CT99: A Novel Nutraceutical Therapy for the Treatment of Diabetes Mellitus

By

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A Thesis
Submitted to the Faculty of Graduate Studies
In Partial Fulfillment of the Requirements
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Faculty of Medicine, University of Manitoba
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ABSTRACT

Although the discovery of insulin in the early 1900's revolutionized the treatment of the diabetic patient the morbidity and mortality caused by this disease of glucose metabolism remains high. As the seventh leading cause of death in Canada and the leading cause of both blindness and lower leg amputations, diabetes affects all organ systems. It is however, the cardiovascular consequences of the disease that continue to result in the majority of diabetic deaths. Although the current regimen of insulin and/or oral hypoglycaemic therapy delays the onset of complications current treatment regimens provide little promise for long-term efficacy or cure. Therefore, the search for improved methods to control blood sugars continues.

One of the most promising new approaches to diabetic therapy is the use of vanadium. First discovered in 1985 to effectively lower blood glucose in rat models this transition metal and many synthetic compounds containing vanadium have been proven effective hypoglycaemic agents. Most of these agents however retain the side effect profile of the original vanadium compounds. Most notably these include severe gastrointestinal toxicity, appetite suppression and high mortality rates as a result. If these side effects could be controlled or eliminated vanadium possesses the potential to be an excellent alternative to current therapy.

The use and acceptance of nutraceuticals and alternative medicines is rapidly increasing and these products can be used to control many health problems. Therefore we approached the issue of vanadium toxicity with the hypothesis that the addition of a

nutraceutical agent with broad range effects and anti-diarrheal action to a vanadate compound may reduce toxicity and provide an efficacious therapy for diabetes. The developed compound is named CT99.

In acute studies CT99–treatment lowered diabetic blood glucose levels to non-diabetic levels within 16 hours. This equalled the effectiveness of a conventional vanadate/water solution. Importantly, the accumulation of vanadium in plasma and major organs was equal to or significantly lower than levels accumulated with a treatment solution made of vanadium and water. In chronic trials CT99 was effective in reducing blood glucose levels for months at a time without the requirement of further treatment. Animals treated for up to one year revealed little evidence of toxicity. Water and food intake in diabetic animals treated with CT99 were normalized, as was body weight. Similarly, evidence of liver, kidney or serum lipid abnormalities was absent. Cardiac function was also improved vs. untreated diabetic animals. Most importantly CT99-treatment significantly reduced the occurrence of diarrhea and death in diabetic animals vs. the vanadate/water solution. These results apply to both Type 1 and Type 2 diabetes.

Clearly, CT99 possesses excellent potential as a therapeutic agent against diabetes. The compound is orally administered, whereas insulin requires injections several times daily. CT99 acutely and chronically controls blood glucose. The control of blood sugar status was so strong that the common complications of diabetes, namely ocular disease, cardiac disease and lipid abnormalities were eliminated. With diabetes approaching epidemic proportions this has strong implications in reducing morbidity and mortality while reducing the enormous health care costs of the disease.

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DEDICATION

To Tara

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REVIEW OF LITERATURE

I. Diabetes

1. Definition:

Diabetes mellitus is a group of metabolic diseases characterized by carbohydrate intolerance associated with a reduced capacity to utilize ingested glucose resulting in hyperglycemia. The disease is multifactorial in origin resulting from a combination of genetic, environmental and autoimmune mechanisms. Diagnosis depends on symptoms of excessive urination (polyuria), increased thirst (polydipsia), increased food intake (polyphagia) and weight loss but requires high blood glucose levels defined as fasting glucose levels > 7 mM or random plasma glucose levels > 11.1 mM [(1)].

2. Classification:

The expert committee on the diagnosis and classification of diabetes mellitus revised the classification and terminology of diabetes in 1997 [(2)]. Currently, the two major forms of the disease are known as Type 1 and Type 2 diabetes, formerly referred to as insulin dependant diabetes mellitus (IDDM) and non-insulin dependant diabetes mellitus (NIDDM) respectively. In Type 1 diabetes deficient production of pancreatic insulin by either an autoimmune process or an idiopathic mechanism reduces uptake of glucose by the liver and peripheral tissues. Conversely, in Type 2 diabetes relatively high circulating levels of insulin are ineffective for glucose uptake because of insulin receptor and post-receptor defects. Type 2 diabetes has a stronger genetic basis than Type 1 and is also associated with obesity and abdominal fat. Gestational diabetes mellitus (GDM) is

defined by the occurrence of glucose intolerance at any time during pregnancy irrespective of diabetic status post-pregnancy. Several other diabetes subgroups exist including specific genetic diseases causing diabetes (glucokinase deficiency, mitochondrial diseases, Down's syndrome, etc), pancreatic disease associated diabetes, endocrinopathies, infectious disease and drug or chemically induced diabetes.

3. Epidemiology:

The burden of diabetes continues to grow and today approximately one million Canadians have the disease with over 60,000 new cases being diagnosed annually [(3)]. The prevalence of diabetes increases with age as 3% of the under 34 year-old population and > 10% of the over 64 year-old population are diabetic. Greater than 90% of diabetics have the Type 2 variant. Although all ethnic groups are affected with diabetes it is important to note that the Aboriginal population has a 3-fold higher occurrence of the disease than the remaining population. Diabetes as a whole accounts for the seventh leading cause of death in Canada (5,447 deaths in 1996), however, the actual number of deaths related to diabetes is as much as five times higher than that considering that cardiovascular failure in a diabetic person is classified as a cardiac disease death and not a diabetic death at this time. The impact on society can also be estimated by the nearly 25,000 potential years of life lost annually due to the disease. Overall, with direct medical costs, disability, work loss and premature death, it is estimated that diabetes costs Canadians over $9 billion (US) annually [(3)].

4. Etiology and Pathogenesis:

i) Type 1 Diabetes

Several mechanisms are involved in the onset of Type 1 diabetes. An autoimmune role in the development of the disease has been suggested, while genetic influences are highly suspected yet not completely understood. Similarly, viral, nutritional and toxic mechanisms may also play a part. However clear evidence remains absent. Autoimmunity is suggested by the presence of immune cells, namely T and B lymphocytes, plasma cells and macrophages within the β-cells of the pancreas [4,5]. This inflammatory response selectively destroys insulin-producing cells of the pancreas and has been termed insulitis. Interestingly, this infiltration does not affect the α,δ or PP endocrine cells within the islets or any of the exocrine pancreas [6]. Attack of the β-cells appears to result from the abberant expression of altered human leukocyte antigens (HLA's) on the cell surface [7]. These altered receptors activate T cells and thereby act as an antigen destining the insulin producing cells for destruction. The altered HLA molecules also provide a clue to the genetic predisposition towards diabetes, as the major histocompatability complex (MHC) is the area of the genome producing the HLA molecules. The finding that the production of a HLA DQβ chain with an aspartic acid at residue 57 resulted in protection from diabetes further substantiates the role of the HLA molecules [8]. Further evidence of genetic involvement is shown by the increased chances (30-50%) of an identical twin contracting the disease than a non-identical twin of a diabetic [9]. Several antibodies involved in the pathogenesis of Type 1 diabetes have also been found. These include islet cell antibody (ICA), islet cell surface antibody (ICSA) and insulin autoantibodies (IAA). ICA's are detected in > 70% of diabetic patients but

less than 1% of non-diabetic controls and may have potential in screening programs as titres are highest early in the disease and decrease significantly with disease progression [10,11]. The IAA protein is also positive in about half of newly diagnosed cases while other antibodies with high specificity continue to be studied [12-14]. Viral infection may also influence the development of diabetes as α interferon and anti-coxsackie virus antibodies have been found more often in diabetic patients [15,16]. Therefore it appears risk factors for diabetes include family history, autoimmune diseases, serum antibody markers and potentially certain viral infections.

