered that diabetes induces its own neuro-pathological effects on the hippocampus therefore as an antidiabetic drug it becomes imperative to study the effect of dioxidovanadium (V) on the hippocampus in a diabetic animal model.

8. Aims

The aims of this study are divided into 2 sub-studies with each being presented as a manuscript:

1. The aim of the first study is therefore to investigate the effects of dioxidovanadium on blood glucose concentration and oxidative stress in the hippocampus of healthy male Sprague Dawley rats over an acute phase.

2. The second study investigated the chronic effects of dioxidovanadium on oxidative stress formation and selective markers associated with learning and memory in STZ-induced diabetic male Sprague Dawley rats.

9. Objectives

The objectives of the first study are as follows:

1. To investigate the effect of dioxidovanadium on blood glucose concentrations in healthy Sprague Dawley rats over an acute period of 10 days.
2. To assess the effects of dioxidovanadium on the oxidative status in the hippocampus of healthy Sprague Dawley rats.

The objectives of the second study are as follows:

1. To investigate the effect of dioxidovanadium on blood in glucose concentration in STZ-induced diabetic rats.
2. To investigate the effects of dioxidovanadium on learning and memory, using the Morris water maze.
3. To investigate the effect of dioxidovanadium on the oxidative status in the hippocampus of STZ-induced diabetic rats.
4. To assess the effects of dixidovanadium on the formation of pathological markers in the hippocampus, affecting memory.

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CHAPTER 2: MANUSCRIPT 1

"The acute effects of dioxidovanadium on blood glucose concentration and oxidative stress in the hippocampus of healthy male Sprague Dawley rats"

Background

Bolus insulin, the gold standard treatment for type 1 diabetes mellitus, has shown to successfully improve glycaemia in patients however not without undesirable effects to certain organ systems such as the brain. Certain areas of the brain such as the hippocampus have a high energy requirement to perform engaged cognitive tasks such as memory and learning. However due to hypoglycaemic episodes elicited by the bolus insulin treatment, neurons are starved and unable to function. These complications have warranted the search for alternative treatment for diabetes. In recent years, vanadium has been acknowledged for its anti-diabetic effects. Vanadium is a transition metal with varying oxidation states which allows it to participate in multiple biological systems and perform physiological functions such as glucose lowering effects in a hyperglycaemic state. It can freely cross the blood brain barrier and favours delivery to the hippocampus, olfactory bulb and cerebellum. However organic vanadium salts have been associated with toxic accumulation often associated with toxicity and neurodegeneration. In our laboratory, we have synthesized dioxidovanadium (V) by attaching organic ligands, pyridine and benzimidazole, to the metal that improve its bioavailability, prevent accumulation and utilize its therapeutic properties to attenuate hyperglycaemia. The complex has been shown to improve renal, cardiac and red blood cell function in a diabetic model. However, the toxicity of this drug on the hippocampus have not been investigated. Therefore, this study sought to investigate the acute effects of dioxidovanadium on oxidative stress in the hippocampus of non-diabetic male Sprague Dawley rats.

The manuscript in chapter 2 is titled **"The acute effects of dioxidovanadium on blood glucose concentration and oxidative stress in the hippocampus of non-diabetic male Sprague Dawley rats"** and is authored by Dayanand Y, Sibiya NH, Khathi A, Booysen I and PS Ngubane and has been prepared for submission to **The Canadian Journal of Diabetes** and formatted according to the journal guidelines to authors for publication. (see Appendix B)

Title: "The acute effects of dioxidovanadium on blood glucose concentration and oxidative stress in the hippocampus of non-diabetic male Sprague Dawley rats"

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Key Message: Research has shown that vanadium is an insulin mimetic, anti-diabetic metal. However, it has previously been shown to be toxic to the hippocampus by inducing oxidative stress and therefore neuronal injury. In this study we have synthesized dioxidovanadium by attaching organic ligands pyridine and benzimidazole, that improve potency and reduce toxicity. Therefore, this study sought to investigate the anti-hyperglycaemic effects of dioxidovandium (V) on oxidative stress in the hippocampus of healthy Sprague-Dawley rats.

Key words: Dioxidovanadium, oxidative stress, antioxidants, hippocampus, anti-hyperglycaemia

Word count: Abstract: 257, Manuscript:2566

Abstract

Objectives: Conventional treatments for diabetes such as bolus insulin have shown to result in hypoglycaemic episodes thus affecting the high glucose requirement needed for hippocampal function which warranted the investigation of alternative treatments. In our laboratory, we have synthesized dioxidovanadium (V) in order to eliminate toxic effects of naturally occurring vanadium whilst improving its potency, however we are yet to establish its effect on the hippocampus. Therefore, in this study we investigated the acute effects of effects of dioxidovandium (V) on oxidative stress in the hippocampus of healthy Sprague-Dawley rats.

Methods: 18 Healthy Sprague-Dawley rats were administered with the vanadium complex (40mg/kg) orally at time 0 (ND-VAN). The control group (ND) (18 rats) received the vehicle only (DMSO solution p.o.). 3 rats from each group were sacrificed at time intervals (1 hour, 2 hours, 6 hours, 24 hours, 5 days and 10 days) after administration at time 0. Blood glucose concentration was recorded prior to sacrificing. The hippocampus was harvested and the following biochemical markers were analysed: MDA, GPx1, TNF-α.

Results: The glucose concentrations between ND and ND-VAN were not significantly different at all the time intervals over the 10-day experiment. Furthermore, MDA was not significantly higher in ND-VAN in comparison to ND group. GPx1 levels in the ND-VAN group were not significantly lower than the ND group. There was also no significant difference observed in the concentration of TNF-α between groups.

Conclusion: The vanadium complex dioxidovanadium (V) did not show any hypoglycaemic effects over an acute period of time. We can also conclude there was no increase in oxidative stress by dioxidovanadium (V). These results in part may suggest that dioxidovanadium does not induced toxicity to the hippocampus acutely.

Introduction

In recent studies, insulin has been shown to facilitate underlying neuronal pathways and promote neuronal function, thereby playing a role in cognition such as learning and memory; therefore, a decline in cognitive function can be observed in patients living with diabetes who are deficient of insulin or insulin action [1]. Despite the current success of exogenous insulin treatment, it has not been considered an ideal drug for the treatment of diabetes. Harmful side effects associated with insulin therapy include hypoglycaemic episodes and insulin resistance in the brain resulting in glucose-deprived (energy-deprived neurons) and reduced synaptic plasticity in neuronal pathways, respectively [2, 3]. These effects further promote various cognitive defects such as memory loss [2]. Such challenges associated with conventional treatment have warranted the investigation of alternative treatments that lower blood glucose concentration and are favourable to organ systems such as the brain. Vanadium has been recognized for its potential use as a therapeutic agent for tuberculosis, anaemia, cancer, and diabetes due to its blood glucose lowering effects [4].

Current research has shown increasingly favourable results on the antidiabetic properties of vanadium, thereby making it a viable treatment for patients suffering from diabetes [5, 6]. The insulin-mimetic effects of vanadium can be traced to the activation of various proteins in the insulin signalling pathway, such as tyrosine kinase, Phosphoinositide 3-kinase (PI3K), and Protein kinase B (AKT) [7-9]. Studies have shown that vanadium manipulates alternative pathways other than the insulin signalling pathway, such as activating mitogen-activated protein kinase (MAPK) or phosphorylation of cytosol kinases instead of membrane kinases, thereby promoting the improved metabolism of glucose [10]. Its therapeutic effects also come from forming hydrogen peroxide, a reactive oxygen species, which has been shown to possess insulin-mimetic effects in cells [10, 11].

Vanadium entry into the brain is uninhibited, and it can freely pass the blood-brain barrier [12]. However, delayed clearance of vanadium from the brain and continuous exposure has been previously reported to result in toxic accumulation [13]. Due to this pro-oxidant effect, there is a depletion of the brain's antioxidant stores, specifically glutathione [14]. Delivery of vanadium is favoured to areas such as the olfactory bulb, prefrontal cortex, and hippocampus, and therefore, these areas succumb to toxic effects such as neuroinflammation and neurodegeneration associated with increased reactive oxygen species diminished antioxidants [12, 15].

In an attempt to increase the bioavailability of vanadium, utilize its therapeutic effects and alleviate accumulative toxicity associated with vanadium, scientists have been synthesizing and investigating organic vanadium compounds [6]. These vanadium compounds consist of a central vanadium element complexed with organic ligands that possess their own beneficial qualities [16]. A more stable and potent form of vanadium is produced, which can be manipulated for medicinal purposes [17, 18].

In our laboratory, we have synthesized a vanadium complex dioxidovanadium (V), (cis-[VO_2(obz)py] Hobz=2-hydroxyphenyl-1H-benzimidazole and py =pyridine]) that has been shown to possess anti-hyperglycaemic effects, promote glucose uptake in skeletal muscle and the liver and improve cardiac and red blood cell function in a diabetic animal model [7, 16, 19, 20]. However, the effects of this vanadium complex in the hippocampus have not been investigated. It is essential to understand the effects of the treatment in the hippocampus without the factor of the disease to attribute any results obtained during the study solely to diodioxvanadium (V). Therefore, we sought to investigate the effects of this vanadium complex on blood glucose concentration and hippocampal oxidative stress in a healthy animal model.

Methods

Dioxidovanadium synthesis

Vanadium complex; Dioxidovanadium[$VO(Hpbyz)_2SO_4.H_2O$] (Hpbyz = 2-pyridylbenzimidazole), synthesized using the UV–Vis, Emission, EPR, IR, V- and H NMR spectroscopy and crystal X-ray diffraction by Prof. Booysen at UKZN Pietermaritzburg campus chemistry department.

Animal housing

Healthy male Sprague-Dawley rats (250-300g) bred in the Biomedical Research Unit at the University of Kwa-Zulu Natal (South Africa) were housed individually in Makrolon polycarbonate metabolic cages (Techniplast, Labotec, South Africa). All animal procedures and experimental conditions were approved by the Animal Research Ethics Committee of the University of KwaZulu-Natal which conforms to the principles and guidelines of Canadian Council on Animal Care (ethics no. **AREC/014/020M**). Acclimatization (duration: 5 days) was conducted before the study. The animals experienced a 12hr day:12 hr night cycle and were kept under standard laboratory environment (constant temperature and humidity) with free access to water and rat chow *ad libitum* (Meadow Feeds, Pietermaritzburg, South Africa). There was close monitoring for pain and discomfort using criteria in the UKZN institutional animal ethics committee's humane endpoint document.

Drug administration and sample collection

36 healthy male Sprague-Dawley rats were used for this experiment. The test group (18 rats) were administered with the vanadium complex (40mg/kg) orally at time 0. The control group (18 rats) received the vehicle only (DMSO solution p.o.). Rats (n=3 as per ethical recommendations) from each group were euthanized at 1, 2, 6 and 24 hours', 5 and 10 days' post-administration via decapitation. Hippocampi were then collected, weighed and snap-frozen in liquid nitrogen. The collected hippocampi

were then stored at -80°C in the Ultra Bio Freezer (Snijers Scientific, Tilburg, Netherlands) until biochemical analysis.

Biochemical analysis

MDA analysis assay

To assess lipid peroxidation in the hippocampus 40 mg of tissue was homogenized in 400µL of 0.2% phosphoric acid followed by centrifuging for 10 min at 400 x g. Thereafter, 400µL 2% phosphoric acid was added to 400µL of the homogenate and separated equally into 2 glass tubes. Subsequently 400 µL of thiobarbituric acid (TBA)/butylated hydroxytoluene (BHT) was added into one glass tube (sample) and 3mM of HCL was added to the second glass tube (blank). To provide an acidic pH of 1.5 200 µL of 1 M HCl was added to all glass tubes and was heated at 100°C for 15 min. The solutions were then allowed to cool to room temperature. The cooled solutions were supplemented with 1.5 mL of butanol and vortexed for 1 min. After allowing the solutions to settle into 2 distinct phases, the top layer (butanol solution) was decanted into Eppendorf tubes and centrifuged at 13200 x g for 6 min (digicen 21R, orto alresa). A BioTek mQuant spectrophotometer (BioTek, Johannesburg, South Africa) was used to check absorbance at 532 nm (reference 600 nm) using 96-well micro titer plates. This absorbance will be used to calculate MDA concentration using Beer's Law.

$$\text{Concentration of MDA (mM)} = \frac{\text{Average Absorbance}}{\text{Absorption coefficient } \left(156\ \text{mmol}^{-1}\right)}.$$

GPx and TNF α analysis

A sandwich ELISA kit (Elabscience and Biotechnology, WuHan) was used following the manufacturer's instruction to assess the above-mentioned biochemical markers in the hippocampus. Hippocampal tissue was weighed and homogenized in phosphate buffered saline (PBS) and centrifuged for 10 min at 5000 x g (digicen 21R, orto alresa). 100 Standards or samples (100 µL) were added to the wells and incubated for 90 min at 37°C. Post 90 min, the liquid was discarded and 100 μL of biotinylated detection antibody (Ab) was added to each well and incubated for 60 min at 37°C. After incubation the liquid was aspirated and subjected to 3x washes using wash buffer. A 100 μL of horse radish peroxidase (HRP) conjugate was added and allowed to incubate for 30 min at 37°C. The plate was then subjected to 5 x washes of substrate reagent (90µL) was added followed by a 15 min incubation period at 37°C. 50 μL A stop (50 µL) solution was then added and a BioTek mQuant spectrophotometer (BioTek, Johannesburg, South Africa) was used to assess absorbance at 450 nm. Concentrations were extrapolated from the respective standard curves.

Statistical analysis

All data was expressed as means ± SD. Statistical comparisons were conducted on GraphPad Prism Instat Software (version 8.00, GraphPad Software, San Diego, California, USA). After confirming normal distribution of data, one tailed Student t- tests were used to analyse all parameters assessed followed by the Tukey-Kramer post hoc test for analysis of differences between groups. Values of $p<0.05$ were considered statistically significant.

Results

Figure 1 represents the blood glucose concentrations in the non-diabetic untreated (ND) and non-diabetic dioxidovanadium (40mg/kg) treated (ND-VAN) groups of animals over a period of 10 days to accommodate for maximum vanadium clearance. These concentrations for each group were recorded prior to sacrificing. It is noteworthy that dioxidovanadium did not show a significant hypoglycaemic effect in comparison to the normal untreated group over all experimental time intervals. Figure 2 presents the malondialdehyde (MDA) analysis in the hippocampus of both ND untreated (n=3) and ND-VAN (40mg/kg) groups (n=3) over all experimental time intervals. There was no significant difference in MDA concentration between the two groups throughout the experimental period. Figure 2 also shows the levels of GPx1 produced in the hippocampus of ND (n=3) and ND-VAN (40mg/kg) (n=3) groups over all experimental time intervals. There was no significant difference in GPx concentration between both the groups. The levels of GPx1 in both groups dropped during time 6 hours and remained lowered in ND during 24 hours. Figure 3 shows the levels of TNF-α in the hippocampus of ND (n=3) and ND-VAN (40mg/kg) (n=3) over all experimental time intervals. There was no significant decrease between the two groups over all time intervals.

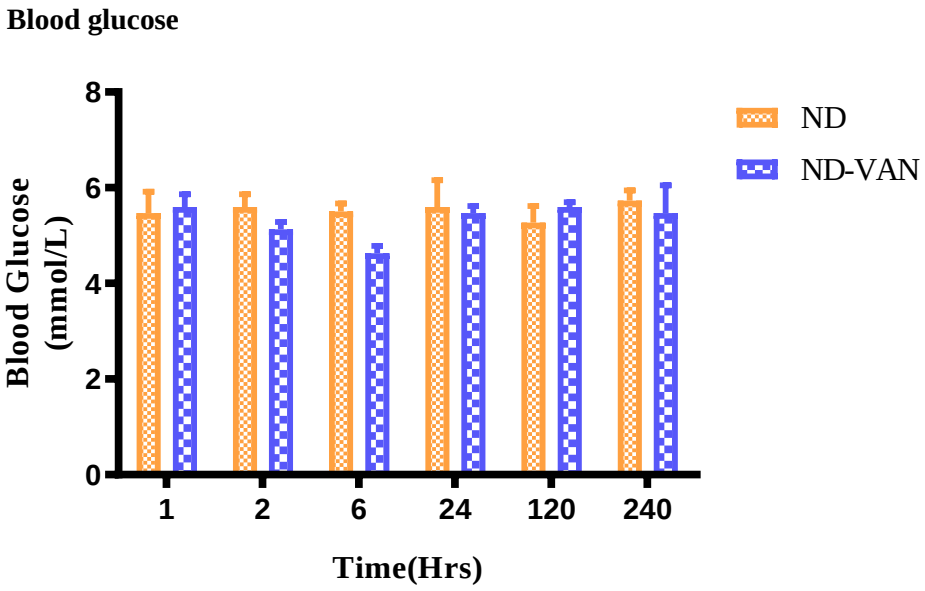
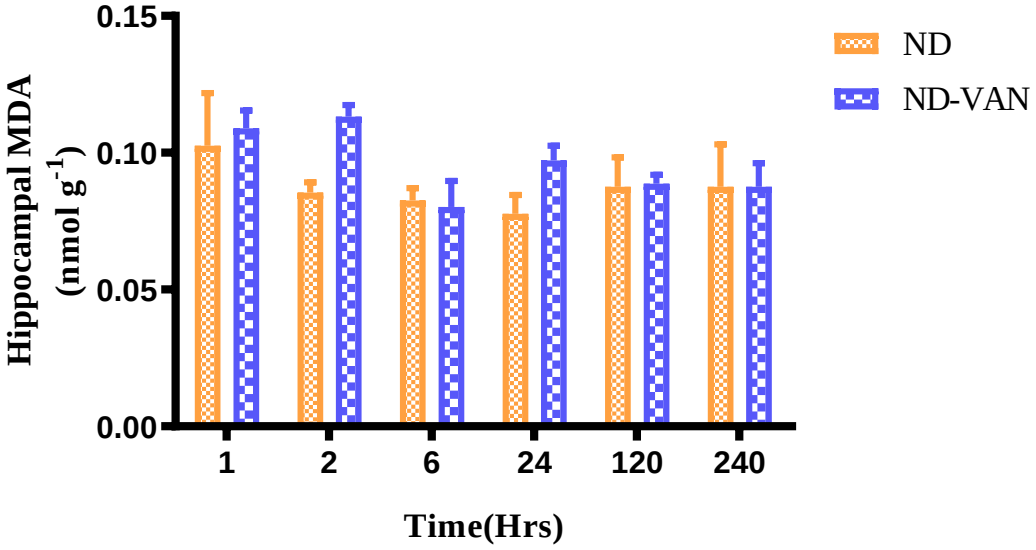


Figure 1 shows the glucose concentrations measured over 6 time intervals (1 Hr, 2 Hr, 6 Hr, 24Hr, 5 days, 10 days) in ND and ND-VAN groups. Values are expressed as means±SD.

Oxidative stress markers



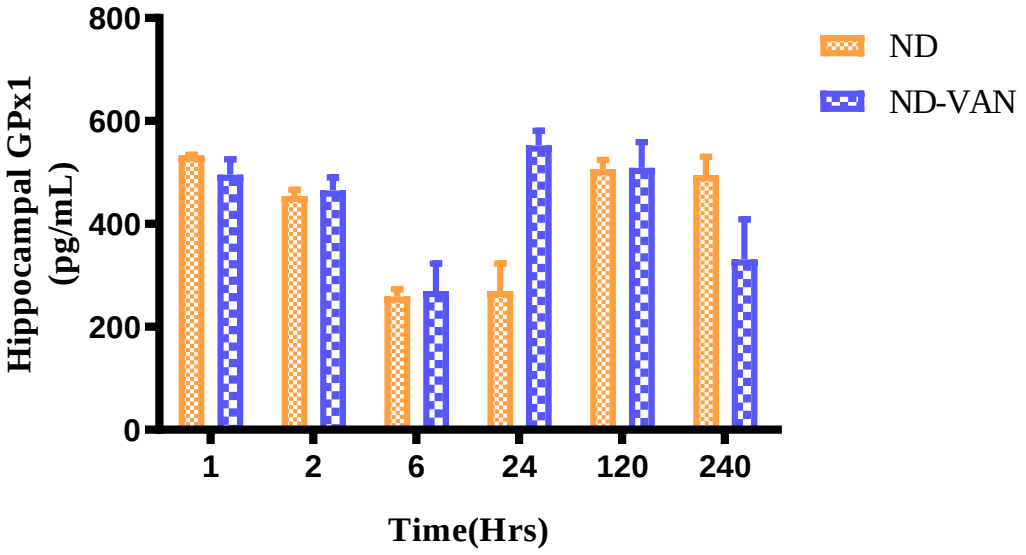


Figure 2 presents the concentration of hippocampal MDA and GPx1 produced in ND and ND-VAN groups over experimental time intervals (1 Hr, 2 Hr, 6 Hr, 24Hr, 5 days, 10 days). Values are expressed as means±SD.

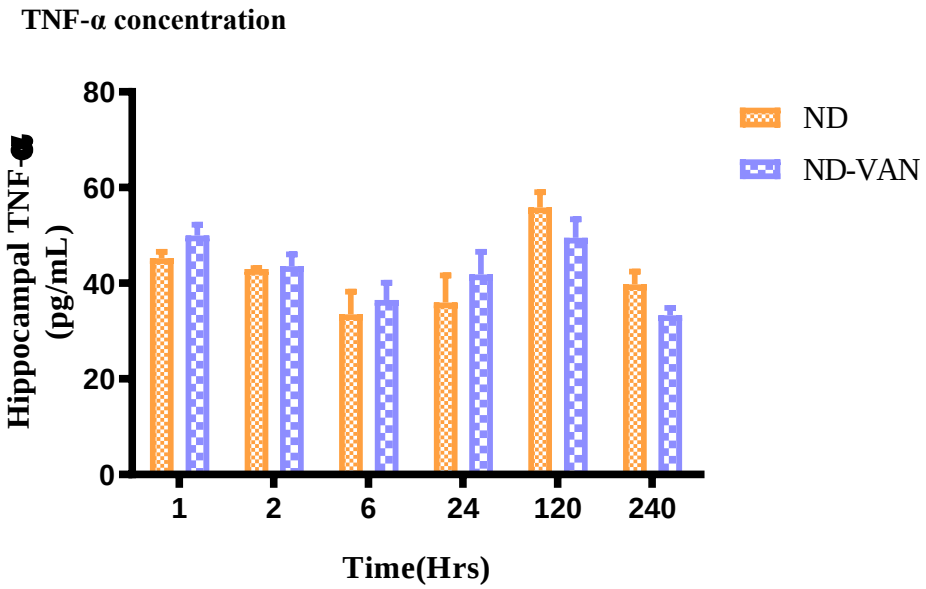


Figure 3 presents the levels of TNF-α produced in the hippocampus in the ND and ND-VAN groups over the 6 time intervals (1 Hr, 2 Hr, 6 Hr, 24Hr, 5 days, 10 days). Values are expressed as means±SD.

Discussion

The brain requires approximately 20% of the daily glucose intake as glucose is its primary energy source [21]. The metabolism of glucose in brain cells, both neuronal and non-neuronal, provides ATP for physiological function and contributes to neurotransmitter production [22]. Neurons require large amounts of energy to drive the sodium potassium (Na^+/K^+) pump to establish membrane potentials [10]. Research has shown that the hippocampus has a high energy requirement to perform engaged behavioural functions [23, 24]. This is supported by the decreased extracellular glucose concentration and increased glucose metabolism during neuronal activity [10, 24]. Cognitive functions such as spatial memory and learning can be attributed to the hippocampus [25].

Vanadium is insulin-mimetic and reduces blood glucose concentrations during hyperglycaemia, and is considered a potential therapeutic agent for diabetes due to the hypoglycaemic side effects of current treatment such as bolus insulin [26, 27]. We have synthesized dioxidovanadium (V) by adding organic ligands, 2-hydroxyphenyl-1H-benzimidazole and py=pyridine to attenuate vanadium toxicity and improve its therapeutic attributes [16, 19]. According to studies performed on vanadium, complete clearance occurs in 3 phases, the rapid, intermediate, and slow phase, which has been shown to occur at times 1hr, 26 hrs, and 10 days respectively, therefore, we designed the experiment to assess the effects of this vanadium complex at time intervals over 10 days to accommodate for various clearance phases [28, 29]. As reflected by our results of this vanadium complex administered to healthy rats did not show a significant decrease in glucose concentration throughout all time intervals after administration. Therefore, we speculate a compensatory mechanism associated with vanadium administration thereby preventing hypoglycaemia in non-diabetic animals. A study with corresponding results investigated the effects of Bis(maltolato)oxovanadium(IV) on non-diabetic fatty Zucker rats, where the concentration of plasma insulin had decreased after administration thereby preventing hypoglycaemia [30]. Furthermore, we deduce that this vanadium complex will not result in hypoglycaemic associated cognitive decline after exposure.

Another mechanism by which naturally occurring vanadium has been shown to reduce blood glucose concentration is through hydrogen peroxide (H_2O_2) production, which has also been shown to mimic insulin by enhancing glucose transport and inhibiting lipolysis [10, 11]. The production of H_2O_2 may occur in 2 possible ways. First is the participation of vanadyl, the tetravalent most occurring vanadium species, in various physiological oxidation reactions [4, 10]. Vanadyl participating in other oxidation reactions in the presence of nicotinamide adenine dinucleotide (NAD) + hydrogen (H) (NADH) is the second pathway that produces H_2O_2 [10]. Despite H_2O_2 having beneficial medicinal effects, accumulative vanadium induces increased production of H_2O_2 which promotes lipid peroxidation [31]. Lipid peroxidation is the reaction between unstable transition metals and polyunsaturated fatty acids

(PUFA) through hydrogen abstraction [32]. Continuous removal of hydrogen from cellular components such as the cell membrane results in the formation of lipid hydroperoxides, thereby inducing oxidative stress in cells and cellular injury [32, 33].

The brain is an organ that is highly sensitive to oxidative stress due to its high content of PUFA [33]. Vanadium delivery to the brain has been confirmed by the LA-ICP-MS results of research done by Folarin et al., which showed evidence that vanadium crosses the blood brain barrier (BBB) and enters the brain parenchyma after oral administration [29]. Furthermore since vanadium favours delivery to areas such as the hippocampus, it is considered toxic and results in neurodegeneration [33, 34]. Malonaldehyde (MDA) is the by-product of lipid peroxidation and is considered a key marker in cognitive diseases such as Alzheimer's and Dementia therefore, we measured MDA in an attempt to reflect the concentration of lipid peroxidation induced by our drug [31]. As presented in figure 2, the concentration of MDA was not significantly higher than our control group. Furthermore, we can assume from this result that dioxidovanadium is more stable and non-toxic to the hippocampus than its naturally occurring forms. This can be traced to the addition of the ligand to vanadyl sulphate which prevents the constant changing of oxidation states thereby preventing pathological interactions of dioxidovanadium (V) with biological systems [16].

In order to affirm that dioxidovanadium (V) did not catalyze the formation of H_2O_2 through other metabolic pathways, we investigated the levels of a major antioxidant in the brain, Glutathione peroxidase (GPx1). GPx1 is responsible for forming water from H_2O_2 [35]. In pathological conditions where H_2O_2 is produced in large amounts, the levels of GPx1 diminish to neutralize H_2O_2 [35]. According to our results in figure 2, the levels of GPx1 did not significantly decrease in the dioxidovanadium (V) treated group compared to our control group, thereby confirming an undetectable toxicity concentration to the neuronal non-neuronal cells of the hippocampus.

A group of glial cells termed microglia are responsible for immunity in the central nervous system [36]. Microglia are the most sensitive to neuronal abnormalities and cellular injury, which promote their activation and proliferation [36]. They are also known to produce ROS in these circumstances [37]. This contributes to the oxidative stress in the hippocampus and is detected by pro-inflammatory markers such as TNF-α, produced during microglial activation [37]. However, vanadium is an anti-inflammatory agent, and as reflected by figure 3, there was no significant increase in TNF-α therefore, we can speculate that there was no cellular injury to induce the activation of microglia and promote the release of TNF-α.

Conclusion

In conclusion, the administration of dioxidovanadium (V), {cis-[VO2(obz)py] Hobz=2-hydroxyphenyl-1H-benzimidazole and py =pyridine]} complex to healthy male Sprague-Dawley rats did not promote hypoglycaemia. We can also deduce this complex does not possess the pro-oxidant and anti-oxidant

depleting effects that is attributed to naturally occurring vanadium as reflected by normal MDA and GPx1 concentration. We speculate that dioxidovanadium (V) did not promote cellular injury which was reflected by normal levels of TNF-α. Furthermore, we can conclude that dioxidovanadium (V) is non-toxic to the hippocampus when administered acutely to a healthy animal model.

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Authors' contributions

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; gave final approval of the version to be published; and agree to be accountable for all aspects of the work.

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Bridge

The first study demonstrated that dioxidovanadium had no hypoglycaemic effect on the hippocampus in a non-diabetic animal model. In addition to this, dioxidovanadium did not promote lipid peroxidation or antioxidant depletion. This related to no significant increase in neuroinflammation in the hippocampus. Therefore, this study concluded that dioxidovanadium treatment was non-toxic to the hippocampus in a non-diabetic animal model which lead to the hypothesis that dioxidovanadium is non-toxic to the hippocampus in the presence of disease and may prevent memory impairment associated with diabetes which led to the second study that investigated the effects of dioxidovanadium on the hippocampus and learning memory in an STZ-induced diabetic animal model.

CHAPTER 3: MANUSCRIPT 2

"The chronic effects of dioxidovanadium on selected markers associated with hippocampal dysfunction in male STZ-induced diabetic rats."

Prologue

The progression of diabetes mellitus often increases the risk of its coexistence with cognitive disorders such as memory loss and therefore has been considered a risk factor for developing diseases such as dementia and Alzheimer's. The key pathological markers induced by diabetes which are prevalent in memory impairment are oxidative stress, increased inflammation and increased concentration of amyloid beta and hyper-phosphorylated tau in the hippocampus thereby providing a strong correlation between these diseases. Bolus insulin is the gold standard treatment for chronic hyperglycaemia however this has shown to exacerbate neuronal damage associated with diabetes and therefore warrants the search for alternative treatment. Vanadium has been acknowledged for its antidiabetic effects however its delivery to the hippocampus results in toxic accumulation. In order to alleviate the toxic effects and enhance the therapeutic properties of vanadium, we have synthesized dioxidovanadium through the attachment of organic ligands. These function as chelators and prevent accumulation often associated with toxicity which ultimately reduces learning and memory. Dioxidovanadium (V) has previously been shown to improve glycaemic control and possess reno-protective and cardio-protective effects. Investigations on toxicity and oxidative stress in an acute study demonstrated that dixodivanadium (V) was not associated with increased oxidative stress and therefore considered non-toxic to the hippocampus of non-diabetic male Sprague Dawley rats since there was no depletion of anti-oxidant GPx1 and no cellular injury reflected by TNF-α. However, the effects of this vanadium complex, chronically on cognitive tasks such as learning and memory and neuronal health in the hippocampus which are yet to be investigated. Therefore, this study aimed to investigate the chronic effects of dioxidovanadium (V) on the learning and memory function in the hippocampus of STZ-induced diabetic rats.

The manuscript in chapter 3 is titled **"The chronic effects of dioxidovanadium on selected markers associated with hippocampal dysfunction in male Streptozotocin-induced diabetic rats."** and is authored by Dayanand Y, Sibiya NH, Khathi A, Booysen I and PS Ngubane and has been prepared for submission to the **Journal of Neuroendocrinology** and formatted according to the publication guidelines for authors. Please refer to guidelines in Appendix B.

Title: The chronic effects of dioxidovanadium on selected markers associated with hippocampal dysfunction in male Streptozotocin-induced diabetic rats.