ii) Type 2 Diabetes

In this form of diabetes two distinct defects coexist. Peripheral tissues, namely muscle and adipose tissue, fail to respond appropriately to circulating insulin and thus the term insulin resistance. Simultaneously, the pancreas fails to respond to this insulin resistance by releasing higher levels of insulin in order to compensate. Therefore the basis of this form of diabetes is more complex than its Type 1 counterpart. Both genetics and environment are believed to be crucial in the insulin resistant transformation. The genetic influences on Type 2 diabetes are strong. This is apparent with the high rates of familial aggregation and even higher rates of disease in identical twins of Type 2 diabetic patients. Offspring of two Type 2 diabetic parents have nearly an 80% rate of disease development while any first degree relative with diabetes doubles the odds of a patient developing diabetes [17-19]. Genetic mutations in the insulin gene have been described but are extremely rare [20,21]. Therefore it has been suggested that insulin defects in themselves are not involved in the pathogenesis of Type 2 diabetes [22]. The insulin

receptor has also been studied as the site of potential insulin resistance. In genetic defects of the insulin receptor however, insulin resistance is much more severe than in Type 2 diabetes and this has been determined as the cause of severe hyperglycemia in Rabson-Mendenhall syndrome, leprechaunism and other rare disorders [23-25]. As the involvement of insulin and its receptor are unlikely in Type 2 diabetes, this leaves an enormous number of downstream possibilities in the insulin cascade. This signalling pathway is reviewed in section iii). It is hypothesized that many of these pathways do play a role in diabetes. Firstly, it has been shown that the insulin receptor is down regulated in diabetes and this may account for some of the insulin resistance [26]. Secondly, the most well described substrate of the insulin receptor, insulin receptor substrate-1 (IRS-1) has been found to have genetic changes resulting in 30% less glucose uptake in response to insulin [27-30]. Although it is clear that genetics play a major role in the etiology of Type 2 diabetes, it is obvious that much of its involvement remains to be elucidated. Compounding the genetic basis of this disease there is a great deal of evidence to suggest environmental and lifestyle issues plays a part in diabetes development. Marked differences exist in worldwide prevalence rates. These differences range from approximately 50% prevalence in Arizona Pima Indians to 0% in New Guinea [31,32]. Further evidence of enviromental involvement in the etiology of Type 2 diabetes results from data showing migrational and rural-urban differences within the same races. Chinese migrants in Mauritius for example have an 8 fold higher chance of developing diabetes than do their mainland compatriots while large differences also exist between urban Figians and rural Indians [31,32]. Western populations currently have much higher rates of diabetes but with the migration and urbanization patterns in the far East it is

expected that worldwide diabetes prevalence will increase dramatically. Although not completely understood, the reasons for these ethnic and geographical differences appear to be lifestyle in origin. Diets high in fat and physical inactivity are closely associated with the development of insulin resistance while central obesity remains one of the strongest risk factors [33,34].

iii) Insulin Signalling Pathway

Because diabetes is such a complex disease involving the pancreas, liver and peripheral tissues that stems from multiple disorders of insulin production, insulin receptor activation and insulin signalling, a focussed examination of the insulin mediated cascade of actions is necessary to appreciate the complexities of the disease. The anabolic hormone insulin produces a variety of cellular effects by initiating a complex cascade of phosphorylation and dephosphorylation reactions involving several intermediate enzymes. Insulin, produced in the endocrine pancreas by the B-cells of the islet of langerhans is produced initially as a single chain of amino acids and is cleaved into two polypeptide chains (A and B) bound together by two disulphide bonds [35,36]. In response to elevated blood glucose or a high ratio of glucagon: insulin, insulin is released into the bloodstream. Circulating insulin contacts insulin–dependant tissues (primarily muscle and fat) and initiates its effects by binding to the insulin receptor (IR). This receptor is a tetrameric protein composed of two identical α chains and two identical β chains **(Figure 1)** [37-39]. The α chains located on the extracellular surface of the plasma membrane bind insulin [40]. Through an allosteric conformational change in the transmembrane domains of the β chains, a signal is transmitted intracellularly [41]. The intracellular segments of the

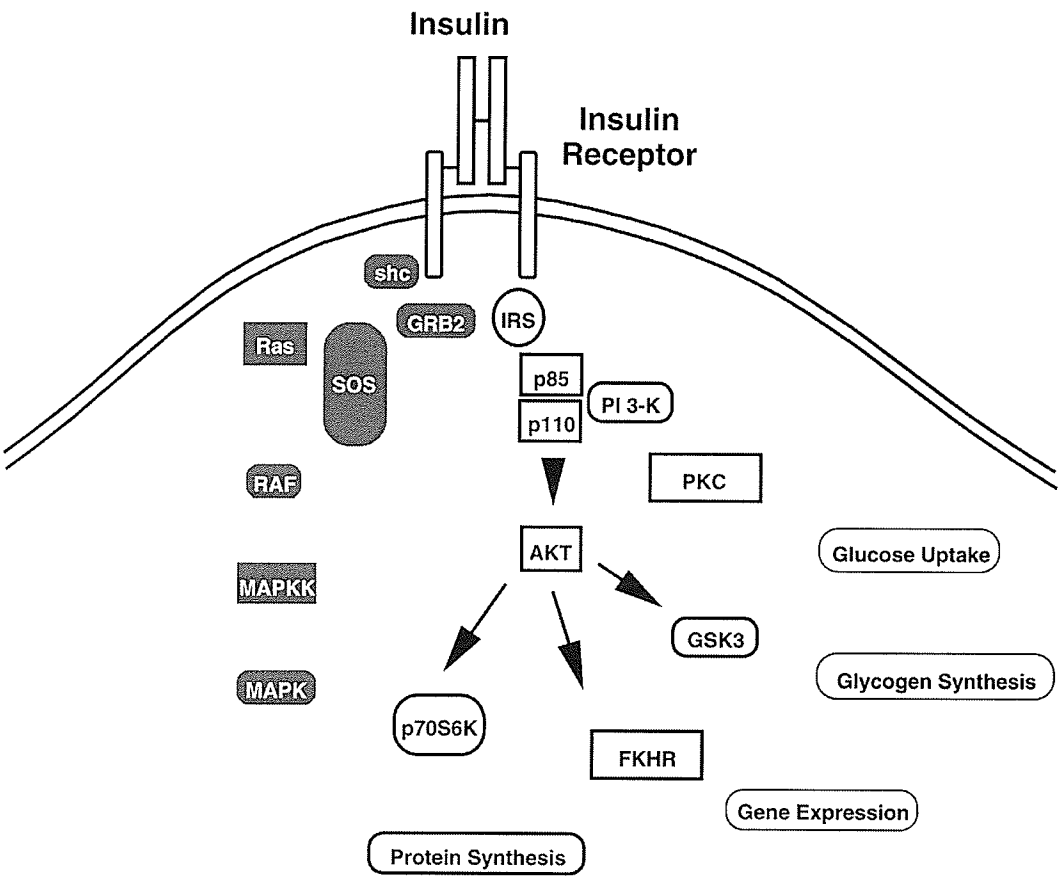


Figure 1. Insulin signalling pathway. Insulin binds to the insulin receptor, a tyrosine kinase that autophosphorylates increasing its own activity. Insulin receptor substrate proteins (IRS's) and shc activate the GRB-2/SOS controlled MAPK pathway (black) while phophatidylinositol 3-kinase (PI 3-K) and its targets stimulate PKC, PKB, glycogen synthase kinase and FKHR transcription factors (grey).

Ref: Kido Y, Nakae J, Accili D. Clinical review 125: The insulin receptor and its cellular targets. J Clin Endocrinol Metab. 2001 Mar;86(3):972-9.

β chains are members of the protein tyrosine kinase family of receptors and upon activation specific tyrosine regions of the β chains are autophosphorylated [42]. Simultaneously, an intracellular insulin receptor substrate (IRS-1) protein is phosphorylated. In addition, several other IRS-1 like proteins have been identified which potentially play a role in the transmission of the insulin signal [43]. These proteins include IRS-2 and Gab1 with similar actions to IRS-1 and the more tissue specific varieties IRS-3 (fibroblasts, liver and adipose tissue) and IRS-4 (embryonic kidney cells). The ability of all of these proteins to bind the phosphorylated insulin receptor lies in a common amino acid sequence known as the SH2 domain (named after the Src homology regions found in the Src proto-oncogene protein) found to recognize phosphorylated tyrosines [44]. An SH3 domain is also common to the proteins but the function of it is as yet undetermined.

Phosphorylated IRS-proteins continue the kinase cascade by associating with a similar SH2 domain of phosphatidylinositol 3-kinase (PI-3K) and others [44,45]. The class 1a PI-3K's have been identified as the subclass responsible for insulin mediated effects [46]. Activation of PI-3K by IRS-1 results in the phosphorylation of phosphatidylinositol bisphosphate (PIP2) producing phosphatidylinositol triphosphate [PI(3,4,5)P3]. This reaction is essential in many of the global effects of insulin as documented by wortmannin inhibition of the reaction [47,48]. The stimulation of glucose uptake, cell proliferation, protein synthesis, and membrane ruffling are all responses dependent on PI-3K's activity [43,49-52]. Furthermore PI-3K is responsible for gene expression controlling glucose-6-phosphatase, hexokinase II, glucose-6-phosphate dehydrogenase and GLUT4 production. Inhibiton of PI-3K with wortmannin or LY294002 blocks insulin stimulated glucose uptake [50]. Protein synthesis is also delicately controlled at the level of PI-3K.