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Author Disclosures

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Abstract

Diabetes mellitus (DM) is metabolic disorder resulting from impaired glucose homeostasis. Chronic hyperglycaemia experienced in patients living with diabetes promote an array of secondary complications such as memory impairment. Current therapeutic options such as bolus insulin are shown to exacerbate memory impairment induced by diabetes. Therefore, scientists are seeking an alternative therapeutic option that can circumvent the undesired effect. Vanadium complexes have become increasingly favourable as an antidiabetic treatment due to its ability to lower blood glucose concentration in a hyperglycaemic environment. However naturally occurring vanadium salts are considered neurotoxic therefore in our laboratory we have synthesized vanadium complex, dioxidovanadium (V), with the aim of effectively reducing blood glucose concentrations and eliminating toxicity. This study therefore sought to investigate the chronic effects of dioxidovanadium (V) on the learning and memory function in the hippocampus of STZ-induced diabetic rats. Streptozoticin (STZ) was used to induce diabetes in 18 male Sprague-Dawley rats. Diabetic rats were divided into untreated, vanadium complex treated (40 mg kg^{-1} p.o) and insulin treated (0,175 mg kg^{-1} s.c). A group of non-diabetic animals were considered as an absolute control. A non-diabetic dioxidovanadium (V) treated group was also included. The insulin group was treated daily. Vanadium treated groups, both non diabetic and diabetic were administered with treatment twice every third day to allow for efficient clearance and prevent accumulation. Blood glucose concentration was recorded weekly and Morris water maze (MWM) was conducted on the 5^{th} week of the study. After 5 weeks, the animals were sacrificed where the hippocampal tissue was harvested for MDA, GPx1, TNF-α, amyloid β and pTau analysis. The results in the untreated diabetic group reflected a significantly higher blood glucose concentration in comparison to the absolute control. Dioxidovanadium (V) treated diabetic rats experienced a significant decrease in blood glucose concentration, however the drug did not lower blood glucose in dioxidovanadium (V) treated normal rats. Vanadium treated diabetic groups displayed a decrease in oxidative stress indicated by decreased MDA and increased GPx1 when compared to the untreated diabetic group. There was no significant change in TNF-α in all groups except insulin treated diabetic rats which was significantly increased compared to the diabetic control. Amyloid beta concentration was significantly decreased in the diabetic control in comparison to the non-diabetic control. The vanadium treated diabetic groups were not significantly different from the diabetic control. The concentration of amyloid beta in the diabetic dioxidovanadium (V) group was not significantly different from the diabetic untreated group which interestingly was significantly lower than the non-diabetic control. The non-diabetic dioxidovanadium (V) followed the same trend as the diabetic control which was significantly lower than the absolute control. The concentration of pTau was not significantly different in all groups. There was a significant memory decline observed in all diabetic groups compared to the non-diabetic group recorded in the probe test. Dioxidovanadium (V) significantly improved glycaemic control and reduced oxidative stress in the hippocampus of a diabetic animal model. The

effect of the complex on inflammation, Aβ and pTau could not be conducted due to the short duration of the study which did not allow for the pathological production of these markers.

Key words: Diabetes, Oxidative stress, Amyloid beta, neuroinflammation, tau tangles, dioxidovanadium

Introduction

Impaired glucose homeostasis resulting from chronic hyperglycaemia is a key characteristic of the metabolic disorder, diabetes mellitus [1, 2]. Gluco-toxicity and alternative metabolic pathways induced by diabetes play a crucial role in the development of microvascular and macrovascular complications in patients living with diabetes [3]. As a macrovascular organ sensitive to glycaemic control, the brain is shown to be 1.5 times more susceptible to dysfunction in a hyperglycaemic environment [4, 5]. The mechanism of memory consolidation is still unclear however the formation of neuronal pathways in the hippocampus are responsible for carrying out engaged cognitive tasks such as learning and memory [6]. According to previous studies diabetes is strongly associated with the development of diseases such as Alzheimer's and Dementia [7].

Pathological events that have been utilized in the detection of these cognitive diseases include oxidative stress via reactive oxygen species generation and antioxidant depletion, increased inflammation, Amyloid beta (Aβ) plaque formation, and hyperphosphorylation of tau proteins aggregating to produce neurofibrillary tau tangles [8]. These processes mediate neurotoxicity and induce neurodegeneration of neurons in the hippocampus [9]. Although neurons are genetically similar, the structure of neurons present in the hippocampus is different and are more prone to experiencing neuron destruction which is directly proportionate to the loss of function [10]. Chronic hyperglycaemia facilitates the development of these markers which has led scientists to believe that there is a correlation between diabetes and cognitive dysfunction [5]. Insulin signalling has recently been discovered to play significant roles in memory formation and neuronal health [8, 11]. The presence of insulin in the hippocampus promotes the production of antioxidants and reduce oxidative stress [11]. Insulin is also able to eliminate accumulation of hyper-phosphorylated tau (pTau) and Aβ by preventing hyperphosphorylation and inducing degradation respectively [12].

In spite of bolus insulin successfully lowering blood glucose concentration, there are complications associated to the dose required to restore normo-glyceamia [13]. According to research, the administration of bolus insulin is 8 times the amount of insulin that the pancreas would produce [14]. The ramifications of this have increased the possibility of patients experiencing insulin-resistance and episodes of hypoglycaemia [15]. This may exacerbate the neurodegenerative effects of diabetes. Studies show that the brain is affected by hypoglycaemia more severely than hyperglycaemia [13]. The brain is dependent on a minute to minute supply of glucose to form adenosine triphosphate (ATP) [16]. This drives the Na^+/K^+ pump and establishes membrane potentials required to form action potentials [17]. Insufficient glucose in the hippocampus would result inefficient neuronal communication and contribute to memory impairment [9, 18].

Therefore, scientists are seeking for alternative treatment and the transition metal vanadium is becoming greatly recognized for its antidiabetic and other medicinal properties [19]. However naturally organic

vanadium has been considered neurotoxic due to unrestricted transport through the blood brain barrier, ultimately accumulation in areas such as the hippocampus [20]. To improve the bioavailability of the vanadium, manipulate its antidiabetic effects and eradicate the toxicity associated with vanadium, organic ligands have been complexed with the metal [21, 22]. These ligands behave as chelators that promote the safe removal of vanadium, stabilize the oxidation states and improve its potency as an antidiabetic therapeutic agent [23]. In our laboratory, we have synthesized dioxidovanadium (V) by incorporating Hobz=2-hydroxyphenyl-1H-benzimidazole and py =pyridine ligands to achieve the desired medicinal benefits and eliminate toxicity. This vanadium complex has shown to successfully reduce blood glucose concentration in diabetic animal models and improve cardiac and renal function [23-25]. However, the effects of this vanadium complex on the hippocampal health and learning and memory are yet to be elucidated. Therefore, the aim of this study was to investigate the chronic effects of dioxidovanadium (V) on the learning and memory function in the hippocampus of STZ-induced diabetic rats.

Methods

Dioxidovanadium synthesis

Vanadium complex; Dioxidovanadium[$VO(Hpbyz)_2SO_4.H_2O$] (Hpbyz = 2-pyridylbenzimidazole), synthesized and characterized using the UV–Vis, Emission, EPR, IR, V- and H NMR spectroscopy and crystal X-ray diffraction by a research group led by Prof. Booysen at UKZN Pietermaritzburg campus chemistry department.

Animal housing

Healthy male Sprague-Dawley rats (250-300g) bred in the Biomedical Research Unit at the University of Kwa-Zulu Natal (South Africa) were housed individually in Makrolon polycarbonate metabolic cages (Techniplast, Labotec, South Africa). Acclimatization (duration: 5 days) was conducted before the study for animal stabilization in a new environment. The animals experienced a 12hr day: 12 hr night cycle and were kept under standard laboratory environment (constant temperature and humidity) with *ad libitum* access to water and rat chow (Meadow Feeds, Pietermaritzburg, South Africa). The animal experimental design was reviewed and approved by the Animal Research Ethics Committee of the University of KwaZulu-Natal (AREC/014/020M). There was close monitoring for pain and discomfort according to criteria in the UKZN institutional animal ethics committee's humane endpoint document.

Induction of diabetes

Non-diabetic male Sprague Dawley rats (18) were injected with a single intraperitoneal injection of STZ (60 mg-kg-1). STZ was prepared in a citrate buffer with a pH of 4.5. Blood glucose concentrations

in all groups were examined 7 days' post-induction using the tail-prick method and OneTouch select glucometer (Lifescan, Mosta, Malta, United Kingdom). STZ-induced rats with greater than 18 mmol/l glucose concentration were confirmed as stable diabetic.

Experimental Protocol

1. Administration of treatment

The study consisted of 5 experimental groups with n=6 in each group. The groups were divided into non-diabetic control (ND), diabetic control (DC), insulin treated (INS), vanadium treated (D-VAN) and non-diabetic vanadium treated (ND-VAN). The untreated groups received the vehicle only (2% DMSO solution p.o.). The untreated and vanadium treated groups (40 mg kg^{-1} p.o) were administered the specific treatment using an 18-guage gavage needle (Kyron Laboratories (Pty) LTD, Benrose, South Africa) twice every third day at 9:00 am and 3:00 pm. The insulin treated group (0.175 mg kg^{-1} s.c) were treated twice daily for 5 weeks. Blood glucose measurements were recorded weekly by OneTouch select glucometer (Lifescan, Mosta, Malta, United Kingdom) using the tail-prick method.

2. Behavioural studies

2.1 Morris water maze (MWM): Assessment of learning and memory

The effects of dioxidovanadium (V) was assessed using the Morris water maze during the last week of treatment (Week 5). Basic swimming speed and willingness of rats were observed the day prior to the test. The maze included an open circular pool (150 cm in diameter and 60 cm in height) with a black interior filled halfway with warm water and containing a fixed hidden small clear escape platform 27 cm away from one distal cue. Rats were trained to locate the hidden platform. Each rat was allowed to search for the platform for 2 min, and rest on the platform for minimum of 1 min once found. The rats that fail to locate the platform in given time were assisted manually to the platform where they spent 1 min before returning them to respective cages. Each rat received four learning trials in the four quadrants (N, S, E, and W) created with two perpendicular lines each day for five consecutive days. Latency in times for locating the platform and mounting were calculated. The rats were given a rest day on the sixth day (prior to probe test day). On the seventh day (probe test day), the platform was removed from the pool and the pool was divided into 4 quadrants based on the cues. The cue that the platform was placed in proximity to, was referred to as the goal quadrant. Rats were randomly placed into a quadrant and allowed to swim freely for 2 min. Boris software was used to blind score the amount of time each rat spent in the goal quadrant. The rats were then returned to their cages, which was placed in proximity of a heater, after being towel dried. The water used during the training and test day was changed daily.

3. Tissue harvesting

The animals were terminated at the end of 5 weeks via decapitation. The brain removed and hippocampus dissected out, weighed and snap-frozen in liquid nitrogen. The samples were then stored at -80°C in a Bio ultra-freezer (Snijers Scientific, Tilburg, Netherlands) until biochemical analysis.

4. Biochemical analysis

For the GPx1, Amyloid beta and pTau a sandwich ELISA kit (Elabscience and Biotechnology, WuHan) was used following the manufacturer's instruction to assess the above-mentioned biochemical markers in the hippocampus. Hippocampal tissue was weighed and homogenized in phosphate buffered saline (PBS) and centrifuged for 10 min at 5000 x g (digicen 21R, orto alresa). 100 µL of standards or samples were added to the wells and incubated for 90 min at 37°C. Post 90 min the liquid was discarded and 100 µL of biotinylated detection antibody (Ab) was added to each well and incubated for 60 min at 37°C. After incubation the liquid was aspirated and subjected to 3x washes using wash buffer. 100 µL of horse radish peroxidase (HRP) conjugate was added and allowed to incubate for 30 min at 37°C. The plate was then subjected to 5 x washes and 90 µL of substrate reagent was added followed by a 15 min incubation period at 37°C. 50 µL of stop solution was then added and a BioTek mQuant spectrophotometer (BioTek, Johannesburg, South Africa) was used to check absorbance at 450 nm. Calculation of results were then conducted.

5. MDA analysis assay

To assess lipid peroxidation, 40 mg of hippocampal tissue was homogenized in 400µL of 0.2% phosphoric acid followed by centrifuging for 10 min at 400 x g. Thereafter, 400µL 2% phosphoric acid was added to 400µL of the homogenate and separated equally into 2 glass tubes. Subsequently 400 µL of thiobarbituric acid (TBA)/butylated hydroxytoluene (BHT) was added into one glass tube (sample) and 3mM of HCL was added to the second glass tube (blank). To provide an acidic pH of 1.5 200 µL of 1 M HCl was added to all glass tubes and was heated at 100°C for 15 min. The solutions were then allowed to cool to room temperature. The cooled solutions were supplemented with 1.5 mL of butanol and vortexed for 1 min. After allowing the solutions to settle into 2 distinct phases, the top layer (butanol solution) was decanted into Eppendorf tubes and centrifuged at 13200 x g for 6 min (digicen 21R, orto alresa). A BioTek mQuant spectrophotometer (BioTek, Johannesburg, South Africa) was used to determine absorbance at 532 nm (reference 600 nm) using 96-well micro titer plates. This absorbance will be used to calculate MDA concentration using Beer's Law.

$$\text{Concentration of MDA (mM)} = \frac{\text{Average Absorbance}}{\text{Absorption coefficient } \left(156\ \text{mmol}^{-1}\right)}.$$

Statistical analysis

Statistical analysis was conducted using GraphPad Prism version 8 (version 8.00, GraphPad Software, San Diego, California, USA) and was expressed as mean ± standard deviation(SD). Two-way ANOVA followed by the Tukey-Kramer post hoc test was used to determine the statistical significance between the means of the two independent groups. A p-value < 0.05 was considered statistically significant.

Results

1. Blood glucose

The blood glucose concentrations in the non-diabetic groups (ND and ND-VAN (40mg/kg)) as well as the diabetic groups, both untreated (DC) and treated (D-VAN (40mg/kg) and INS) were recorded during the last week of treatment. It is noteworthy that there is no significant difference in blood glucose concentration between the ND and ND-VAN groups. As expected the diabetic control group was significantly higher than ND $^{\theta}$ (ND vs DC, $p<0.05$). Importantly there was a significant decrease observed between in D-VAN treated group in comparison to the DC group $^{\#}$ (DC vs D-VAN, $p<0.05$). There was a significant decrease in glucose concentration observed in insulin-treated group when compared to DC and DC-VAN.

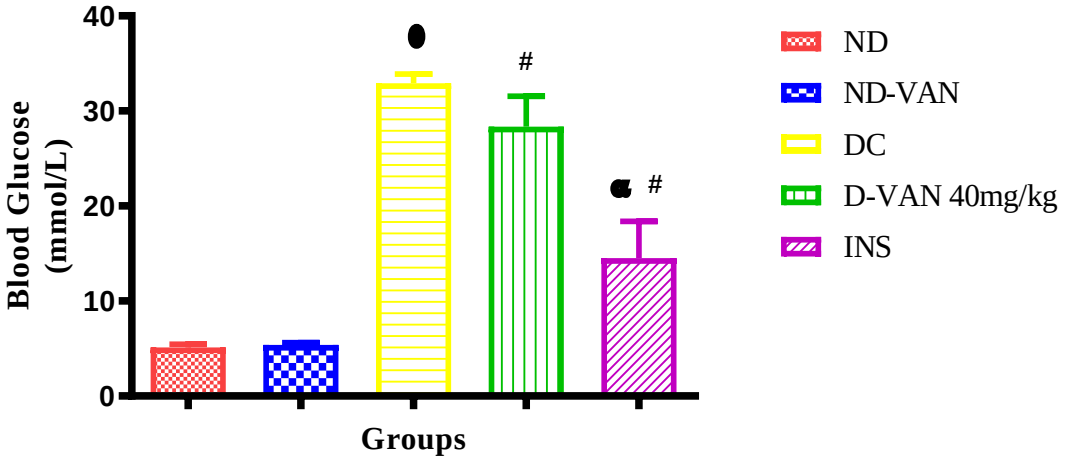

Figure 1 presents the blood glucose concentration measured during week 5 of treatment in all experimental groups (ND, ND-VAN, DC, D-VAN, INS). Values are expressed as means±SD (n= 6 in each group). θ $p<0.05$ in comparison to ND. # $p<0.05$ in comparison to DC. α $p<0.05$ when compared to D-VAN.

2. Morris water maze task

Latency times of the MWM and time spent in the goal quadrant during the probe test (Fig.2), in all experimental groups (ND, ND-VAN, DC, D-VAN and INS) (n=6) were calculated during the last week of treatment. Interestingly latency time ND-VAN $^{\theta}$ was significantly lower than ND (ND vs ND-VAN, $p<0.05$) on day 1. There was no significant difference between ND and DC on day 1. No significant decrease in latency times were observed in D-VAN and INS in comparison to DC. On day 2, there was no significant difference between ND-VAN and ND Latency times were significantly higher in DC when compared to ND $^{\theta}$ (ND vs DC $p<0.05$). DC latency times was not significantly different from D-VAN and INS. On day 4, ND-VAN and DC was significantly higher when compared to the ND group $^{\theta}$ (ND vs ND-VAN, ND vs DC, $p<0.05$). There was no significant difference between the DC group and treatment groups VAN and INS. It is interesting to note that there was no significant difference between the ND and ND-VAN groups in time spent in the goal quadrant during the probe test. As expected DC expressed significantly lesser time spent in the goal quadrant compared to the ND $^{\theta}$ (ND vs DC, $p<0.05$). There was no significant increase in time spent in goal quadrant in D-VAN and INS when compared to DC.

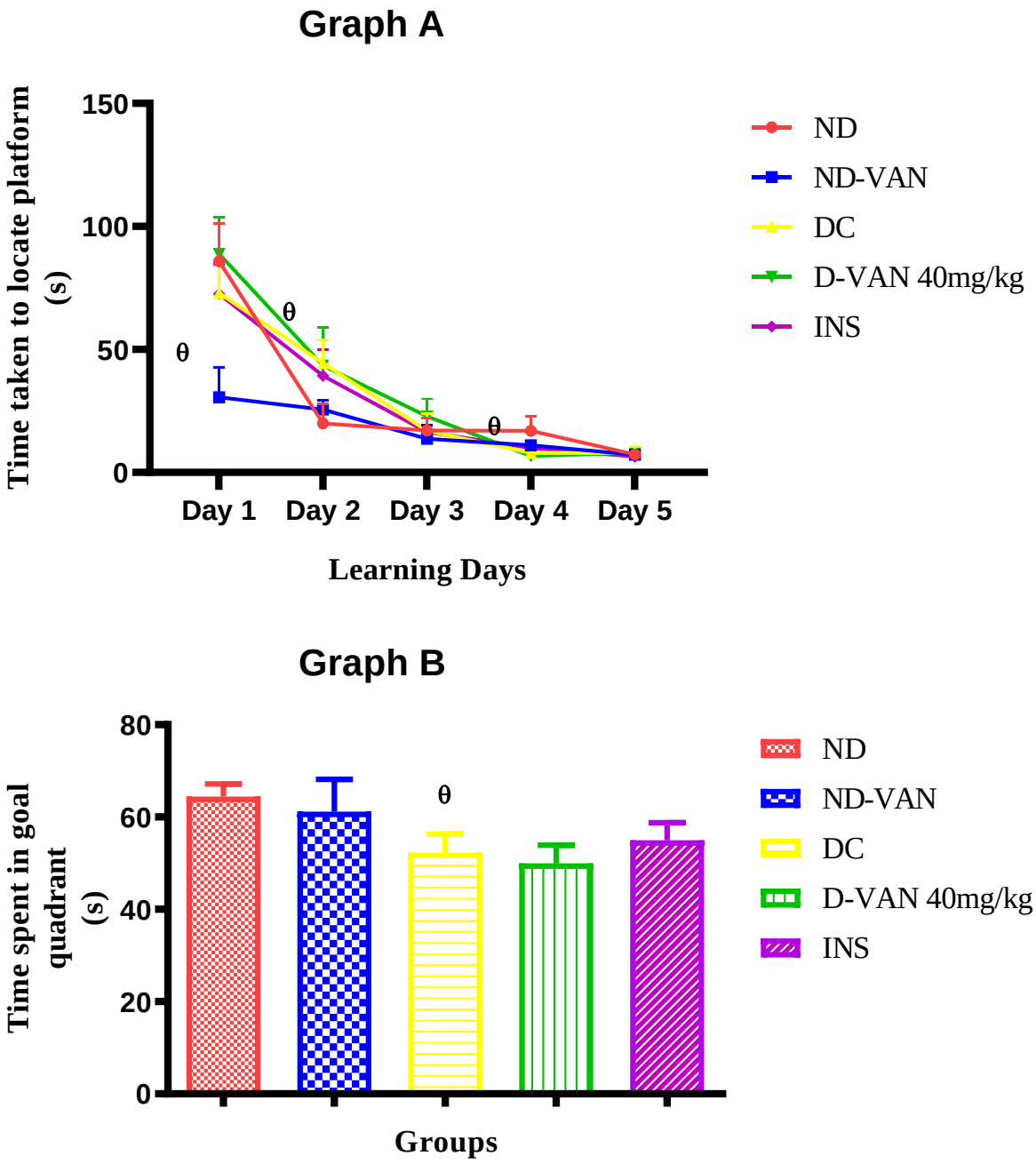

Figure 2 denotes graph A which shows the latency times measured in all experimental groups (n=6) over 5 days of training conducted. The figure also includes graph B showing the probe test results which is calculated from time spent in the goal quadrant in all experimental groups (n=6) during the 7th day of the MWM task used to test for rate of memory consolidation. Values are expressed as means±SD. θ $p<0.05$ in comparison to ND.

3. Hippocampal weights

The weights of the hippocampus were recorded post sacrificing of all experimental groups (ND, ND-VAN, DC, D-VAN, INS) (n=6). There was no statistical significant difference between ND and ND-VAN hippocampal weight. Interestingly however, DC hippocampus weighed significantly lesser than ND $^{\theta}$ (ND vs DC, $p<0.05$). There was no significant difference between DC and the treatment groups (D-VAN and INS).

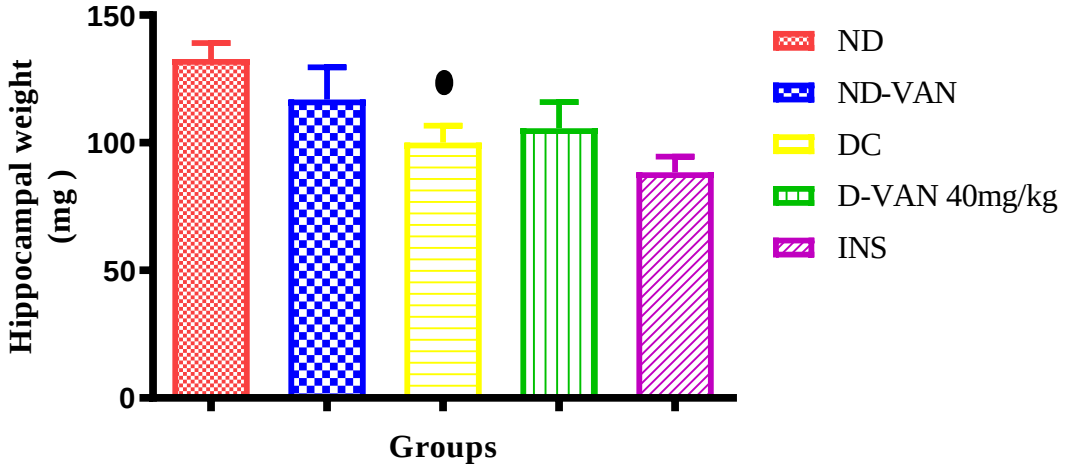

Figure 3 represents the weights of hippocampi of animals in each experimental group. (ND, ND-VAN, DC, D-VAN, INS). Values are expressed as means±SD. θ $p<0.05$ in comparison to ND. There were significant decreases in the diabetic groups when compared to the ND group. It is notable that the D-VAN was greater than DC hippocampal weight however not significantly.

4. Oxidative stress markers

4.1 *MDA analysis*

The concentration of malonaldehyde (MDA) produced in the hippocampus of experimental groups (ND, ND-VAN, DC, D-VAN, INS) (n=6) was measured. It is notable that there was no significant difference between ND and ND-VAN however a slight decrease is observed in ND-VAN. There was a significant increase in DC when compared to ND group $^{\theta}$ (ND vs DC, $p<0.05$). Interestingly there was a significant decrease between treatment groups VAN and the DC group $^{\#}$ (DC vs D-VAN, $p<0.05$). The INS group also showed a significant decrease when compared to the DC group $^{\#}$ (DC vs INS, $p<0.05$).

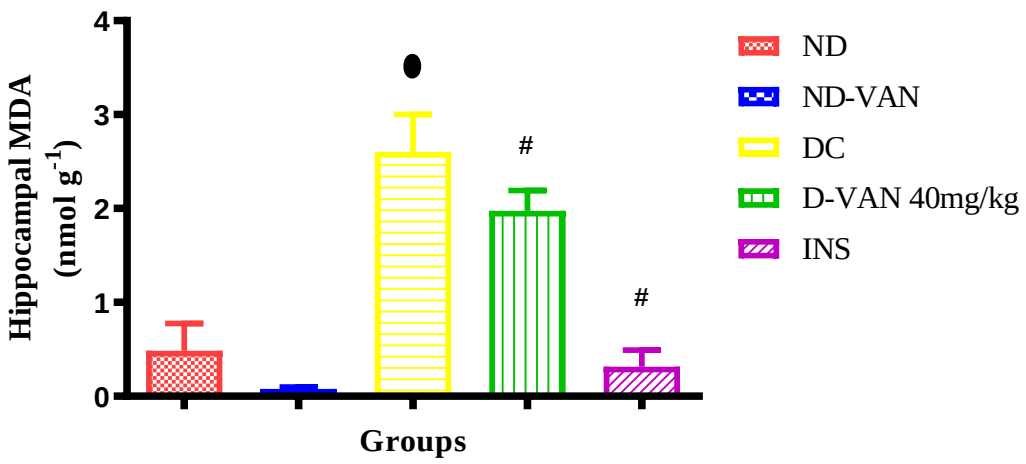

Figure 4 depicts the concentration of MDA produced in the hippocampus of all experimental groups (ND, ND-VAN, DC, D-VAN, INS). Values are expressed as means±SD. θ $p<0.05$ in comparison to ND. # $p<0.05$ when compared to DC.

4.2 GPx1 concentration

The concentration of GPx1 concentrations were also measured in all experimental groups. There was no significant difference between ND and ND-VAN. The concentration of GPx1 of the DC group was significantly lower in concentration in comparison to the ND group $^{\theta}$ (ND vs DC, $p<0.05$). It is also noteworthy that the D-VAN was significantly higher than DC $^{\#}$ (DC vs D-VAN, $p<0.05$) and INS group was significantly lower than the D-VAN group $^{\alpha}$ (D-VAN vs INS, $p<0.05$).

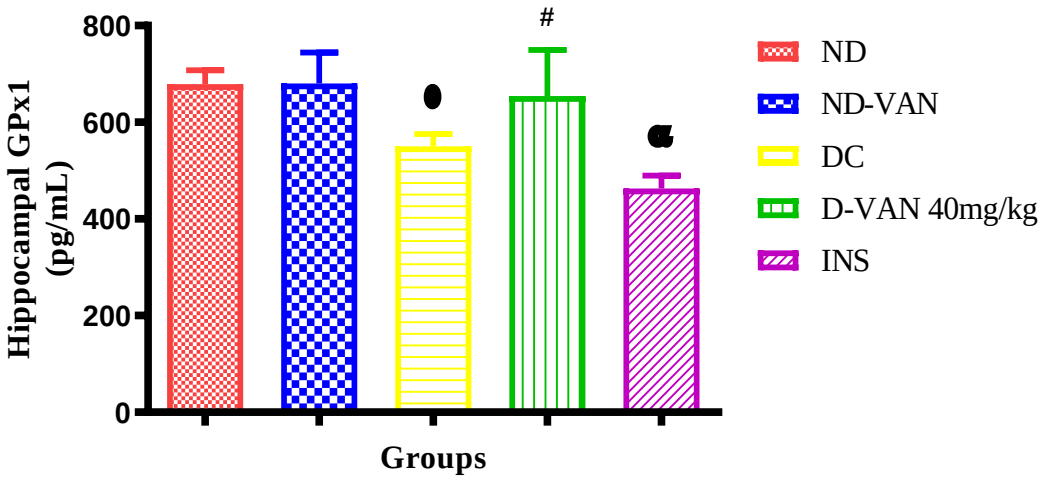

Figure 5 presents the levels of GPx1 produced in the hippocampus of all experimental groups (ND, ND-VAN, DC, D-VAN, INS). Values are expressed as means±SD. # $p<0.05$ in comparison to DC. α $p<0.05$ when compared to D-VAN. θ $p<0.05$ in comparison to ND.

5. TNF-α

The concentration of TNF-α in the hippocampus of all the experimental groups (ND, ND-VAN, DC, D-VAN and INS) (n=6) were measured. There was no significant difference expressed in ND-VAN and DC, when compared to the ND group. There was no significant difference observed between DC and the treated groups D-VAN. TNF-α concentration in INS was significantly higher than DC # (DC vs INS, $p<0.05$).

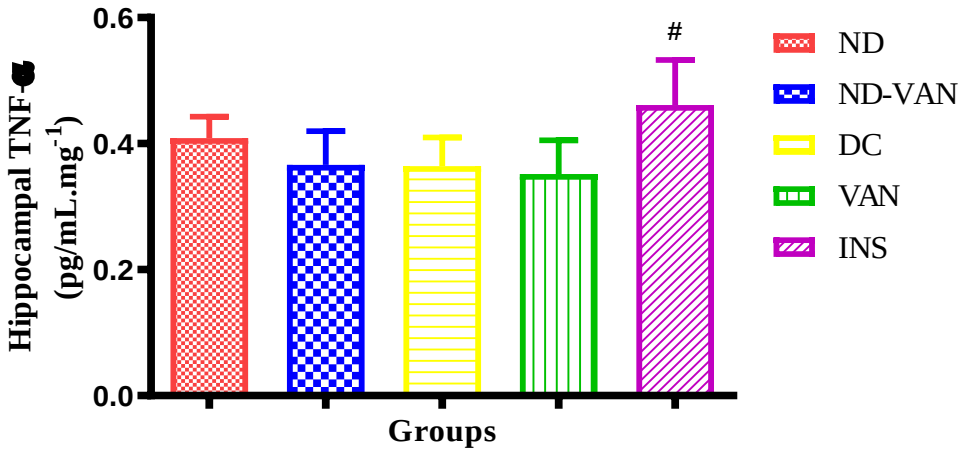

Figure 6 represents the concentrations of TNF-α in all experiment groups (ND, ND-VAN, DC, D-VAN, INS) (n=6). Values are expressed as means±SD. # $p<0.05$ in comparison to ND.

6. Amyloid beta (1-42)

Figure 7 represents the concentration of hippocampal amyloid beta (1-42) formation measured in ND, ND-VAN, DC, D-VAN, INS groups. The concentration of amyloid beta in ND-VAN and DC groups were significantly lower when compared to the ND group $^{\theta}$ (ND vs ND-VAN, ND vs DC, $p<0.05$, Fig.7). D-VAN and INS were not significantly different when compared to DC.

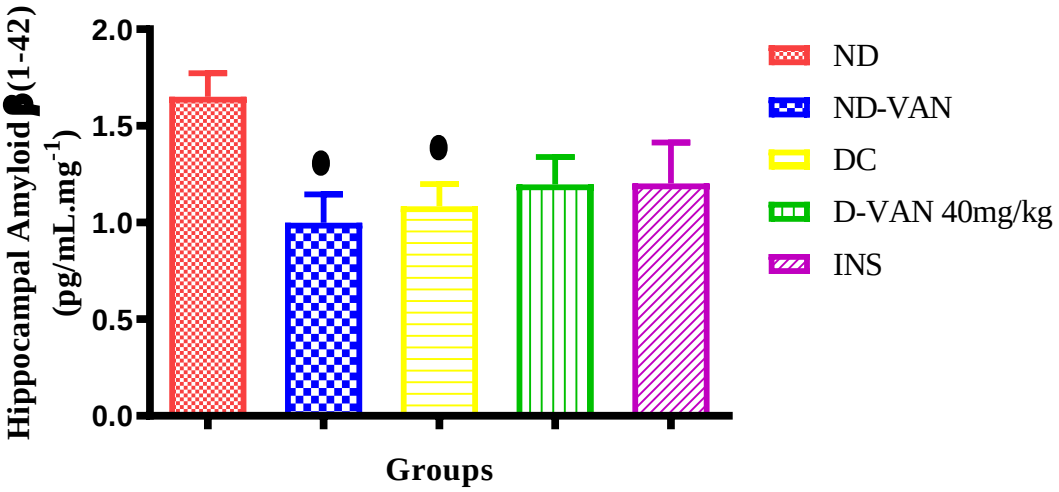

Figure 7 represents the concentrations of Amyloid beta (1-42) in all experiment groups (ND, ND-VAN, DC, D-VAN, INS) (n=6). Values are expressed as means±SD. θ $p<0.05$ in comparison to ND.