The phosphorylating activation of both initiating and elongation factors involved in mRNA translation are both regulated at the PI-3K level [53-55]. Although crucial to GLUT4 activity PI-3K does not function alone in the process of glucose transport. PI-3K mediates many of its effects through protein kinase B (PKB), p70 S6 kinase and atypical protein kinase C's (PKCγ and PKCδ).

PKBα activation by insulin is a result of a complex interaction between both PI-3K and PIP3 and the PH domain of PKBα itself [43,56-59]. One apparent consequence of this reaction is the shuttling of PKBα to the plasma surface and multi-site phosphorylation. This has been shown to upregulate and increase translocation of the GLUT4 transporter to the plasma membrane in muscle and adipose tissue while other cell types may vary in response to PKBα specifically. The effect of PKBα on glycogen synthase activity is an area of debate. Glycogen synthase is activated through a dephosphorylation reaction involving the inhibition of glycogen synthase kinase-3 (GSK-3), which appears to be PKB dependent, and the concomitant activation of PP1G (glycogen bound form of protein phosphatase 1) by p90 rsk2 [60,61].

p70 S6 Kinase activation through multi-site phosphorylation normally requires both PI-3K and PKBα [62]. However, evidence of alternative pathways exist in which insulin directly activates it or the mTOR (mammalian target of rapamycin) pathway produces it. It appears p70 S6 kinase is not involved in GLUT4 mediated glucose uptake, since rapamycin does not inhibit the effects [63]. However, synthesis of the GLUT1 transporter has been shown to involve the enzyme, postulating a role for it in the extended effects of insulin [64,65]. The PI-3K, PKB, mTOR pathway also provides an essential framework for many of the protein synthesis effects of insulin. Translation

initiation factors are controlled by phosphorylation reactions. As such the components of the insulin cascade play prominent roles in control of translation. Insulin stimulation phosphorylates initiation factors thereby activating translation. With inhibition of this pathway, through wortmannin or rapamycin treatment, phosphorylation is inhibited. Conversely, overexpression of PKB results in increased protein synthesis via activation of initiation factors [66].

The atypical PKC enzymes are activated in response to PI-3K, PIP3, PIP2 and PDK1 and elicit a separate pathway, independent of the PKB-P70 S6 kinase pathway. Multiple investigations have proven the involvement of the PKC's in cellular glucose uptake. With overexpression of wild type PKC's GLUT4 translocation was elevated while overexpression of dominant negative mutants partially inhibited GLUT4 translocation and glucose uptake [67,68].

The mitogen activated protein kinase (MAPK) pathway also plays a role in the insulin-signalling cascade as well, however its importance has yet to be determined. Complex cascades of events initiated by the IR activate MAPK resulting in phosphorylation of p90 ribosomal S6 kinase and phospholipase A2 (PLA2) **(Figure 1)**. MAPK may be important in the process because of its interactions with PI-3K as GTP-loaded Ras interacts directly with PI-3K. It appears the MAPK pathway may provide glucose transport assistance through elevating GLUT3 transporter levels in prolonged insulin treatment but not via effects on the GLUT4 transporter [64,65]. Evidence for MAPK involvement in glycogen synthase activity was diminished with the finding that PD98059, an inhibitor of MEK, was unsuccessful in blocking insulin stimulated activation of glycogen synthase [69]. Current evidence supports a role for the insulin

dependent MAPK pathway in protein synthesis however. PD98059 inhibition of MEK blocks the activation of initiation factor eIF-4E a protein important in the recognition of mRNA by ribosomes [70]. Other initiation factors including eIF-2B however appear to be activated by PI-3K independent of the MAPK pathway [71]. It is clear that a combination of several pathways is required for these processes.

Many of the steps implicated in insulin-dependent glucose transport, glycogen synthase and protein synthesis are controlled by phosphorylation. Therefore, one may assume that in addition to the many kinases involved in phosphorylation, protein tyrosine phosphatases (PTP's) capable of dephosphorylating the same steps could have equally integral roles in these processes. Although kinases have been the focus of research to date, some phosphatases have been identified which play a role in the cascades above. PTPase 1B has been shown to dephosphorylate the IR and SH-PTP2 can reduce the phosphorylation of IRS-1 [72,73]. By blocking these steps respectively, each could potentially interfere with the cellular actions of insulin. These enzymes provide potential locations for pharmacologic intervention. Vanadium compounds are an example of protein tyrosine phosphatase inhibitors (PTPI), substances capable of blocking the PTP action and thereby improving the response of tissues to insulin [74,75].

5. Complications:

Diabetes is a multi-systemic disease. In addition to adversely affecting carbohydrate metabolism, it also causes cardiac disease, renal failure, blindness, peripheral neuropathy and is the leading cause of lower limb amputations. In fact, diabetic patients aged 35-64 are 6 times as likely to suffer a stroke or heart attack than

non-diabetics while the senior diabetic population is twice as likely. Diabetes also represents the leading cause of blindness and the major inciting factor for dialysis in Canada. In only 15 years (1981-1996) the percentage of renal failure patients as a consequence of diabetes has risen from 16% to 28% [3].

6. Current Therapeutic Approach:

i) Diet and exercise

The primary therapy for the diabetic patient continues to be lifestyle modifications. Although the necessity for insulin in Type 1 diabetes is absolute, a healthy diet combined with a good body mass index can reduce the amount of medication needed. In Type 2 diabetes, an early diagnosis combined with exercise, weight control and a proper diet can completely postpone or reduce the need for medication. In addition to controlling the glycemic status, these changes can provide beneficial effects to blood pressure and lipid levels which when combined with an abstinence from smoking will reduce the major complications of diabetes, macro and microvascular disease. The difficulty of remaining on such a strict regimen cannot be underestimated. Unfortunately, most patients end up on either insulin or oral hypoglycaemic agents.

ii) Insulin

Several biosynthetic varieties of insulin are produced by recombinant DNA technology and are used in the treatment of both Type 1 and 2 diabetic patients [76]. These compounds are biologically equivalent to the human hormone with identical polypeptide structures and immunogenicity. Each insulin preparation is chosen for its specific profile

of action. Regular (R) insulin acts rapidly with a short duration and is best suited for use during acute hyperglycaemic episodes including diabetic coma, pre-coma and surgical procedures. NPH and lente (L) insulins are intermediate acting medications with moderate onsets of action and duration used commonly in patients. Ultralente insulin (U) is a long acting analogue with hypoglycaemic activity greater than 24 hours. Mixtures of all these insulins are also available in different quantities. Acting directly on tissue insulin receptors, these medications temporarily control blood glucose levels by increasing the uptake of carbohydrates, increasing the storage of glycogen in the liver and converting glucose to fat in adipose tissues. Appropriate doses reduce glycosuria and prevent both diabetic coma and diabetic ketoacidosis. The only method of administration of these substances is via intramuscular injection. As a result, patients risk infection if the skin surface is not properly decontaminated prior to injection. Skin irritation and local allergic reactions are other possible side effects. The potential for any insulin compound to cause severe hypoglycemia is a concern and doses must be cautiously monitored in order to properly reduce blood glucose levels, especially under stress conditions such as exerecise, illness and missed meals where insulin requirements change significantly.

iii) Sulfonylureas

This group of drugs is currently the front line treatment of Type 2 diabetes when both diet and exercise fail to control glycemic status. Included in this category of hypoglycaemic agents are glyburide, glipizide, gliclazide, chlorpropamide, acetohexamide and tolbutamide. Although the mechanism of action of all sulfonylureas (SU) is the same, the pharmacokinetics of the above drugs differs significantly [77]. Each

is chosen based on its profile of half-life, duration of action and time to peak concentration [76]. The SU's are administered orally and function by increasing pancreatic β-cell activity and thereby improving insulin release. Interaction of the SU with a specific SU receptor on the B-cell membrane results in the closure of voltage dependant K^+ channels and consequently a rise in intracellular Ca^{2+} secondary to membrane depolarization. Additionally, the drugs improve peripheral glucose uptake and reduce hepatic glycogenolysis through ill-defined mechanisms furthering their hypoglycaemic effects [78]. These agents are not of use in Type 1 diabetes because of the necessity for functioning B-cells. In Type 2 diabetes the drugs are very effective early in the course of the disease and especially when fasting glucose levels are < 11.2 mM. However, prolonged treatment with sulfonylureas results in progressively less responsiveness and often demands the addition of either insulin or another class of oral hypoglycaemics. The susceptibility of elderly and underweight Type 2 diabetic individuals to severe hypoglycaemic episodes is the major risk associated with these medications while weight gain in patients on SU's is a significant concern especially considering that most Type 2 diabetic patients are already overweight. Common side effects include nausea, heartburn, epigastric fullness, mild diuresis and skin reactions. Therapy with sulfonlyureas is cautioned in patients with adrenal disease and contraindicated in pregnancy, thyroid disease, and both renal and hepatic failure because metabolic degradation of the drugs involves both the kidney and liver [76].