7. pTau analysis

The concentration of pTau production was measured in ND, ND-VAN, DC, D-VAN, INS groups. There was no significant difference between ND and ND-VAN or DC. pTau concentration in D-VAN and INS was not significantly different when compared to DC

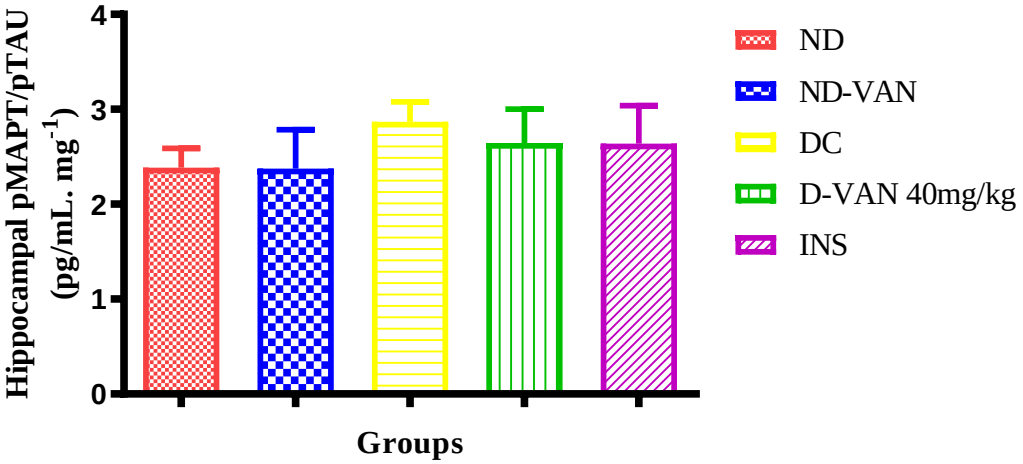

Figure 8 presents the concentration of pTau in all experiment groups (ND, ND-VAN, DC, D-VAN, INS) (n=6). Values are expressed as means±SD.

Discussion

Diabetes mellitus is a metabolic disease characterized by chronic hyperglycaemia resulting from insulin deficiency or insulin resistance [26, 27]. STZ is a naturally occurring toxic agent that destroys pancreatic beta cells via DNA strand crosslinking and generation of free radicals [23, 28]. For the purpose of this study, STZ was used in order to establish a hyperglycaemic animal model in order to study the effects of dioxidovanadium on learning and memory in the hippocampus during chronic hyperglycaemia. Chronic hyperglycaemia can be confirmed by a significant increase in glucose concentration in our diabetic control (DC) in comparison to the glucose concentration of the non-diabetic control (ND). A hyperglycaemic, insulin deficient environment in the hippocampus favours mitochondrial dysfunction, neuro-inflammation and reactive oxygen species production resulting in neuro-degeneration [29]. Therefore, diabetes has often been associated with impaired cognitive tasks such as learning and memory and diseases such as dementia and Alzheimer's.

Among various therapeutic options for diabetes, bolus insulin is considered the gold standard treatment. Insulin plays a pivotal role in cognitive tasks such as learning and memory through the influence of insulin signalling on the hippocampus [30]. However, despite the efficacy of insulin, the treatment in lowering blood glucose concentration as seen in this study, long term exposure of bolus insulin may result in hypoglycaemic episodes and insulin resistance thereby exacerbating the cognitive defects associated with diabetes [18]. Amongst others, these side effects have warranted the search for alternative treatment. In our experiment, we did not observe hypoglycaemia in the insulin treated diabetic group, this may be due to occurrence of hypoglycaemia occurring in acute episodes.

Vanadium is a transition metal that has shown to possess insulin mimetic effects and significantly lower blood glucose concentration [31, 32]. However, due to its unrestricted access to the brain, naturally occurring vanadium salts were shown to accumulate in various areas of the brain such as the hippocampus and promote the formation of reactive oxygen species resulting in neurodegeneration [33]. In our laboratory, we have synthesized vanadium complex, dioxidovanadium (V) complex, (cis-[VO2(obz)py] {Hobz=2- hydroxyphenyl-1H-benzimidazole and py =pyridine]}) by attaching organic ligands pyridine and benzimidazole that function as chelators to improve the efficacy of lowering blood glucose concentration and attenuate toxicity associated with the drug [34, 35]. As reflected by our results, the vanadium complex had significantly lowered blood glucose concentration in a diabetic animal model in comparison to our diabetic control. We can also deduce that this complex does not alter glycaemic control in a healthy animal model during chronic administration as blood glucose concentration of non-diabetic animals treated with vanadium did not exhibit a significant decrease when compared to the glucose concentration of the non-diabetic control. We speculate that the antidiabetic effects of this vanadium complex may be anti-hyperglycaemic and not hypoglycaemic thereby preventing side effects associated with insulin. Examples of anti-hyperglycaemic actions used by current treatment are the inhibition of hepatic glucose production by biguanides [36]. Studies have shown that oral administration of vanadium and vanadium complexes in healthy animals results in decrease of plasma insulin, thereby preventing hypoglycaemia [37]. However, the anti-hyperglycaemic mechanism of action by which dioxidovanadium (V) lowers blood glucose concentration requires further investigation.

Oxidative stress can be described as the imbalance between oxidants and antioxidants [38]. During chronic hyperglycaemia, the rate of glucose metabolism by physiological pathways is exceeded by the rate of glucose entry into neurons resulting in shunting glucose to other metabolic pathways thereby giving rise to sorbitol formation, advanced glycation end products (AGE) production and protein kinase C (PKC) activation [39]. These pathways induce the formation of reactive oxygen species (ROS) and disrupt antioxidant formation [39, 40]. The concentration of ROS increases lipid peroxidation in cells. Lipid peroxidation is the reaction between ROS and polyunsaturated fatty acids present in membranes thus altering the fluidity and permeability of the membrane often resulting in cellular injury or cell

destruction [41, 42]. Since the brain possesses a high amount of polyunsaturated fats and it is highly susceptible to lipid peroxidation and therefore neuronal loss. This can be supported by our hippocampal weight results which depict a smaller hippocampus in the diabetic groups when compared to the non-diabetic animals. Previous toxicology testing of vanadium on the hippocampus indicated that organic vanadium administration resulted in CA1 hippocampal neuronal loss and therefore a decrease in weight in healthy animals [43]. It is important to highlight that the hippocampal weight in the brains of our ND-VAN group did not display a significant decrease in hippocampal weight in comparison to the non-diabetic untreated control group (ND).

Neurons in the brain are structurally diverse with hippocampal neurons being the most sensitive to neurodegeneration [10]. Long term potentiation, synaptic plasticity and memory consolidation are compromised [44]. A by-product of lipid peroxidation is malonaldehyde (MDA), highly reactive three-carbon dialdehyde used as a biomarker for oxidative stress [45]. According to our results the MDA concentration in the hippocampus of a diabetic model is significantly higher than a healthy animal, this confirms the strong correlation between oxidative stress and diabetes. Currently, the mechanism by which vanadium lowers blood glucose concentration is via 2 major pathways [46]. Research has shown that vanadium is an insulin mimetic and participates in the insulin signalling pathway by inhibiting tyrosine phosphatase tyrosine receptors, phosphorylating phosphoinositide 3-kinases (PI3K) and protein kinase b (AKT) thereby facilitating glucose uptake [47]. By promoting insulin signalling pathway and facilitating physiological metabolism of glucose, this prevents the shunting of excess glucose to pathologic metabolic pathways mentioned above, thereby preventing the generation of ROS and oxidative stress.

The second mechanism by which naturally occurring vanadium exerts blood glucose lowering effects is through the production of hydrogen peroxide (H_2O_2) [46]. The formation of H_2O_2 is induced by the tetravalent form of vanadium, vanadyl, participating in oxidation reactions in the presence of nicotinamide adenine dinucleotide (NAD) + hydrogen (H) (NADH) [46]. Studies have shown that H_2O_2 has the ability to improve glucose transport and inhibits lipolysis [48]. However, H_2O_2 is a reactive oxygen species thus characterizing naturally occurring vanadium as a pro-oxidant drug. Since vanadium has been shown to freely cross the blood brain barrier and favour deposition in areas such as the hippocampus, it has been associated with the promotion of oxidative stress in the hippocampus and therefore considered neurotoxic[20, 39]. It is therefore expected that the combined effects of diabetes and vanadium on the brain further increases MDA concentration hence it is important to highlight that the concentration of MDA in our diabetic vanadium treated group (D-VAN) was significantly lower than our diabetic control (DC). Furthermore, the concentration of MDA in the non-diabetic vanadium treated group was visibly lower however not significantly different from the non-diabetic control, suggesting that dioxidovanandium (V) does not induce memory impairment through lipid peroxidation in the hippocampus. Previous studies conducted the effects of vanadium complexes such as

bis(ethylmaltolato)oxovandium (IV) as a possible treatment option for AD, indicated that the complex improved the spatial memory and contextual memory of AD mice by inhibiting the pathogenic pathways thereby suggesting that oral administration of dioxidovanadium may directly target the hippocampus rather than solely promoting the decrease of MDA secondarily by lowering blood glucose concentration [49].

Another mechanism by which both diabetes and organic vanadium salts promote oxidative stress is through the depletion of major antioxidants such as Glutathione peroxidase (GPx1) [50]. GPx1 is responsible for neutralizing reactive oxygen species such as H_2O_2 [51]. In our study, the induction of diabetes by STZ causes a significant decrease in the untreated diabetic control group (DC) in comparison to healthy animals in the non-diabetic control (ND). The administration of dioxidovanadium (V) to diabetic animals resulted in a significant increase in GPx1 concentration when compared to the diabetic control (DC). Since MDA and GPX1 concentrations in the non-diabetic group were not significantly different from the absolute control, we speculate that increased GPx1 concentration in the D-VAN can be attributed to a protective mechanism against oxidative stress.

Research has indicated that there is an increase in inflammation in the brain due to the cell injury mediated by oxidative stress caused by diabetes [52]. Microglial cells detect the increase in oxidative stress and promote the release of inflammatory cytokines such as Interleukin-1 (IL-1), Interleukin-6 (IL-6) and Tumour necrosis factor alpha (TNF-α) [52]. TNF-α is an inflammatory cytokine produced during acute inflammation which mediates various signalling events that promote neuronal necrosis or apoptosis of neurons [53]. Increased neuron destruction in the hippocampus will alter its function and may result in memory impairment [54]. The results obtained in this study as shown in figure 6, were not consistent with literature as diabetic animals (DC and D-VAN) were not significantly higher in comparison to the non-diabetic group.

This may be due to the fact that cognitive dysfunction occurs as at a later stage of diabetes and begins at only 4 weeks in an animal model. Our study was only conducted over 5 weeks, due to ethical constraints hence there was no detectable increase in TNF-α in the diabetic control in comparison to the non-diabetic control at this point in our study [55]. Furthermore, studies have shown that increases in TNF-α in the hippocampus of diabetic patients results from drastic changes in blood glucose concentration rather than a chronic hyperglycaemic state [56]. This may be associated with hypoglycaemic drugs that may result in a cycle between hyperglycaemia and hypoglycaemia which may explain the increase in TNF-α concentration in the hippocampus of the insulin treated diabetic group.

In recent studies, diseases such as Dementia and Alzheimer's disease (AD) are shown to be implicated in diabetic individuals [44, 57]. A marker of advanced memory impairment, amyloid β was discovered to accumulate and form amyloid plaques neurons of the hippocampus in diabetic individuals thereby

supporting the hypothesis that diabetes promotes memory dysfunction [58, 59]. According to figure 7, the results presented in this study did not support this hypothesis. This may be due to the nature of amyloid β (Aβ). Despite driving the pathology behind AD, Aβ is produced under physiological circumstances from the cleavage of Amyloid precursor protein (APP) [8, 60]. APP plays an essential role in neuronal differentiation, neuronal migration and synapse formation [60]. The removal of Aβ degradation is facilitated by insulin degrading enzymes (IDE) [61]. IDE has a greater affinity for insulin, however the rate of insulin hydrolysis is much slower than Aβ [62]. In STZ models, which may be considered analogous to type 1 diabetes, it has been shown that, contrasting to type 2 diabetes, the concentration of IDE increases. Therefore, IDE is considered a potential target for the treatment of AD [62, 63]. This suggests that in the absence of insulin, at the initial stages of cognitive impairment, increased IDE is available to degrade accumulating Aβ, which was observed in our diabetic control (DC) whereas in a normal animal such as those in ND Aβ would be competing with insulin resulting in decreased degradation. Although there was no significant difference between DC and D-VAN in Aβ concentration, a study conducted by He. et al., states that vanadium complexes further promoted the degradation of Aβ by increasing the expression of peroxisome proliferator-activated receptor gamma and IDE which can be reflected by our non-diabetic dioxiodvanadium (V) treated group (ND-VAN) [64]. This suggests that dioxidovanadium (V) may protect against accumulation of Aβ at later stages of cognitive impairment however since the duration of the experiment may only allow for early cognitive defects further research on this complex

Another marker that is present in neurons in the hippocampus of patients with diabetes and is considered as a hallmark in the pathology of memory impairment is hyper-phosphorylated tau (pTau) [65]. Like Aβ, tau is a protein present in neurons in physiological conditions [66]. Tau proteins are abundant in neurons and responsible for stabilizing microtubules thereby providing axons with flexibility [67]. This is achieved through isoforms and phosphorylation of tau, however in diabetes the function of tau is compromised and the protein is hyper-phosphorylated [66]. This is facilitated by impaired AKT signalling resulting in the activation of glycogen synthase kinase 3 beta (GSK3β) which then hyperphosphorylates tau proteins [68]. pTau then accumulates in neurons and facilitates the formation of neurofibrillary tangles [29]. In spite of our study period being too short to observe a significant difference between DC and ND, there is a visible increase in DC compared to ND suggesting that as the disease progresses, the concentration of pTau will increase further. Previous studies on vanadium complexes have reflected that vanadium indirectly reduces pTau formation by regulating AKT signalling, however there was no significant decrease noted in groups treated with vanadium (D-VAN and ND-VAN) albeit the visual difference in concentrations [64]. This may also be attributed to the lack of time for effective action of the drug.

According to our learning period of the MWM task, the duration of time taken to find the platform by all groups were not significantly different, this may be due to different neuronal circuits involved in

learning and memory. The significant differences noted in day 1 and day 2 in the diabetic animals in comparison to the non-diabetic animals may be attributed to the presence of disease which indicates the degeneration of functional neuronal matter in the hippocampus caused by gluco-toxicity. Despite the contradicting results for Aβ and pTau, there was significant memory impairment in the diabetic animals (DC) in comparison to the non-diabetic animals (ND) as seen in our result of the probe test in MWM task. Suggesting that memory dysfunction may be of a different etiological mechanism.

A possible pathological pathway for this study is via the induction of oxidative stress, as we have seen is significantly increased in our diabetic control group, and oxidative stress facilitated increase of acetylcholinesterase [69]. Acetylcholinesterase is an enzyme that promotes the clearance of acetylcholine in neuronal synapses through hydrolyses [70]. Acetylcholine release in hippocampal neurons mediates hippocampus-dependant learning. Therefore under environments with increased oxidative stress and neuronal loss, such as in diabetes, memory is compromised [69, 71]. We therefore speculate that an increased oxidative stress in the diabetic animals has mediated the increased production of acetylcholinesterase thereby affecting memory consolidation [72].