iv) Biguanides

Metformin is a recent addition to the oral hypoglycaemic agents used in Type 2 diabetes treatment [79]. Its mechanism of action is poorly identified but appears to involve improving the peripheral effects of insulin possibly via increasing the expression of insulin receptors peripherally, while reducing the hepatic glucose output. It has often been used as an adjuvant therapy with one of the SU's when responsiveness to those agents diminishes but is more recently being used as a monotherapy. Its ability to reduce blood glucose levels is similar to the SU's but is not associated with hypoglycaemic events. And while SU's may result in weight gain, the UKPDS [80,81]. An uncommon but serious side effect of the medication is lactic acidosis, while gastrointestinal side effects are common including nausea, bloating, vomiting, diarrhea and flatulence. Metformin is contraindicated in acidotic states, renal and hepatic impairment, pregnancy and excess alcohol ingestion [76].

v) α-glucosidase inhibitors

These medications slow the absorption of carbohydrates post-prandially thereby buffering the immediate rise in blood glucose levels following large meals. These substances have less effect on fasting blood glucose levels [82]. Specific drugs included in this group are acarbose and miglitol. Both substances competitively inhibit the GI tracts pancreatic amylase and brush border α-glucosidase thus blocking the hydrolysis of carbohydrates [83]. No effect on insulin or insulin receptors exists and therefore the drugs are popular to combine with other antihyperglycemic medications in Type 2 diabetes treatment. Circulating lipid levels have also been shown to decrease moderately with

treatment [84]. As a single treatment in addition to diet and exercise most side effects are limited to gastrointestinal problems (diarrhea, abdominal pain and flatulence). In combination with other medications various side effects have been observed although most are rare [76]. Contraindications to these drugs include most GI diseases.

vi) Thiazolidinediones (TZD's)

Members of this family including rosiglitazone and pioglitazone exhibit a paradox in that they improve glycemic control while reducing circulating insulin levels. It is suggested that these effects may in fact prolong β-cell survival. The anti-hyperglycemic activity is achieved primarily through increasing the sensitivity of muscle and adipose tissue to insulin while reducing hepatic gluconeogensis [85]. The TZD's are agonists for peroxisome proliferator-activated receptor-gamma (PPARγ) which when activated on muscle and adipose tissue membranes causes activation of insulin responsive genes that alter glucose transport and utilization via the GLUT-4 transporter [86]. Lipid profiles in treated patients also show improvements with HDL levels rising and triglyceride levels falling. These drugs are used both alone and in combination for the treatment of Type 2 diabetes. Adverse effects have included edema and weight gain similar to the SU's. They are contraindicated in diabetic patients with severe congestive heart failure and hepatic failure [76].

vii) Non-sulfonylurea Secretagogues

Repaglinide and nateglinide provide anti-diabetic action through a mechanism similar to their SU counterparts but have different pharmacokinetic properties. With

significantly shorter half-lives these substances stimulate insulin secretion briefly and if taken with meals provide excellent post-prandial glucose control [87,88]. Although this requires a strict dosing regimen, the chances of a severe hypoglycaemic event is reduced versus that caused by the SU's. Weight gain however remains a concern as with the SU's. Contraindications to these medications include hepatic and renal impairment [76].

II. Vanadium

1. Basic Chemistry:

Vanadium is a group 5 transition metal and is a trace element naturally present in both water and soil [89]. The element exists in three oxidation states of which vanadyl (IV) and vanadate (V) are the most physiologically active. In biological systems the balance between these states is maintained as a function of the pH, vanadium binding ligand availability and access to oxidation from the air [90]. The oxidation states (III) and (IV) are cationic in solution while (V) is anionic. In complexing with an infinite array of potential ligands, vanadium can exist as monomers, oligomers and polymers. Further complexity is found with vanadium existing as insoluble precipitates and soluble solutions when only slight modifications of pH are made. Such diversity with basic chemistry continues to hinder the interpretation of vanadiums action *in vivo*. The basic chemistry of vanadium is extensively reviewed elsewhere [90].

2. Physiologic Functions:

Although the precise physiologic actions of vanadium are unclear it seems evident that the substance is required in the body as a trace mineral [91,92]. Dietary deficiencies of the substance in animal studies have produced a variety of effects. Reproductive difficulties include reduced fertility, higher rates of spontaneous abortion and decreased milk production. Skeletal abnormalities included leg deformations, foot inflammation and generalized musculoskeletal pain [93]. Vanadium also appears important in thyroid, iron, glucose and lipid metabolism [94]. Both pancreatic amylase and lactate dehydrogenase, two enzymes involved in the metabolism of glucose from starches were altered by vanadium deficiency. It is estimated that a daily vanadium intake of approximately 10ug in humans is sufficient to eliminate these effects. This dietary intake is easily achieved with a balanced diet [95]. The highest levels of vanadium (> 40ng/g) can be found in shellfish, cereals, mushrooms and fish [96,97]. Even with a diet rich in these foods it is postulated that the maximum intake of vanadium in the daily diet would not exceed 30ug. No toxicity has been noted at these levels and several over the counter supplements currently contain vanadium in similar quantities. The total body pool of vanadium is approximately 200ug [98].

Vanadium is not readily absorbed from the intestinal tract normally [95,99]. Less than five percent of the ingested amount is taken into the blood under physiologic conditions. However, the administration of vanadium salts, including those most often used in insulin-mimesis, increase the bioavailability of the element by raising the absorption another five percent [100,101]. Vanadium reaching the vascular system undergoes several modifications. In the acidic environment of the stomach and duodenum

most vanadium is transformed to the vanadyl (IV) state [102]. That remaining in the (+V) state as vanadate is more readily taken up into the intestinal lining cells through a poorly understood anion-transport system, possibly a phosphate pathway [103]. Within the vasculature, the majority of vanadate is transformed to vanadyl and both are carried in association with transferrin and ferritin (IV only) [104,105]. Both species are shuttled to bone which acts as the primary reservoir for vanadium within the body. The excretion of vanadium is primarily a renal function with smaller concentrations found in bile and feces [98,106,107].

In addition to the effects of vanadium deficiencies mentioned above many direct actions of the substance are documented. Vanadium is exploited as a potent inhibitor of protein phosphotyrosine phosphatases [108,109]. Also it has been shown to have growth factor like effects [110-112]. This is not surprising since as a phosphatase inhibitor vanadium is able to alter the levels of protein tyrosine kinases (PTK's). In doing such, second messenger systems dependant on kinase activity can be dramatically transformed. These changes have been found to be both mitogenic and cytotoxic depending on cellular conditions [113,114]. Mitogenic effects are extremely dose specific over narrow concentration ranges while cytotoxicity is noted with peroxovanadium treatment and not vanadium salts [115-118]. In acting on cellular proliferation proto-oncogene induction has also been noted. Many insulin-like effects are replicated with vanadium treatment. These include increasing glucose transport, anitlipolytic activity in adipocytes, increased hepatic glycogen synthesis and cellular uptake of potassium [119].

3. Insulin-mimetic Actions:

Vanadium's insulin-mimetic effect is the best studied of its cellular actions. Almost all metabolic effects of insulin can be reproduced without insulin using vanadium species. As outlined above insulin produces its cellular effects through the action of a specific tyrosine kinase receptor that activates a cascade of protein kinases. In contrast, vanadium acts independently of the insulin receptor [120-123]. The additions of cell permeable inhibitors to the insulin receptor block all effects of insulin but none of the same vanadium effects [124]. IRS-1, immediately downstream of the insulin receptor in the cascade of insulin phosphorylation is also therefore not activated by vanadium. PI3-kinase is another second messenger activated by insulin. Its actions are diverse but include the phosphorylation of the PI pathway and the production of phospholipids **(Figure 1)**. The specific inhibition of PI3-kinase with wortmannin also blocks the cellular effects of insulin [47,48]. Similarly, vanadiums effect on glucose metabolism is blocked with wortmannin [125]. However, the antilipolytic effects of insulin but not vanadium were blocked during wortmannin treatment. Vanadiums insulin-mimetic action is currently under intense investigation. Shecter and colleagues have identified two potential sites of activation. The activity of both an intracellular cytosolic protein tyrosine kinase (CytPTK) and a membrane bound PTK are increased with vanadium administration. Insulin has no effect on either [120,125]. Inhibition of the 53 kD CytPTK with nM doses of staurosporine blocked the glucose oxidation and lipogenesis effects of vanadium without blocking insulin effects [126]. All vanadium mediated insulin-like effects are not accounted for by this mechanism since the same treatment did not abolish glucose transport changes. Therefore the potential for insulin and vanadium to act synergistically, through

separate pathways, promises to be an area of much research in the treatment of diabetes. The ability of vanadium to increase glucose uptake, glycogen synthesis and inhibit lypolysis lends it to potential therapeutic use in the treatment of both Type 1 and Type 2 diabetes mellitus.

4. Vanadium Salts:

Evidence for the essentiality of vanadium and its hypoglycaemic potential in diabetes therapy is growing [127,128]. Vanadium's use in the treatment of diabetes stems from the first trial of sodium metavanadate. Administered to Type 1 diabetic rats over a four-week period it restored blood glucose to non-diabetic control levels [129]. Subsequently, various vanadate (or vanadium salt) solutions have been exploited for their anti-diabetic properties. Irrespective of the salt solution used it was shown that sodium orthovanadate, sodium metavanadate and vanadyl sulphate all possess equal hypoglycaemic effects [130]. Urine volume, glycosuria and glucose tolerance were equal in the three vanadate treated diabetic rat groups. Surprisingly, diabetic rats treated with vanadyl sulphate for three weeks remained normoglycemic 13 weeks after termination of the treatment [131].

One of the more common uses in vitro for vanadium compounds are as inhibitors of protein tyrosine phosphatases (PTPases). The role of liver PTPases in vivo is speculated to account for some the insulin mimetic effects of these agents. This enhanced action of protein kinases in the insulin-signalling cascade is a proposed mechanism of vanadate action in diabetes [132]. In fact significant decreases in PTPase activity were found in vanadate treated diabetic animals while markedly increased numbers of hepatic

insulin receptors were also apparent [133]. Streptozotocin (STZ) induced Type 1 diabetic rats have reduced islet cell content and insulin secretion. However, with vanadate treatment these diabetic animals responded by increasing islet cell size and insulin content to near control levels [131]. However, the insulin secretion of these same cells was improved only 12% over the untreated diabetic rats. Also, it was shown that other hormones involved in glucose homeostasis including glucagon, corticosterone and noradrenaline are minimally affected by vanadate [134]. The mechanism of vanadium action in diabetes is therefore complex and multifactorial and is reviewed elsewhere [135-137]. Carbohydrate transport is reduced in diabetes primarily through a reduction in GLUT transporter volume and activity. Vanadate treatment has been shown to increase GLUT expression and transcription nearly back to non-diabetic levels [138]. Similarly, cardiac myocyte glucose oxidation and GLUT4 expression are reduced in diabetes. Vanadate treated myocytes responded with normal levels of glucose oxidation and improved GLUT4 expression [139,140]. In addition to the improved glucose response of vanadate treated animals, GLUT-5 expression is also increased and fructose transport is improved [141].

The lipid profile of the diabetic patient is altered drastically from the non-diabetic. The cholesterol and triglyceride changes partially account for the increased rates of atherosclerotic disease and myocardial infarction found in the diabetic population. Ten weeks of vanadate treatment to diabetic mice improved serum total cholesterol and triglyceride levels in combination with their hypoglycaemic actions [142]. Only 21 days of vanadate treatment was necessary to reverse the diabetic lipid profile in Type 1 diabetic rats. This included total lipids, cholesterol and triglycerides [143,144].

The leading cause of death in the diabetic population is cardiac in origin [145,146]. As a result intense investigation has been placed on the cardiac effects of vanadium compounds. Cardiac performance has been shown to improve with vanadate treatment in diabetes [129]. One study attributed some of this effect to vanadate's ability to reverse diabetic hypothyroidism [147]. The free radical generating enzymes within the heart and vasculature show increased activity in diabetes. With vanadium treatment, diabetic rat levels of glutathione peroxidase, catalase and superoxide dismutase were corrected to near normal, while glycoproteins, plasma lipid peroxide and erythrocyte membrane phospholipids were restored to normal [148,149]. Cardiac lipoprotein lipase, reduced in diabetes and potentially atherogenic has also been restored to normal [150]. Vascular integrity has also been improved with vanadate treatment as evidenced by normalization of endothelin levels in STZ diabetic treated rats [151].

The multisystem effects of diabetes are not limited to the heart, vasculature and serum lipids however. Intestinal alterations in diabetes have also been analysed with respect to vanadium treatment. Increased sodium-dependent glucose transport and Na,K-ATPase activity in Type 1 diabetic rats was corrected with vanadate treatment while the decreased activity of intestinal 6-phosphofructo-1-kinase and 6-phosphofructo-2-kinase were also restored [152-155]. Carbohydrate metabolism relies heavily on the release of pancreatic amylase into the intestine and this is interrupted in diabetes. Vanadate induced normoglycemia was combined with the restoration of amylase transcription and activity in diabetic rats [156,157].

Many biochemical parameters altered in the diabetic state have been assessed to determine the effects of vanadate treatment on general metabolism, liver and renal

function. Renal and hepatic glucose-6-phosphatase and fructose-1,6-bisphosphatase activities were returned to normal levels with vanadate [143,158]. Additionally it was found that plasma glutamic pyruvic transaminase, glutamic oxaloacetate transaminase [142], liver arginase activity and expression [159,160], pyruvate kinase expression and tyrosine aminotransferase gene expression [161] were positively affected with vanadium treatment. Increased mRNA synthesis of P-enolpyruvate carboxykinase (PEPCK) and others gluconeogenic enzymes implies improved glucose utilization by the liver [161,162]. Other hepatic indices improved with vanadate include tryptophan-niacin metabolism [163], hexokinase activity [164], hepatic lipase activity [150], glucose-6-phosphate [165] and protein C activity [166]. As much as hepatic and cardiac consequences abound in diabetes, so do renal dysfunction and kidney failure. Blood urea nitrogen (BUN), a clinical index of renal disease, increases in diabetes and is reduced in vanadium treated rats [142]. Renal sorbitol, urinary albumin, IgG excretion and kidney size were also reduced with treatment [167,168].

This data suggests vanadium exhibits a remarkable spectrum of anti-diabetic properties, and these are the reasons for its continued interest in the diabetic research community. However, vanadium salts have been and currently are heavily scrutinized for their toxic side effects [169,170]. These include gastrointestinal toxicity, diarrhea, dehydration and vanadium accumulation in vital organ systems [129,169,171-177]. In fact, the severity of this toxicity has resulted in vanadate-induced death in animal models [178].

Appetite suppression and reduced water intake were some of the first effects noted with vanadium treatment in diabetes. Reduced water intake in diabetic animals is another sign of effective anti-diabetic action. The removal of glucose, an osmotic diuretic from the circulation reduces both polydipsia and polyuria. However, appetite suppression

is more concerning as many researchers have noticed reduced body weight gain while treatment persisted [179,180]. The accumulation of vanadium in various organs has also been documented [176,181]. Although these studies have defined bone as the major reservoir for vanadium, no toxic effects have been found functionally or pathologically to date. While liver and kidney are also sites for vanadium storage toxic effects on these organs are still debated [144,166].

In diabetic pregnancy, it has been shown that serum vanadium levels are much higher than expected, resulting in nearly 50% mortality in the dams. As well, reproductive capacity was decreased as evidenced by reduced live offspring [182,183]. A complete review of developmental and reproductive effects is cited [170].

In vitro effects of vanadium also point to potential toxicity. Cell culture exposure results in increased proliferation and differentiation of cells and is therefore potentially carcinogenic [184]. The mitogenic effects of the substance include activation of several proteins involved in phosphorylation including c-jun and junB [185,186]. By contrast vanadium was also shown to possess cytotoxic effects, attributed to the action of vanadate on PTPases. Furthermore vanadium has been shown to inhibit cell adhesion and induce protooncogene expression [184,187].

The role of vanadium salts in the treatment of diabetes has undergone significant advances in the last 20 years. Beneficial effects on the diabetic state range from its hypoglycaemic actions down to effects on specific liver and plasma enzymes as outlined. However, it remains clear that the unknown long-term effects of vanadium accumulation and the known toxic complications of its treatment require further investigation prior to instilling this regimen into the drug repertoire for human diabetics.