It is essential to highlight that targeting the increase of acetylcholinesterase is a mechanism used by many therapeutic agents such as donepezil, rivastigmine and galantamine, in Alzheimer's disease. Thus, preventing an increase in acetylcholinesterase activity results in improved acetylcholine signalling [73]. This suggests that administration of vanadium complexes may improve memory consolidation indirectly, by reducing the oxidative stress, a key factor associated with acetylcholinesterase increase. Furthermore, since dioxidovanadium lowered MDA concentration significantly and increased GPx1 concentration in the hippocampus of D-VAN, the performance of the animals in the probe test may be the result of diabetes and not of dioxdiovanadium (V). This can be supported by the non-diabetic animals treated with vanadium which showed no significant decrease in memory impairment in comparison to the ND group suggesting that dioxidovanadium (V) treatment does not promote memory dysfunction. Since the treatment of dioxidovanadium did not possess detrimental effects on the hippocampus in non-diabetic animals, reduced lipid peroxidation, and lowered glycaemic levels in diabetic animals without toxic effects, we deduce that treatment of a diabetic patient with dioxidovanadium (V) does not exacerbate dysfunction in cognitive tasks such as memory and learning. Also we can speculate that by improving antioxidant status and restoring normoglyceamia and therefore lowering oxidative stress, it may protect against the development of cognitive dysfunction in patients suffering from diabetes and requires further investigation on the effect of dioxidovanadium (V) treatment on learning and memory in diabetes, to possibly eliminate the requirement of additional therapeutic agents used to treat secondary pathological effects on the hippocampus caused by diabetes.

Conclusion

We can conclude by stating that dioxidovanadium significantly lowers blood glucose concentration in a diabetic animal model whilst attenuating oxidative stress in the hippocampus by reducing lipid peroxidation and increasing antioxidant GPx1 in the brain of hyperglycaemic environment. Chronic hyperglycaemia did not result in an increase in TNF-α therefore further investigation is warranted on the effects of dioxidovanadium (V) on inflammation. We speculate that the results for Aβ and pTau were the result of a short study duration and the effects of dioxidovanadium (V) on these markers requires more research. We infer that the memory impairment present in the diabetic dioxidovanadium treated group was solely attributed to diabetes pathology since the non-diabetic dioxidovanadium (V) group displayed no significant memory impairment thereby affirming that dioxidovanadium is non-toxic to the hippocampus and does not exacerbate memory impairment in a diabetic animal model. We also advocate for the further investigation of dioxidovanadium on learning and memory as it may emerge a potential single treatment option of the coexisting diabetes and memory impairment induced by diabetes.

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CHAPTER 4: SYNTHESIS

Diabetes mellitus is a metabolic disorder characterized by a chronic hyperglycaemic condition (1). Patients with this condition are 93% more likely to develop cognitive disorders such as memory impairment (2). Research has shown a strong correlation between diabetes, Alzheimer's and dementia (3). This can be attributed to the detrimental effects of diabetes on the hippocampus (4). The hippocampus is primarily associated with memory consolidation (5). Hippocampal neuronal health and function are susceptible to changes in blood glucose concentration and therefore are significantly affected in hyperglycaemia (4). Neurons have a constant glucose requirement to produce the ATP needed to perform their functions, however, in diabetes, the glucose uptake exceeds the rate of physiological, metabolic pathways, thereby shunting excess glucose to alternative pathways to be metabolized (6). These pathways favour the generation of polyols, advanced glycation products (AGE), and activated protein kinase c (PKC) (7-9). This results in reactive oxygen species formation, depletion of antioxidants resulting in oxidative stress, neuroinflammation, plaque formation, neuronal injury, and neurodegeneration, which are considered the hallmark of cognitive disorders (3, 10).

Current treatment of diabetes, such as bolus insulin, does not accommodate the development of these secondary diseases, instead exacerbates these conditions through unwarranted episodes of hypoglycaemia and insulin resistance (11, 12). These induce further harmful effects as glucose is the sole fuel source of the brain, which is required to perform neuronal and non-neuronal functions, and insulin plays a significant role in facilitating memory formation (13, 14). These undesired effects have probed scientists into seeking alternative therapeutic options for diabetes treatment. Transition metals have varying oxidation states, allowing them to react with multiple biological processes in the body (15). Vanadium is one such transition metal that exhibits this trait and has been greatly investigated for its anti-diabetic qualities (16). Naturally occurring vanadium salts cross the blood-brain barrier freely and favours delivery to areas such as the hippocampus (17). However, vanadium administration results in the formation of reactive oxygen species, and continuous exposure results in the accumulative deposition of vanadium in the hippocampus, which promotes an imbalance of oxidants and antioxidants, resulting in oxidative stress and destruction of neurons (17). In our laboratory, we have synthesized a vanadium complex dioxidovanadium (V) through organic ligands' attachment to alleviate toxic accumulation and improve its potency as an anti-diabetic drug (18). These ligands act as chelators that prevent vanadium accumulation by facilitating efficient excretion (19). They also provide stability and enhance bioavailability (20).

Since vanadium is considered neurotoxic, it is essential to understand the effects of the complex on the brain without disease acutely; therefore, the first study aimed to investigate the effects of the complex

on oxidative stress generation and inflammation in a healthy animal model in comparison to an untreated control group using MDA analysis and GPx1 and TNF-α concentrations (17). Furthermore, vanadium is an anti-diabetic drug associated with insulin-mimetic effects shared dioxidovanadium however the effects of this drug on blood glucose concentration in non-diabetic rats have not been investigated prior to this study (21). Therefore, blood glucose concentrations were monitored in both groups prior to sacrificing to provide insight on the effect dioxidovanadium (V) on non-diabetic animals. Vanadium clearance occurs in 3 stages, and therefore, the study was conducted following the clearance times to assess the effects of vanadium on the above markers at different concentrations before it is excreted (22, 23). Analysis of the blood glucose concentration data revealed that dioxidovanadium did not significantly decrease blood glucose concentration compared to the untreated control group thereby unveiling that the administration of dioxidovanadium does not cause hypoglycaemic effects. Findings of this study correlated with the results of research done on organic vanadium salts and vanadium complexes which displayed the compensatory mechanism of decreased insulin release indicated by decreased plasma insulin (24-26). We can therefore eliminate the risk of hypoglycaemic episodes in a diabetic individual (27).

A unique mechanism by which vanadium reduces blood glucose concentration is the generation of H_2O_2 (28). H_2O_2 has been shown to have therapeutic effects against diabetes by improving glucose uptake and inhibiting lipolysis, thereby inhibiting gluconeogenesis (29). However, H_2O_2 is responsible for the generation of oxidative stress. H_2O_2 reacts with increased amounts of polyunsaturated fats in neuronal membranes, causing lipid peroxidation to alter membrane permeability and nullifying its protective benefits (30, 31). This promotes neuronal injury and MDA production, which was used as an indicator for lipid peroxidation in this study (32).

Interestingly administration of dioxidovanadium complex in the non-diabetic animals did not promote an increase in MDA concentration compared to the normal untreated animals suggesting that dioxidovanadium does not promote the induction of lipid peroxidation. In order to confirm that this vanadium complex did not favour the imbalance of ROS and antioxidants through depletion of antioxidants, we assessed GPx1, a major antioxidant present in the hippocampus, responsible for neutralizing H_2O_2 (33). Vanadium salts deplete GPx1 concentration by inducing excess H_2O_2 however, from the results of study 1, the concentration of GPx1 did not significantly differ from the control group suggesting that dioxidovanadium (V) does not induce oxidative stress as its analogue, vanadium salts (34). Favourable results in the first study may suggest that this novel complex did not induce toxicity in the hippocampus over an acute period. Unsuccessful results in the first study would rule out dioxidovanadium (V) as a safe therapeutic option for diabetes as the hippocampus is subjected to hyperglycaemia induced toxicity in diabetes.

Despite the promising results observed in the first study diabetes is a chronic condition, and vanadium was shown to accumulate toxically over long periods (23) it was therefore essential to test the compatibility of the drug with the hippocampus over a chronic time in the presence of the disease. As a drug that reduces hyperglycaemia it was also beneficial to understand the effects of dioxidovanadium (V) on diabetes induced memory impairment, often reflected by oxidative stress, neuroinflammation, amyloid-beta and pTau as it may improve or protect against the formation on these markers. STZ is a drug used to induce chronic hyperglycaemia by destroying pancreatic beta cells, similar to the pathology of type 1 diabetes, and thus was used to create a diabetic animal model in the second study in order to investigate the anti-diabetic effects of dioxidovanadium its effects on oxidative stress using MDA and GPx1, inflammation TNF-α and the pathological proteins (35) Amyloid-beta and pTau, present in memory disorders. The study also included the Morris water maze to assess the collective effect of the above mention biochemical markers on learning and memory in dioxidovanadium (V) treated diabetic animals. Since the accumulative toxic effects of vanadium salts are associated with long-term exposure, the study included a dioxidovandium (V) non-diabetic group (23). This allowed any unfavourable results or decline in memory function in the dioxiovanadium (V) group to be attributed to diabetes and not the administration of dioxidovanadium.

Expectedly the complex lowered blood glucose concentration significantly in the diabetic group treated with dioxidovanadium (V) however it is noteworthy that the non-diabetic group treated with vanadium did not show a significant decrease in glycaemia. This confirms the results obtained in the first study and favours the speculation that the dioxidovanadium (V) mechanism of action may not cause hypoglycaemia. Interestingly, the concentration of MDA in the dioxidovanadium (V) treated diabetic group was significantly lower than the diabetic control, and the GPx1 concentration was significantly higher than the diabetic control. This suggests that dioxidovanadium enhances protective mechanisms against oxidative stress induced by diabetes (34). The non-diabetic complex (ND-VAN) treated group confirms that MDA production was not enhanced by dioxidovanadium, and any increase observed in the diabetic complex treated group (D-VAN) can be solely attributed to diabetes since the MDA concentration of ND-VAN was not significantly different from the non-diabetic absolute control, another result that corresponded with the results obtained in the first study.

In the hippocampus, microglial cells, which are the immune cells of the nervous system, are responsible for inducing inflammation under diabetic conditions, however, in contrast to this, the results from the second study showed no significant difference in the concentration of TNF-α, an inflammatory cytokine, except in the insulin-treated positive control (36, 37). This may be associated with findings from certain studies that state an increase in TNF-α is associated with drastic fluctuations in glycaemic levels rather than a stable chronic condition, as seen in diabetes (38, 39). Furthermore, since bolus insulin causes hypoglycaemic episodes, this may have resulted in the increased release of TNF-α, which may have responded to the interchanging of hyperglycaemic and hypoglycaemia (38).

Aggregated Amyloid-beta and pTau have been used to diagnose neurological conditions such as Alzheimer's and dementia (2, 40). Amyloid beta (Aβ) is a protein produced from the cleavage of amyloid precursor protein required for synapse formation and neuroplasticity (41, 42). Its removal from the hippocampus is facilitated by insulin-degrading enzymes (IDE) (43). However, in diabetes, these proteins aggregate and form plaques in neurons (44). This disrupts neuronal functioning in the hippocampus and results in memory impairment, which our study did not observe (45). This may be due to the duration of the study. This protein is expressed in the late stages of disease progression, which the 5-week period did not accommodate (45, 46). Contrasting to what was expected, the normal untreated control group had the highest concentration of Aβ, and the diabetic groups both treated and untreated were not significantly different from each other. A possible explanation is a relationship between STZ-induced animals and IDE (43). STZ induced animal models present with increased IDE concentrations (43). In the absence of insulin, the sole purpose of IDE is then to degrade Aβ. The dioxidovanadium non-diabetic group also displayed a lower concentration of Aβ in comparison to the non-diabetic group. Research has shown that vanadium complexes have reduced the formation of Aβ, thus providing a reason for this result (46). Tau proteins are highly essential for neuronal micro-stability and are also expressed in a physiological state however, in diabetes, these proteins become hyper-phosphorylated (pTau) (40, 47). This renders tau functionless and results in the formation of pTau tangles contributing to neuron pathology and inhibiting memory consolidation however, as explained above, the duration of the study did not accommodate for the disease progression to allow for the formation of pTau, and therefore there was no significant difference observed in all test groups (48). Further research is required to unveil the effects of dioxidovanadium on these proteins in a diabetic animal model.

There was no significant change observed in latency times on the last day of training, possibly due to the neuronal pathways for learning being different from memory retention. Although, despite the unexpected concentrations of Aβ and pTau, the MWM task had shown a significant decrease in memory consolidation, as seen in the probe test, in the diabetic animals compared to the non-diabetic animals. This suggests a different aetiology behind memory impairment in these diabetic animals. Acetylcholine is a major neurotransmitter in memory formation in the hippocampus, and acetylcholinesterase is responsible for clearing the neurotransmitter from synapses to avoid prolonged action potentials (49). Inhibiting acetylcholinesterase is a mechanism used by certain drugs to treat Alzheimer's (49, 50). Studies show that an increase in oxidative stress, as seen in our diabetic animals, promotes the release of acetylcholinesterase (51, 52). This may explain the memory decline observed in the diabetic animals as the concentration of MDA was greater than the non-diabetic animals. Since dioxidovanadium had begun to reduce oxidative stress in the diabetic animals treated with dioxidovanadium, we can speculate that treatment may indirectly prevent the increase of acetylcholinesterase over a more extended treatment period (34). The direct effects of dioxidovanadium (V) on acetylcholinesterase requires

further investigation. These results show that this vanadium complex is compatible and does not subject the hippocampus to toxic effects. It also advocates for the further investigation of this vanadium complex on learning and memory as it may improve these cognitive tasks, thereby preventing the need for additional pharmaceuticals to address secondary effects of diabetes on the hippocampus.

Conclusion

The vanadium complex, dioxidovanadium (V) lowers blood glucose significantly in a diabetic animal model administration in a non-diabetic animal model does not result in hypoglycaemia over acute and chronic time periods. Dioxidovanadium does not induce lipid peroxidation in the neurons of the hippocampus in acute and chronic periods in the absence or presence of diabetes suggesting that administration of dioxidovanadium is non-toxic to the hippocampus. It also improves antioxidant status by increasing the concentration of GPx1 under chronic hyperglycaemia. Also, by lowering blood glucose concentration and oxidative stress in the neurons of the hippocampus this vanadium complex may indirectly protect against the development of diseases such as Alzheimer's and dementia thereby avoiding use of therapeutic intervention for these secondary effects associated with diabetes.

Study shortfalls and future recommendations

Dioxodivanadium has shown promising results in the treatment of diabetes and shown to be non-toxic to the hippocampus however in order to confirm the direct effect of this vanadium complex on the hippocampus, it may be useful to quantify vanadium accumulation through mass spectrometry studies and observe the brain structure histologically. Further research should be conducted on the effect of this complex on other brain areas associated with learning and memory such as the prefrontal cortex. It has been highlighted in this study that dysfunction in cognitive tasks induced by diabetes may develop from other aetiological mechanisms such as the unwarranted neurotransmitter clearance promoted by oxidative stress. The effects of using an STZ-induced model may also contribute to the different mechanism by which memory is impaired. The authors therefore recommend studying the use of dioxidovanadium under other diabetic animal models to obtain clearer results. The period of the chronic study did not suffice for the development of Amyloid beta and pTau and therefore further research is required on the direct impact of dioxidovanadium (V) on these proteins in a hyperglycaemic setting during a period of 3 months of greater.

Schematic summary of study

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APPENDICES

Appendix A: Ethical Clearance

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

04 August 2020

Ms Yalka Dayanand (215036364)
School of Laboratory Medicine & Medical Sciences
Westville

Dear Ms Dayanand,

Protocol reference number: AREC/014/020M
Project title: The distribution and penetration of dioxidovanadium (V) complex in the brain and its effects on brain glucose metabolism and function in an STZ-induced rat model.

Full Approval – Research Application

With regard to your revised application received on 28 JulyDear thiru,
2020, the Animal Research Ethics Committee has accepted the documents submitted and **FULL APPROVAL** for the protocol has been granted.

Please note: There must be adherence to national and institutional COVID-19 regulations and guidelines at all times. Researchers will be personally responsible and liable for non-adherence to national regulations. If in doubt, please contact the Research Ethics Chair and/or the University Dean of Research for advice.

Please note: Any Veterinary and Para-Veterinary procedures must be conducted by a SAVC registered VET or SAVC authorized person.

Any alteration/s to the approved research protocol, i.e Title of Project, Location of the Study, Research Approach and Methods must be reviewed and approved through the amendment/modification prior to its implementation. In case you have further queries, please quote the above reference number.

Please note: Research data should be securely stored in the discipline/department for a period of 5 years.

The ethical clearance certificate is only valid for a period of one year from the date of issue. Renewal for the study must be applied for before 03 August 2021.

Attached to the Approval letter is a template of the Progress Report that is required at the end of the study, or when applying for Renewal (whichever comes first). An Adverse Event Reporting form has also been attached in the event of any unanticipated event involving the animals' health / wellbeing.

I take this opportunity of wishing you everything of the best with your study.

Yours faithfully

[redacted]

Dr Sanil D Singh, BVSc, MS, PhD
Chair: Animal Research Ethics Committee

/kr
cc Supervisor: Dr Phikelelani Ngubane
cc BRU Manager: Dr Jaca

Animal Research Ethics Committee (AREC)
Ms Karen Reinertsen (Administrator)
Westville Campus, Govan Mbeki Building
Postal Address: Private Bag X54001, Durban 4000
Telephone: +27 (0) 31 260 8850 **Facsimile:** +27 (0) 31 260 4609 **Email:** animalethics@ukzn.ac.za
Website: http://research.ukzn.ac.za/Research-Ethics/Animal-Ethics.aspx

Appendix B: Journal guidelines

CANADIAN JOURNAL OF DIABETES

AUTHOR INFORMATION PACK

TABLE OF CONTENTS

ISSN: 1499-2671

DESCRIPTION

The *Canadian Journal of Diabetes* is Canada's only diabetes-oriented, peer-reviewed, interdisciplinary journal for diabetes health-care professionals.

Published eight times a year, the *Canadian Journal of Diabetes* contains original articles; reviews; case reports; shorter articles such as Perspectives in Practice, Practical Diabetes and Innovations in Diabetes Care; Diabetes Dilemmas and Letters to the Editor. The *Canadian Journal of Diabetes* is distributed as a benefit of membership to all members of the professional section of Diabetes Canada.

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INTRODUCTION

About the Journal

The *Canadian Journal of Diabetes* is Canada's only diabetes-oriented, peer-reviewed, interdisciplinary journal for diabetes health-care professionals. The *Canadian Journal of Diabetes* is the official publication of the Professional Section of Diabetes Canada. The journal invites novel clinical and translational science submissions relevant to diabetes care and education.

Mission

The mission of the *Canadian Journal of Diabetes* is to promote the sharing of interdisciplinary research and evidence-based knowledge, from clinical or translational science to public health and education, which leads to advances in the care of diabetes.

Types of paper

1. Original Research
2. Review
3. Case Reports
4. Practical Diabetes
5. Perspectives in Practice
6. Innovations in Diabetes Care
7. Diabetes Dilemma
8. Letters to the Editor

All article types (with exception of letters to the Editor) require 2 to 3 key messages (for details, please refer to the manuscript preparation section).

The title of all articles should clearly indicate the population and type of diabetes referred to in the article (for example: Eye Color in Adults with Type 2 Diabetes).

Please contact the editorial board prior to submission if you are unable to adhere to the word and reference limits detailed below.

Please note that all of the word counts exclude references, unless otherwise noted.

1. Original Research (≤4000 words): Original research articles report basic science and clinical investigation in areas relevant to diabetes. Authors should take care to clearly establish the link of the work to diabetes, keeping in mind the broad readership of the journal by healthcare providers. Original research articles should include the following subsections: introduction, methods, results, discussion and brief conclusion. Original research articles must include a structured abstract (250 words maximum). Original research articles may be up to 4000 words and contain up to 4 figures and/or tables. Reference list must not exceed 50 references.

2. Review (≤5000 words): Review articles report basic science and clinical investigation in areas relevant to diabetes. Review articles must also include an abstract, although it need not be structured (maximum 250 words). Review articles should provide answers to clinically relevant questions that have not been well-answered to date, or bring readers up to date on useful concepts in a rapidly changing field. Review articles should provide a balanced presentation of the issues and evidence on the topic. Review articles may be up to 5000 words and contain up to 4 figures and/or tables. The reference list should not exceed 75 references. (Please note: Literature reviews conducted using a scientific method, such as systematic reviews and meta-analyses, should be submitted as original research).

3. Case Reports (≤1000 words): Case reports should outline a clinical situation that illustrates unique or atypical features or provide a lesson to be learned that is relevant to diabetes care. Case reports should include a brief introduction, a description of the case and discussion, including relevance, implications and recommendations. Case reports do not require an abstract. Articles in this section should not exceed 1000 words in length and may contain up to 2 figures and/or tables. The reference list should not exceed 20 references. Written informed consent from the patient(s) or their guardians(s) should be obtained before submission.

4. Practical Diabetes(≤2000 words): Articles under this section should be structured like review articles, be well-referenced and focus on any aspect of the care of people with diabetes. Practical Diabetes articles must also include an abstract, although it need not be structured (maximum 250 words). Practical diabetes articles could include review of new resources relevant to the care and education of people with diabetes. Articles in this section should not exceed 2000 words in length and may contain up to 2 figures and/or tables. The reference list should not exceed 25.

5. Perspectives in Practice (≤2000 words): This section provides a format for authors to discuss new programs or services, ideas, insights or practical approaches to diabetes care and education or professional development. Papers in this section should be well-referenced. Articles in this section should not exceed 2000 words in length and may contain up to 2 figures and/or tables. The reference list should not exceed 25 references.

6. Innovations in Diabetes Care (≤700 words): Papers in this section review new resources relevant to the care and education of people with diabetes. They may comment on range and depth of contents, readability level, design, approach, price and graphic elements. Articles in this section should not exceed 700 words in length and may contain 1 figure or table. The reference list should not exceed 10 references.

7. Diabetes Dilemmas (≤850 words): This feature is intended to highlight interesting and challenging cases in diabetes. This may include: diagnostic considerations, a picture to illustrate a clinical feature, management challenges and complications. The case should illustrate an approach to the problem and provide a succinct summary of take-home points. The case presentation should be 250 words (maximum) and clearly demonstrate the clinical diabetes challenge. Alternatively, a picture or illustration can be submitted instead of the case presentation provided it demonstrates the challenge. The case presentation should be followed by a discussion that is 600 words (maximum) outlining the approach to the clinical diabetes challenge. One figure or table may be included. Reference list should not exceed 10 references. Written informed consent from the patient(s) or their guardians(s) should be obtained before submission.

8. Letter to the Editor (≤500 words): Letters to the editor comment on a recently published article (which must be cited in the reference list) and should be submitted within 2 months of printed publication of the article. Letters do not have abstracts and may have a maximum of 5 references. The author(s) of the article under discussion will be invited to respond to the comment letter using the same format guidelines.

1 Abstract (word count) Word Count* References (maximum) Tables/Figures Original Research Required (250) ≤ 4000 50 4 tables or figures Review Required (250) ≤ 5000 75 4 tables or figures Case Reports No ≤ 1000 20 2 tables or figures Practical Diabetes Required (250) ≤ 2000 25 2 tables or figures Perspectives in Practice No ≤ 2000 25 2 tables or figures Innovations in Diabetes Care No ≤ 750 10 1 table or figure Diabetes Dilemmas No ≤ 850 10 1 table or figure Letter to the Editor No ≤ 500 5 N/A Table 1. Summary of Submission Requirements

* excludes abstract and references

BEFORE YOU BEGIN

Ethics in Publishing
For information on ethics in publishing and ethical guidelines for journal publication, refer to: https://www.elsevier.com/publishingethics and https://www.elsevier.com/journal-authors/ethics.

Human and animal rights

Work on human beings that is submitted to the *Canadian Journal of Diabetes* should comply with the principles laid down in the Declaration of Helsinki Recommendations guiding physicians in biomedical research involving human subjects, adopted by the 18th World Medical Assembly, Helsinki, Finland, June 1964 (and successive amendments). The manuscript should contain a statement that the work has been approved by the appropriate ethical committees related to the institution(s) in which it was performed. Studies involving experiments with animals must state that their care was in accordance with institution guidelines.

Patients and Study Participants

Studies on patients or volunteers require ethics committee approval and informed consent, and should be documented in the manuscript.

Patients have a right to privacy. Therefore identifying information, including patient's photographs, pedigree, images, names, initials, or hospital numbers, should not be included in the submissions unless the information is essential for scientific purposes and written informed consent has been obtained for publication in print and electronic form from the patient (or parent, guardian or next of kin). If such consent is made subject to any conditions, Elsevier must be made aware of all such conditions. Written consents must be provided to the journal on request.

Although complete anonymity is difficult to achieve, all efforts towards this goal must be taken. Even where consent has been given, identifying details should be omitted if they are not essential. Masking the eye region in photographs of patients is inadequate protection of anonymity. If identifying characteristics are altered to protect anonymity, such as in genetic pedigrees, authors should provide assurance that alterations do not distort scientific meaning, and editors should so note.

Permissions

Written consent from the author and publisher of any submitted material previously published must accompany the manuscript.

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All authors must disclose any financial and personal relationships with other people or organizations that could inappropriately influence (bias) their work. Examples of potential conflicts of interest include employment, consultancies, stock ownership, honoraria, paid expert testimony, patent applications/ registrations, and grants or other funding. If there are no conflicts of interest, then please state this: Conflicts of interest: None. See also https://www.elsevier.com/conflictsofinterest. Further information and an example of a Conflict of Interest form can be found at: https://service.elsevier.com/app/ answers/detail/a_id/286/supporthub/publishing.

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Use of inclusive language

Inclusive language acknowledges diversity, conveys respect to all people, is sensitive to differences, and promotes equal opportunities. Content should make no assumptions about the beliefs or commitments of any reader; contain nothing which might imply that one individual is superior to another on the grounds of age, gender, race, ethnicity, culture, sexual orientation, disability or health condition; and use inclusive language throughout. Authors should ensure that writing is free from bias, stereotypes, slang, reference to dominant culture and/or cultural assumptions. We advise to seek gender neutrality by using plural nouns ("clinicians, patients/clients") as default/wherever possible to avoid using "he, she," or "he/she." We recommend avoiding the use of descriptors that refer to personal attributes such as age, gender, race, ethnicity, culture, sexual orientation, disability or health condition unless they are relevant and valid. When coding terminology is used, we recommend to avoid offensive or exclusionary terms such as "master", "slave", "blacklist" and "whitelist". We suggest using alternatives that are more appropriate and (self-) explanatory such as "primary", "secondary", "blocklist" and "allowlist". These guidelines are meant as a point of reference to help identify appropriate language but are by no means exhaustive or definitive.

Authorship Criteria and Changes to Authorship

The *Canadian Journal of Diabetes* has adopted the authorship recommendations of the International Committee of Medical Journal Editors (ICMJE) (http://www.icmje.org/recommendations/browse/ roles-and-responsibilities/defining-the-role-of-authors-and-contributors.html). Authorship is thus based on the following 4 criteria:

Substantial contributions to the conception or design of the work; or the acquisition, analysis, or interpretation of data for the work; AND Drafting the work or revising it critically for important intellectual content; AND Final approval of the version to be published; AND Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Authors are asked to include in the manuscript a statement regarding the specific contributions of all authors to the preparation of the manuscript under the heading "Author Contributions". This statement should follow the acknowledgement section. Authors are expected to consider carefully the list and order of authors before submitting their manuscript and provide the definitive list of authors at the time of the original submission. Any addition, deletion or rearrangement of author names in the authorship list should be made only before the manuscript has been accepted and only if approved by the journal editor. To request such a change, the editor must receive the following from the corresponding author: (a) the reason for the change in author list and (b) written confirmation (e-mail, letter) from all authors that they agree with the addition, removal or rearrangement. In the case of addition or removal of authors, this includes confirmation from the author being added or removed.

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Registration of Clinical Trials
* All randomized controlled trials submitted to the *Canadian Journal of Diabetes* whose primary purpose is to affect clinical practice (phase 3 trials) must be registered in accordance with the principles outlined by the International Committee of Medical Journal Editors (ICMJE). ICMJE-approved registries currently include the following: https://www.anzctr.org.au, https://www.clinicaltrials.gov, http://www.ISRCTN.org, https://www.umin.ac.jp/ctr/index.htm, https://www.trialregister.nl, and https://eudract.ema.europa.eu/. Please include the unique trial number and registry name on manuscript submission.

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Patient Consent Form

Studies on patients or volunteers require informed consent, which should be documented within the manuscript. In addition, patient consent form(s) need to be completed and uploaded with the original manuscript. Appropriate consents, permissions and releases must be obtained where an author wishes to include case details or other personal information or images of patients.

Author Disclosures

A statement regarding authors' conflicts of interest must be included in the manuscript. This should be under the heading Author Disclosures. This statement should follow the Author Contribution Statement.
All authors listed in the manuscript are required to submit a completed ICMJE Form for Disclosure of Potential Conflicts of Interest. All authors must disclose possible conflicts of interest at the time of submission. This information will remain confidential while the paper is under review and will not influence editorial decision. The information will be published with the article.

General Points
Manuscripts must be written in English. All materials (including tables) must be typed and double-spaced in Times New Roman 11 pt font. Use 1.0" margins on all sides, and number all pages in the bottom right corner. Separate each paragraph with a blank line. The term "diabetic" should not be used as a noun. Use lowercase for "diabetes", "type 1" and "type 2". Use numerals in the text rather than writing out numbers. Report all measurements in SI units. Report blood glucose in mmol/L. The abbreviation for hemoglobin A1C/glycated hemoglobin is A1C and values should be reported in derived NGSP units (% to one decimal point).

References must be accessible to the readership and should ideally be in English or French. References in other languages may only be used on an exceptional basis.

PREPARATION

Use of word processing software
It is important that the file be saved in the native format of the word processor used. The text should be in single-column format. Keep the layout of the text as simple as possible. Most formatting codes will be removed and replaced on processing the article. In particular, do not use the word processor's options to justify text or to hyphenate words. However, do use bold face, italics, subscripts, superscripts etc. When preparing tables, if you are using a table grid, use only one grid for each individual table and not a grid for each row. If no grid is used, use tabs, not spaces, to align columns. The electronic text should be prepared in a way very similar to that of conventional manuscripts (see also the Guide to Publishing with Elsevier). Note that source files of figures, tables and text graphics will be required whether or not you embed your figures in the text. See also the section on Electronic artwork.
To avoid unnecessary errors you are strongly advised to use the 'spell-check' and 'grammar-check' functions of your word processor.

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Divide your article into clearly defined sections (for example, Original Research articles are divided into Introduction, Methods, Results, Discussion, Conclusion, Acknowledgements, References, Tables, Figures), with each heading written in bold font, and appearing on its own separate line. Each

subsection should be given a brief heading, written in italics, and appearing on its own separate line. Subsections should be used as much as possible. When cross-referencing text, refer to the subsection heading as opposed to simply "the text".

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Introduction: Provide an adequate background, avoiding a detailed literature survey or a summary of the results and state the objectives of the work.

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Model Organisms: "Experiments were conducted in c. elegans strain SP304 (RRID:CGC_SP304)"

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Tools, Software, and Databases: "Image analysis was conducted with CellProfiler Image Analysis Software, V2.0 (http://www.cellprofiler.org, RRID:nif-0000-00280)"

Wiley Author Resources

Manuscript Preparation Tips: Wiley has a range of resources for authors preparing manuscripts for submission available **here**. In particular, we encourage authors to consult Wiley’s best practice tips on **Writing for Search Engine Optimization**.

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5. EDITORIAL POLICIES AND ETHICAL CONSIDERATIONS

Peer Review and Acceptance

The acceptance criteria for all papers are the quality and originality of the research and its significance to journal readership. Papers will only be sent to review (single blinded) if the Editor-in-Chief determines that the paper (original research or review manuscript) meets the appropriate quality and relevance requirements. Wiley's policy on the confidentiality of the review process is available **here**.

MEDLINE evaluates a journal's ethical policy by checking that journals ask submitting authors to provide three things: a declaration of conflict of interest (CoI), confirmation that informed consent was sought from test subjects, and that animal rights were taken into consideration. The reviewer will then check three things during the review:

1. **Policy Exists**: is there evidence in the author guidelines that the journal requires that the appropriate ethical requirements are followed.