5. Modified Vanadium compounds:

In an attempt to reduce toxicity several methods of modifying the chemical structure of vanadium have been attempted. Organo-vanadium complexes were synthesized to increase uptake in the intestinal lining, as vanadium salts are poorly ingested [188]. Bis(maltolato)oxovanadium(IV) is one such species found to achieve anti-diabetic effects at one half the dosage required of vanadium salts, with minimal toxicity [188]. The same group also found naglivan, another organo-vanadium compound, produced antidiabetic effects without overt signs of toxicity [189]. Vanadyl sulphate administration was directly compared to three newer organic complexes by Reul et al with success [190]. All three compounds improved glycemic control and restored glycolytic enzyme function greater than vanadium salt therapy alone. Interestingly, this was independent of intestinal uptake as the same results were achieved with an intra-peritoneal route of administration.

The combination of vanadium compounds with hydrogen peroxide produces potent insulin-mimetic agents known as peroxo-vanadium (pV) complexes. While vanadate is known to function independent of the insulin receptor pV's act on the insulin receptor and insulin receptor substrates [191,192]. By inhibiting the dephosphorylation of the insulin receptor theses substances potentiate the effects of insulin and as such may be useful as insulin adjuvants.

The simplest method of alleviating the toxicity of vanadium compounds in diabetes treatment would appear to be reducing the dosage of vanadate. Recently, it was found much lower doses than previously used were effective in reducing blood glucose when given in combination with benzylamine [193]. By improving GLUT-4 recruitment

and glucose transport in adipose tissue minimal doses of vanadium incapable of acting as a hypoglycaemic alone possessed substantial effects in combination after as little as two-weeks. The synergistic combination of vanadium and magnesium (MgV) was also tested and found to have beneficial effects [194]. The MgV solution improved insulin sensitivity and glycogen synthesis in treated diabetic rats to a greater degree than V alone. Vanadium's pro-oxidant functions are believed to be detrimental in its use as an anti-diabetic agent. With the administration of an anti-oxidant, U-83836E significantly improved diabetic indices were found after 12 weeks of treatment [195]. Polydipsia, polyuria, glycosuria and hyperglycemia are further decreased with combination treatment while HbA1c and cataract formation were also reduced. The use of chelating agents in synergy with vanadium may also be a route to successful diabetic treatment. Tiron chelation of vanadium produced lower vanadium accumulations in both bone and kidney while not inhibiting the anti-diabetic properties of the agent [196,197]. The recent popularity of nutraceuticals, or functional foods led to another method of delivering vanadium. In combination with Trigonella seed powder, a hypoglycaemic material itself, vanadium restored glycemic control and altered enzymes to normal at a lower dosage [198].

6. Human trials:

The use of vanadium in human diabetic patients is in its early stages. Small studies with Type 2 diabetic patients have been run and resulted in moderate success. Improved cholesterol levels and insulin sensitivity as assessed by euglycemic, hyperinsulinemic clamp was achieved but in the absence of blood glucose control [199,200]. Fasting glucose levels have been reduced but with gastrointestinal complications [201].

These side effects were much less severe than those of animal trials and consisted of mild nausea, abdominal pain, gas, diarrhea and vomiting all of which decreased over time [169]. A comprehensive review of these trials is cited [203].

III. Alternative Medicine:

1. Basics:

Over the past ten years alternative medicine in its various forms has dramatically increased in popularity. Chiropractic, acupuncture, homeopathy, massage therapy, and the various naturopathic and herbal remedies available all provide patients with non-conventional alternatives to medical treatment. Increased use of these therapies is attributed to an increase in the proportion of the population using these therapies rather than simply increased visits per patient annually [203]. The use of natural and herbal medicines accounts for a large proportion of this growth with approximately 12.1% of the population using these therapies in 1997 compared to just 2.5% in 1990 [203]. Similarly sales of plant medicines and herbs increased dramatically by 25% per year since the mid 1990's and were estimated at $2.5 billion in the USA in 1996 [204,205]. Clearly in the midst of an aging population with more health problems the acceptance of natural alternatives to Western medicine is occurring. Similarly, research interest into natural, Chinese and diet-derived therapies is also increasing as evidenced by the development of the National Centre for Agri-food Research in Medicine. Some extensively used plant and animal derived products such as St. John's wort, echinacea and glucosamine are used in the

management of common medical conditions such as depression, colds and arthritis respectively.

The gastrointestinal side effects experienced by vanadium treated diabetic subjects are common and many investigations have led to new vanadate compounds and oxidized species of current drugs. In contrast, it seems reasonable that the judicious use of appropriate natural products capable of altering the response of the gastrointestinal tract to vanadium may function to reduce the harmful toxicity of vanadate.

2. Functional Foods and Nutraceuticals:

i) Definition

Functional foods are similar in appearance to conventional foods. They provide health-related benefits (prevention or treatment of disease) beyond those obtained by normal nutrition. Nutraceuticals are defined as naturally derived bioactive compounds that are found in foods, dietary supplements and herbal products that have health promoting, disease preventing or medicinal properties. Nutraceuticals are therefore commonly denoted as the extract or pill form of a functional food concentrating the active component for increased anti-disease activity. Therefore, for example, diets rich in certain fish would be classified as a functional food, the specific omega-3 fatty acids within the fish oils including linolenic acid, eicosapentanoic acid (EPA) and docosahexaenoic acid (DHA) can be delivered as a nutraceutical via capsules [206].

ii) Examples

The breadth of action and variety of functional foods/nutraceuticals used can be appreciated by discussing a few of the more popular agents:

Cranberry juice – used since 1920 for treating and preventing urinary tract infections. The mechanism involves urine acidification and inhibiting bacterial adhesion to the bladder surface [207].

Ginseng – one of today's most popular alternative medications. It is used to promote both physical and mental performance. Although large-scale studies are lacking, smaller scale studies have shown increased resistance to infection and increased energy metabolism [208].

St. John's Wort – available for internal use in capsule form and externally as an ointment or oil. It is currently used for depression, anxiety, menstrual cramps, colds, congestion and fighting infection [209,210].

Although the effects of most functional foods and nutraceuticals are multi-systemic, some are known to possess especially strong effects on the gastrointestinal tract. Anti-diarrheal compounds fitting into the functional food or nutraceutical categories are plentiful but not all those used currently have substantial scientific proof of efficacy **(Table 1).**

iii) Probiotics

One popular class of anti-diarrheal nutraceuticals are the probiotics. These are foods with live microbial species as components which when ingested are suspected of

Table 1: Natural Remedies Commonly Used in the Treatment of Diarrhea.

Acidophilus	Guarana	Rhubarb
Agrimony	Lady's Mantle	Savary
Apple	Meadowsweet	Slippery Elm
Barberry	Mormon Tea	Squaw Vine
Bilberry	Mullein	Tamarind
Blackberry	Nutmeg	Tea - Black
Black Current	Oakbark	Tea - Green
Black Haw	Poppy	Wild Cherry Bark
Blueberry	Psyllium	Wild Strawberry
Borage	Raspberry	Wood Betony
Fennel	Rhatany	Yellow Dock

Reference: Peirce A. The American Pharmaceutical Association Practical Guide to Natural Medicines. First Edition. Stonesong Press. 1999.

enhancing the normal flora of the gut and displacing toxic bacterial species. Common foods within the probiotic genre include yogurt, milk products and kefir.

Acidophilus – Lactobacillus acidophilus bacterial species normally resident within the GI tract can be supplemented with exogenous acidophilus. Eight ounces of yogurt is the equivalent of one enteric-coated capsule. Controversy regarding the efficacy of these supplements continues and may depend on the species of *Lactobacillus* used [211-214].

iv) Prebiotics

The prebiotics on the other hand do not contain live microbes themselves. Rather these functional foods are indigestible food ingredients which selectively stimulate growth of certain normal gut flora. Examples include lactulose, sorbitol, inulin and soy oligosaccharidases [215].

Short Chain Fatty Acids – produced by intestinal bacteria in response to fermented milk decrease plasma cholesterol levels by deconjugating bile acids and inhibiting their resorption distally in the GI tract [215,216].

v) Teas

Teas, both black and green are popular beverages worldwide derived from the leaves of the Camellia sinensis plant with an extremely broad based range of effects from the stimulatory effects of 50mg caffeine in a cup of black tea to the diuretic properties of both. The production of black tea vs. green tea relates to a complex fermentation process altering the chemical composition. Herbalists maintain the uses of these leaf extracts include the treatment of diarrhea, common colds, asthma, congestion, headache and

stroke [210]. Dilution of the leaves in boiling water produces an extract in which the strength correlates with the time of steeping. Diarrhea therapy requires a much stronger extract than that used for other purposes. The components of tea believed to be important in some of these effects include polyphenols, flavanoids, tannins and theophylline/theobromide.

Polyphenols are a large group of related compounds composed of aromatic rings substituted with hydroxyl groups composing up to 40% of tea by weight [217]. Polyphenols include catechins, gallocatechins, flavonols, gallic acid and tannins with several more specific molecules in each group respectively [217].Within the plant they function as a chemical defense against predators [218]. However, more recently tea and its components are recognized as being preventive in diseases ranging from diabetes to cancer to heart disease and renal disease [219]. Specifically green tea has been found to reduce plasma glucose levels in old rats while suppressing intestinal glucose transport. [220,221]. As cardiac pathology, especially myocardial infarctions are so common in diabetes it is important to note the variety of effects teas have on cardiac disease. Coronary artery disease and atherosclerosis are the result of lipid deposition and oxidation within the intimal layer of the vasculature. Any reduction in antioxidants or corresponding increase in reactive oxygen species (ROS) has the potential to result in increased oxidized low-density lipoprotein (oxLDL) and the development of vascular plaques eventually leading to ischemia and heart attacks. The catechins, most notably epicatechin gallate (EGCG) found within tea are potent antioxidants as are tocopherols, both of which can inhibit oxidation of low-density lipoprotein [222-224]. Similarly, these compounds reduce vascular smooth muscle proliferation while limiting peroxidation and

peroxide decomposition products [225,226]. Tea treatment has also reduced plasma cholesterol and triglyceride levels while increasing the activity of superoxide dismutase in treated rats [227,228]. Inflammatory changes and histamine action are also potentially interrupted by the rutin and catechin content of tea [219]. Hypertension is also a common confounding disease especially in Type 2 diabetic obese patients. To maintain a healthy blood pressure sensitive control must be maintained within the vasculature controlling the release of vasoconstrictive and vasodilatory substances. Flavanoids have been found to potentiate the release of vasodilatory substances while catechin may control thromboxane A2 release, a powerful vasoconstrictor [229,230]. The complications of diabetes are not limited to the heart and vasculature however. Renal disease and neurological diseases also increase in prevalence in the diabetic population and teas have been found to have beneficial effects on both of these organ systems. Catechins effect on blood pressure mentioned above has the capacity to improve kidney function in cases of renal hypertension since renal artery stenosis and vascular constriction are crucial in this pathology. Both increased sodium and prostaglandin E2 release are suspected to account for improved renal circulation, delaying renal failure [231,232]. Further evidence of renal protection was the finding of reduced renal disease indices, BUN and creatinine, following tea treatment to rats induced with nephropathy [233]. Presently, the effects of tea on the nervous system include improved alertness, reduced blood pressure centrally, and even slowing of the CNS functional deficits associated with aging [234-236].

The effects of tea compounds and tea itself are well described in cancer research as well. Several reviews are cited focussing on this area of nutraceutical research [219,237-

[240]. The final cited review also identifies some of the effects of tea on skin, eye, infectious and metabolic diseases.

IV. Experimental Models:

1. Type 1 Diabetic Animals:

Animal models of Type 1 diabetes either spontaneously develop diabetes (BB rats, NOD mice) or are induced with the disease via viral, chemical or surgical mechanisms [241-244]. Induction of diabetes through chemical means involves the injection of either streptozotocin (STZ) or alloxan intravenously or intraperitoneally. STZ (2-deoxy-2-(3-methyl-3-nitrosurea)1-d-glucopyranose)(α+β)) is an antibiotic derived from *Streptomyces* bacteria that exerts cytotoxic effects on the pancreatic islets of Langerhans, specifically the β-cells in a poorly understood manner [245]. These mechanisms may include but are not limited to free radical production, DNA destruction, alkylation and the liberation of nitric oxide [246-248]. Alloxan functions similarly but is used more often in rabbit models while STZ is commonly used with rat species [245]. Commonly used species of rat include Sprague Dawley and Wistar animals injected with STZ at a dose > 40 mg/kg, although most researchers choose to use doses in the 60 mg/kg range to ensure hyperglycemia and hypoinsulinemia.

2. Type 2 Diabetes:

The hyperglycaemic, hyperinsulinemic, hyperlipidemic and obese syndrome characterisitic of human diabetes is not easily replicated. In fact, several similar syndromes are defined such as syndrome X or the metabolic syndrome [249]. In these states, the degree of hyperglycemia and specific complications vary. As a result several different categories of Type 2 diabetic animal models exist. These are thoroughly reviewed elsewhere and include rat, mouse and primate models [250]. Rat species often harbour the fatty (fa) or corpulent (cp) genes while mice carry the diabetic (db) or obese (ob) genes. One animal model commonly used currently is the Zucker fatty rat first described in 1961 [251,252]. Although this animal is not overtly hyperglycaemic it does present the other hallmarks of the diabetic state. A subset of this strain, the Zucker diabetic fatty rat (ZDF) was bred from these animals and expresses increased circulating glucose levels [253,254]. The male animals develop overt diabetes with high glucose and insulin levels at approximately 7 weeks of age [255,256]. However, by 12 weeks hyperinsulinemia slowly decreases with even further hyperglycemia. The development of this diabetic state corresponds with pathologic changes to pancreatic islet cells, including a reduction in glucose stimulated insulin secretion. This model simulates the Type 2 human diabetic condition well. The slow development of the condition and the following reduction in insulin secretion are both characteristic of human diabetes.

HYPOTHESIS

A combination of sodium orthovanadate and a nutraceutical compound with known anti-diarrheal properties will eliminate the gastrointestinal toxicity associated with vanadium treatment. In doing so, this orally administered nutraceutical compound will facilitate applications in the treatment of Type 1 and Type 2 diabetes in animals.

MATERIALS AND METHODS

1. Materials:

Product	Supplier
Alanine aminotransferase assay kit	Sigma-Aldrich Canada Ltd. (Oakville, ON) IDEXX laboratories Inc. (Toronto, ON)
Albumin assay kit	IDEXX laboratories Inc. (Toronto, ON)
Alkaline phosphatase assay kit	IDEXX laboratories Inc. (Toronto, ON)
Amylase assay kit	IDEXX laboratories Inc. (Toronto, ON)
Asparatate aminotransferase assay kit	Sigma-Aldrich Canada Ltd. (Oakville, ON) IDEXX laboratories Inc. (Toronto, ON)
Blood urea nitrogen assay kit	IDEXX laboratories Inc. (Toronto, ON)
Bodipy FL C5-HPA	Molecular Probes (Eugene, OR)
Bovine serum albumin	Sigma-Aldrich Canada Ltd. (Oakville, ON)
Calcium chloride	Sigma-Aldrich Canada Ltd. (Oakville, ON)
Cholesterol assay kit	Sigma-Aldrich Canada Ltd. (Oakville, ON) IDEXX laboratories Inc. (Toronto, ON)
Citric acid	Fisher Scientific. (Napean, ON)
Citric acid-trisodium salt	Sigma-Aldrich Canada Ltd. (Oakville, ON)
Creatinine assay kit	IDEXX laboratories Inc. (Toronto, ON)
Dextrose	Fisher Scientific. (Napean, ON)
Ethanol	Fisher Scientific. (Napean, ON)
Fura-2	Molecular Probes (Eugene,OR)
Globulin assay kit	IDEXX laboratories Inc. (Toronto, ON)
HEPES	Sigma-Aldrich Canada Ltd. (Oakville, ON)
Hydrogen peroxide	Sigma-Aldrich Canada Ltd. (Oakville, ON)