2. **Policy is Adequate**: is the policy appropriate for the journal, e.g. a review journal does not need to have a statement on human/animal rights or informed consent.

3. **Policy Consistently Followed**: is there evidence in all the published articles that authors have declared their conflicts of interest and that appropriate procedures were followed when the research was conducted. This will be checked in the final published articles.

It is recommended that all articles include a statement regarding CoI, regardless of whether or not a CoI exists – for example, “The authors have stated explicitly that there are no conflicts of interest in connection with this article.”

There should be robust journal workflows in place to ensure all three criteria are met. Examples of failures would be: a journal that requires authors to declare that institutional review board (IRB) approval was sought for their research, but this is not communicated to the readers of the final article; journals that do require declarations of informed consent, but don't say so in the author guidelines; or journals that only publish statements when conflicts-of-interest were declared, and assume that all readers know omission means that there aren't any conflicts.

Human Studies and Subjects

For manuscripts reporting medical studies that involve human participants, a statement identifying the ethics committee that approved the study and confirmation that the study conforms to recognized standards is required, for example: **Declaration of Helsinki**; **US Federal Policy for the Protection of Human Subjects**; or **European Medicines Agency Guidelines for Good Clinical Practice**. It should also state clearly in the text that all persons gave their informed consent prior to their inclusion in the study. Patient anonymity should be preserved. When detailed descriptions, photographs, or videos of faces or identifiable body parts are used that may allow identification, authors should obtain the individual's free prior informed consent. Authors do not need to provide a copy of the consent form to the publisher; however, in signing the author license to publish, authors are required to confirm that consent has been obtained. Wiley has a **standard patient consent form** available for use. Where photographs are used they need to be cropped sufficiently to prevent human subjects being recognized; black eye bars should not be used as they do not sufficiently protect an individual’s identity).

Animal Studies

A statement indicating that the protocol and procedures employed were ethically reviewed and approved, as well as the name of the body giving approval, must be included in the Methods section of the manuscript. Authors are encouraged to adhere to animal research reporting standards, for example the **ARRIVE guidelines** for reporting study design and statistical analysis; experimental procedures; experimental animals and housing and husbandry. Authors should also state whether experiments were performed in accordance with relevant institutional and national guidelines for the care and use of laboratory animals:

• US authors should cite compliance with the US National Research Council's **Guide for the Care and Use of Laboratory Animals**, the US Public Health Service's **Policy on Humane Care and Use of Laboratory Animals**, and **Guide for the Care and Use of Laboratory Animals**.
• UK authors should conform to UK legislation under the **Animals (Scientific Procedures) Act 1986 Amendment Regulations (SI 2012/3039)**.
• European authors outside the UK should conform to **Directive 2010/63/EU**.

Clinical Trial Registration

The journal requires that clinical trials are prospectively registered in a publicly accessible database and clinical trial registration numbers should be included in all papers that report their results. Authors are asked to include the name of the trial register and the clinical trial registration number at the end of the abstract. If the trial is not registered, or was registered retrospectively, the reasons for this should be explained.

Research Reporting Guidelines

Accurate and complete reporting enables readers to fully appraise research, replicate it, and use it. Authors are expected to adhere to recognised research reporting standards. The EQUATOR Network collects more than 370 reporting guidelines for many study types, including for:

• **Randomised trials**: **CONSORT**
• **Observational studies**: **STROBE**
• **Systematic reviews**: **PRISMA**
• **Case reports**: **CARE**
• **Qualitative research**: **SRQR**
• **Diagnostic / prognostic studies**: **STARD**
• **Quality improvement studies**: **SQUIRE**

• **Economic evaluations**: **CHEERS**
• **Animal pre-clinical studies**: **ARRIVE**
• **Study protocols**: **SPIRIT**
• **Clinical practice guidelines**: **AGREE**

We also encourage authors to refer to and follow guidelines from:

• **Future of Research Communications and e-Scholarship (FORCE11)**
• **National Research Council's Institute for Laboratory Animal Research guidelines**
• **The Gold Standard Publication Checklist from Hooijmans and colleagues**
• **Minimum Information Guidelines from Diverse Bioscience Communities (MIBBI) website**
• **FAIRsharing website**

Species Names

Upon its first use in the title, abstract, and text, the common name of a species should be followed by the scientific name (genus, species, and authority) in parentheses. For well-known species, however, scientific names may be omitted from article titles. If no common name exists in English, only the scientific name should be used.

Genetic Nomenclature

Sequence variants should be described in the text and tables using both DNA and protein designations whenever appropriate. Sequence variant nomenclature must follow the current HGVS guidelines; see **varnomen.hgvs.org**, where examples of acceptable nomenclature are provided.

Sequence Data

Nucleotide sequence data can be submitted in electronic form to any of the three major collaborative databases: DDBJ, EMBL, or GenBank. It is only necessary to submit to one database as data are exchanged between DDBJ, EMBL, and GenBank on a daily basis. The suggested wording for referring to accession-number information is: ‘These sequence data have been submitted to the DDBJ/EMBL/GenBank databases under accession number U12345’. Addresses are as follows:

• DNA Data Bank of Japan (DDBJ): **www.ddbj.nig.ac.jp**
• EMBL Nucleotide Archive: **ebi.ac.uk/ena**
• GenBank: **www.ncbi.nlm.nih.gov/genbank**

Proteins sequence data should be submitted to either of the following repositories:

• Protein Information Resource (PIR): **pir.georgetown.edu**
• SWISS-PROT: **expasy.ch/sprot/sprot-top**

Conflict of Interest

The journal requires that all authors disclose any potential sources of conflict of interest. Any interest or relationship, financial or otherwise that might be perceived as influencing an author's objectivity is considered a potential source of conflict of interest. These must be disclosed when directly relevant or directly related to the work that the authors describe in their manuscript. Potential sources of conflict of interest include, but are not limited to: patent or stock ownership, membership of a company board of directors, membership of an advisory board or committee for a company, and consultancy for or receipt of speaker's fees from a company. The existence of a conflict of interest does not preclude publication. If the authors have no conflict of interest to declare, they must also state this at submission. It is the responsibility of the corresponding author to review this policy with all authors and collectively to disclose with the submission ALL pertinent commercial and other relationships.

Funding

Authors should list all funding sources in the Acknowledgments section. Authors are responsible for the accuracy of their funder designation. If in doubt, please check the Open Funder Registry for the correct nomenclature: **https://www.crossref.org/services/funder-registry/**

Authorship

The list of authors should accurately illustrate who contributed to the work and how. All those listed as authors should qualify for authorship according to the following criteria:

1. Have made substantial contributions to conception and design, or acquisition of data, or analysis and interpretation of data; and

2. Been involved in drafting the manuscript or revising it critically for important intellectual content; and

3. Given final approval of the version to be published. Each author should have participated sufficiently in the work to take public responsibility for appropriate portions of the content; and

4. Agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Contributions from anyone who does not meet the criteria for authorship should be listed, with permission from the contributor, in an Acknowledgments section (for example, to recognize contributions from people who provided technical help, collation of data, writing assistance, acquisition of funding, or a department chairperson who provided general support). Prior to submitting the article all authors should agree on the order in which their names will be listed in the manuscript.

Corrections to authorship

In accordance with Wiley's **Best Practice Guidelines on Research Integrity and Publishing Ethics** and the **Committee on Publication Ethics**' guidance, *Journal of Neuroendocrinology* will allow authors to correct authorship on a submitted, accepted, or published article if a valid reason exists to do so. All authors – including those to be added or removed – must agree to any proposed change. To request a change to the author list, please complete the **Request for Changes to a Journal Article Author List Form** and contact either the journal's editorial or production office, depending on the status of the article. Authorship changes will not be considered without a fully completed Author Change form. Correcting the authorship is different from changing an author's name; the relevant policy for that can be found in **Wiley's Best Practice Guidelines** under "Author name changes after publication."

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All accepted manuscripts are required to publish a data availability statement to confirm the presence or absence of shared data. If you have shared data, this statement will describe how the data can be accessed, and include a persistent identifier (e.g., a DOI for the data, or an accession number) from the repository where you shared the data. Authors will be required to confirm adherence to the policy. If you cannot share the data described in your manuscript, for example for legal or ethical reasons, or do not intend to share the data then you must provide the appropriate data availability statement. *Journal of Neuroendocrinology* notes that FAIR data sharing allows for access to shared data under restrictions (e.g., to protect confidential or proprietary information) but notes that the FAIR principles encourage you to share data in ways that are as open as possible (but that can be as closed as necessary).

Sample statements are available **here**. If published, all statements will be placed in the heading of your manuscript.

Human subject information in databases. The journal refers to the **World Health Medical Association Declaration of Taipei on Ethical Considerations Regarding Health Databases and Biobanks**.

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As part of the journal's commitment to supporting authors at every step of the publishing process, the journal requires the submitting author (only) to provide an ORCID iD when submitting a manuscript. This takes around 2 minutes to complete. **Find more information here**.

OPEN SCIENCE INITIATIVES

Recognizing the importance of research transparency and data sharing to cumulative research, *Journal of Neuroendocrinology* encourages the following open science practices.

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The Open Materials badge recognizes researchers who share their research instruments and materials in a publicly-accessible format, providing sufficient information for researchers to reproduce procedures and analyses of published research studies.

The Open Data badge recognizes researchers who make their data publicly available, providing sufficient description of the data to allow researchers to reproduce research findings of published research studies. An example of a qualifying public, open-access database for data sharing is the Open Science Framework repository. Numerous other data-sharing repositories are available through various Dataverse networks (e.g.,**http://dataverse.org**) and hundreds of other databases available through the Registry of Research Data Repositories (**http://www.re3data.org**). There are, of course, circumstances in which it is not possible or advisable to share data publicly. For example, there are cases in which sharing participant data could violate confidentiality. In these cases, the authors may provide an explanation of such circumstances in the Alternative Note section of the disclosure form. The information the authors provide will be included in the article's Open Practices note.

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Authors will have an opportunity at the time of manuscript submission and at the time of acceptance to inform themselves of this initiative and to determine whether they wish to participate. Applying and qualifying for Open Science badges is not a requirement for publishing with *Journal of Neuroendocrinology*, but these badges are further incentive for authors to participate in the open science movement and thus to increase the visibility and transparency of their research. More information about the Open Practices badges is available from the Open Science Framework wiki.

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8. POST PUBLICATION

Access and Sharing

When the article is published online:

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9. EDITORIAL OFFICE CONTACT DETAILS

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10. GUIDANCE ON USE OF ANTIBODIES

In the broad field of Neuroendocrinology, antibodies are a valuable and commonly used tool to identify expression of a specific protein by immunohistochemistry in tissues which may contain a variety of cell types. However, concern has been raised, evident from journal editorials and articles (1-4), regarding the lack of specificity of some antibodies and inadequate controls applied by authors to verify antibody specificity prior to their use, leading to erroneous information. Journal of Neuroendocrinology is as vulnerable as any other journal to receiving manuscripts in which antibodies have not been characterized with sufficient rigor.

Journal of Neuroendocrinology often receives manuscript submissions in which an antibody has been raised to a mammalian antigen but has been used to localize or quantify protein expression in a different species, for example, a fish or bird. This is understandable to overcome the dearth of antibodies from commercial suppliers raised specifically to proteins from less commonly used species. However, many antibodies appear to be used without sufficient validation of their specificity in the target species.

Often, attempts to verify specificity of an antibody are limited to either omission of the primary antibody and/or pre-absorption with the antigen to which it was raised. However, the former only tests the specificity of the secondary antibody while the latter only confirms that the antibody binds to the antigen to which it was raised. Neither approach demonstrates specificity on the sample. A common strategy employed by authors to suggest specificity is citation of an article where the antibody has been previously used. However, on reference to such citations, it is common for the antibody concerned not to receive any better validation than the aforementioned tests of primary antibody omission or antigen pre-absorption.

To increase confidence in immunohistochemical data, *Journal of Neuroendocrinology*, in line with similar journals, will require adequate validation to be demonstrated if this has not already been established. This may include, but should not be restricted to, a pre-absorption test. The ideal test would be lack of staining in tissue samples from an animal in which the gene of interest has been deleted. However, this is, by-and-large, not feasible, particularly for less commonly used

animal models. An alternative may be to test the antibody on a suitable cell line which does not express the protein of interest, but where the protein can be heterologously expressed. Additional measures could include (i) a Western blot demonstrating only a band(s) for the protein of interest, (ii) coincidence of expression with mRNA encoding the protein or (iii) use of a second antibody raised to a different sequence of the same protein demonstrating colocalization or the same distribution with both antibodies. Furthermore, where cross species reactivity is desired, it may be important to confirm that the target sequence has not significantly diverged from the species to which the antigen was raised. Citation of papers where an antibody has been adequately characterized in accordance with these strategies would be acceptable.

To support readers who may wish to use an antibody described in the Journal, we have also adopted the requirement for authors to provide a full description of the antibody, including the source, catalogue number, the species to which the antigen was raised and research resource identifier (RRID) number (see author guidelines for explanation). If a peptide was the antigen source, the sequence of the antigen used to raise the antibody should be provided, indicating whether the target sequence in the species of interest is different from the peptide of the species of origin. The dilution factor of the antibody used in the study should also be specified. With the implementation of this policy, the Editors of the Journal hope that we can increase the confidence of our readers in the immunohistochemical data presented in *Journal of Neuroendocrinology*.

Validation of uncharacterized antibodies or seeking to use for cross species detection – a combination of more than one from the following:

Antigen pre-absorption test
Western blot
Negative staining in a transgenic knockout animal
Heterologous expression of protein in a negative cell line.
Knockdown of expression in a positive cell line.
Colocalization with another antibody raised to the same protein
Coincident localization with mRNA

1. Saper, CB 2005, An open letter to our readers on the use of antibodies. J. Comp Neurol 493:477-478
2. Gore A 2013 Editorial: Antibody validation requirements for article published in Endocrinology
3. Saper, CB 2009, A guide to the perplexed on the specificity of Antibodies
4 Bourdeaux, J, Welsh AW, Agarwal S, Killam E., Baquero MT., Hanna JA., Anagnostou VK., Rimm DL. 2010 Biotechniques 48:197-209

11. GUIDANCE OF USE OF PCR

PCR analysis: For measurement of mRNA including by microarray analysis and Next-GEN sequencing (RNAseq), confirmation of mRNA expression should be provided using quantitative RTPCR. A statement about the quality and integrity of the RNA must be provided together with the results of electrophoretic analysis of the purity of the PCR products. Full details of the oligonuceoltide primers and PCR protocol must be stated either in the text or in Supplementary Material. The stability of reference genes used for normalization of PCR data must be reported for the experimental conditions described. Where possible, analysis of mRNA levels should be accompanied by assessment of either protein levels or activities.

Microarray analysis and Next Generation Sequencing: Studies involving microarray analysis of mRNA must conform to the “Minimum Information about a Microarray Experiment” (MIAME) guidelines, including deposition of the raw data in an appropriate repository such as Gene Expression Omnibus (GEO) or ArrayExpress, and the Accession number must be stated in the manuscript. Similarly, sequencing data generated by Next Generation sequencing must be deposited in a public repository such as GEO or ArrayExpress before a manuscript can be accepted for publication. The accession number must also be provided in the manuscript.

12. GUIDANCE ON USE OF STATISTICAL ANALYSIS

It is recommended that authors consult a professional statistician for guidance concerning the design and analysis of their work. The design and execution of experiments, including all measures, data manipulations and statistical analysis should be clearly described when reporting

original research. Accordingly, the methods of statistical analysis used should be described, including the statistical analysis packages used, with sufficient detail for a statistician with access to the data to reproduce the results presented. Sample sizes should be detailed, including power calculations if appropriate, with the desired effect size and power, and estimates of variability. Results of statistical tests should be standardized e.g. for ANOVA, report the F value, degrees of freedom, and probability: $F(x,x,) = x.xx$, $P = 0.xxx$. Three decimal places are usually adequate for reporting statistics. Sample sizes should be given in the Methods section, in Figure captions and, where appropriate, in the Results section, especially where studies contain a number of different treatment groups. The reader should be able to easily determine the sample size for each data set presented. If tables of statistical outcomes are appropriate, these should be provided as supplementary data.

Author Guidelines updated February 2019