Insulin elisa kit	American Lab Products (Windham, NH)
Ketamine	Mid-West veterinary Labs (Winnipeg, MB)
Magnesium chloride	Fisher Scientific. (Napean, ON)
Nitric acid	Sigma-Aldrich Canada Ltd. (Oakville, ON)
Phosphatidic acid	Sigma-Aldrich Canada Ltd. (Oakville, ON)
Potassium chloride	Sigma-Aldrich Canada Ltd. (Oakville, ON)
Sodium chloride	Sigma-Aldrich Canada Ltd. (Oakville, ON)
Sodium citrate	Sigma-Aldrich Canada Ltd. (Oakville, ON)
Sodium orthovanadate	Sigma-Aldrich Canada Ltd. (Oakville, ON)
Sprague Dawley rats	Central Animal Care Services, University of Manitoba (Winnipeg MB)
Streptozotocin	Sigma-Aldrich Canada Ltd. (Oakville, ON)
Total bilirubin assay kit	IDEXX laboratories Inc. (Toronto, ON)
Total protein assay kit	IDEXX laboratories Inc. (Toronto, ON)
Triglyceride assay kit	Sigma-Aldrich Canada Ltd. (Oakville, ON) IDEXX laboratories Inc. (Toronto, ON)
Triton X-100	Sigma-Aldrich Canada Ltd. (Oakville, ON)
Uric acid assay kit	IDEXX laboratories Inc. (Toronto, ON
Vanadium references	SCP Science (Baie D'Urfé, QC)
Vettest 8008	IDEXX laboratories Inc. (Toronto, ON)
Xylazine	Mid-West veterinary Labs (Winnipeg, MB)
Zucker diabetic fatty rats	Genetic Models Inc. (Indianapolis, IN)

II. Methods:

1. Animals:

Male Sprague Dawley rats weighing 175-200 g were obtained from Central Animal Care Services (University of Manitoba, Winnipeg, MB). Zucker diabetic fatty rats and Zucker non-diabetic control animals were obtained from Genetic Models Inc. (Indianapolis, IN). Animals were housed two per cage on woodchip bedding in polycarbonate cages, and offered free access to both food (lab diet 5P00, Prolab) and water. A 12:12 light:dark cycle was employed using 600-1800 hrs as the light cycle. The animals were maintained at 20°C with 50% humidity throughout the study. Animals were sacrificed with a single intraperitoneal injection of a 9 mg/ml ketamine:0.9 mg/ml xylazine cocktail. Blood was collected by exsanguination and centrifuged briefly to obtain plasma. Plasma was stored at -20°C for subsequent analysis. Internal organs were surgically excised and snap frozen in liquid nitrogen. These were then maintained at -80°C.

2. Insulin-dependent diabetic model:

Rats were lightly anaesthetized and tail vein injections of streptozotocin (STZ) were used for diabetic induction. STZ at a dosage of 55mg/kg body weight was diluted in a citrate buffer vehicle (pH = 4.5) and injected in a single dose. Control animals received an injection of buffered vehicle alone. Animals were allowed to adjust to their diabetic state for 4 days prior to treatment.

3. Blood Glucose Analysis:

Four days post-STZ administration, blood glucose levels were assessed using a Bayer glucometer elite® testing system in all animals. A distal tail snip generated the 5 µl quantity of blood necessary for analysis. Subsequent daily glucose levels were done by removing the scab formed on the tail. Daily testing was performed from 0900 hrs – 1100 hrs. Glucose tolerance testing is performed by the oral administration of a 3 g/kg glucose load using 40% glucose solution [257]. Blood glucose is measured prior to and 30, 60, 120, 240 and 360 minutes post glucose.

4. Treatment of Diabetes:

A plant based nutraceutical extract was combined with sodium orthovanadate at a concentration of 20 mg vanadate/ml nutraceutical. This decoction was named CT99. Because of patent restrictions, the exact composition of CT99 cannot be revealed at this time. For the majority of all experiments the sodium orthovanadate was identical coming fro the same lot of vanadium produced at Sigma®. Where indicated however, different lot numbers of vanadate were used and the manufacturer notes these to be of different purities. CT99 was maintained at room temperature without access to light for 5 hrs prior to use. A water/vanadate preparation was also used to treat the diabetic animals. Sodium orthovanadate was suspended in double distilled water at a concentration of 20 mg/ml and stored without exposure to light at room temperature for 5 hrs prior to animal treatment. At 1600 hrs daily, any animal with a blood glucose level > 10 mM was orally gavaged with the appropriate treatment solution. Rats with daily blood glucose levels < 10 mM were considered normoglycemic and not treated that day. Animal treatment

groups included CT99-treated diabetics (CT99-D), water/vanadate-treated diabetics (water/van-D), diabetic (D) and non-diabetic animals (ND) each treated periodically with water or nutraceutical alone (no vanadate). Each animal received 2 ml of treatment solution, corresponding to a total vanadium dose of 40 mg.

5. Biochemical Analysis:

Biochemical diagnostic kits were used for assessment of alanine aminotransferase (ALT), aspartate aminotransferase (AST) triglycerides (TG), and cholesterol levels. A Vet test 8008® spectrophotometric system was used for albumin, alkaline phosphatase, amylase, blood urea nitrogen (BUN), creatinine, globulin, total bilirubin, total protein and uric acid analysis. The ALT, AST, TG and cholesterol assays were also performed with this system. Urine specific gravity was assessed with a hand refractometer while plasma insulin levels were measured with an ELISA assay [258].

6. Myocardial Contractility:

i) Perfusion with Phosphatidic Acid

Rat cardiomyocytes mounted on glass coverslips were mounted in a Leiden chamber heated to 37°C with a Medical Systems PDMI-2 Open Perfusion Micro-Incubator (Greenvale, NY). Cells were perfused with a HEPES buffered solution bubbled with 100% oxygen. The control perfusion buffer contained 140 mM NaCl, 6mM KCl, 1mM $MgCl_2$, 1.25mM $CaCl_2$, 10mM dextrose and 6mM HEPES (pH 7.4) and 0.02% BSA. Myocytes were equilibrated for 10-15 min prior to treatment with phosphatidic acid (PA) at concentrations of 10,25 and 50μM respectively. Cells were

perfused with PA for a period of 15 minutes and were paced at 0.5 Hz with duration of 200ms using platinum electrodes.

ii) Cardiomyocyte Contractile Performance

Active cell shortening (unloaded) and changes in resting cell length were measured using a video edge detection system (Cresent Electronics, Sandy, UT) coupled to a Pulnix monochrome CCD camera at a capture rate of 60 Hz in as described in detail elsewhere [259].

iii) Measurement of Cellular Ca^{2+}

The Ca^{2+} sensitive dye fura-2 was used as an intracellular indicator of Ca^{2+}. Myocytes adherent to laminin-coated glass coverslips were loaded for 15 min at 37°C washed prior to experiments. These were placed in a Leiden chamber that was mounted on a Nikon Diaphot microscope with a 40x epiflourescent objective. Cells were excited at 340 and 380 nm with an emission of 505nm. The fluorescence was collected on a SPEX flourolog spectrofluorometer and quantitated ratiometrically with photomultiplier tubes and a Pentium computer as described in detail previously [259].

iv) Measurement of the Movement of PA

The movement of PA in the cardiomyocyte was measured using the fluorescent indicator 2-(4,4-diflouro-5,7-dimethyl 4-bora-3a,4a diaza-indecend-3 pentanoyl) –1-hexadecanoyl-sn-glycero-3-phosphate (ß-BODIPY FL C_5- HPA). Briefly a mixture of unlabelled PA and BODIPY PA (10:1 ratio) at a $[25\mu M]_{final}$ was added to cells with

fluorescence measured over time. Cells were excited at 488nm and the emission at 525nm was monitored. Cells were placed in a heated chamber as used in calcium and contractile measurements. Images of fluorescence were captured with Bio-Rad MRC 600 confocal microscope equipped with a Krypton/Argon ion laser (American Laser Corp., Salt Lake City, UT).

7. Vanadium level Determinations:

i) Sample Preparation

Plasma samples were diluted 1:1 with 0.25 M sodium citrate solution containing 1% Triton X-100. These solutions were directly assayed for vanadium. Weighed aliquots of frozen tissue samples were digested in three stages: the first using 4 mL concentrated HNO_3, the second using a combination of 2 mL HNO_3 and 30% H_2O_2 and finally 2 mL HNO_3. All digestions were carried out at 130 °C until the sample was completely dry. After the third drying, 1% HNO_3 was added to the digests and heated at 80 °C for 1 hr. Once cool, the samples were volumed and analysed. Dilutions were made as required.

ii) Standards

Standard solutions of vanadium at varying concentrations were prepared from a Certified Reference Standard of vanadium (1 mg/mL, SCP Science). Calibration curves were generated before and after sample sets. *Standards used for Plasma Analysis.* Standard solutions from 0-100 μg/L were made up in a 0.125 M sodium citrate (Fisher Scientific) solution containing 0.5% Triton X-100 (BDH). *Standards used for Tissue Analysis.* Standard solutions from 0-100 μg/L were made up in 1% HNO_3.

iii) Vanadium Analysis

Vanadium concentrations were measured using a Polarized Zeeman Graphite Furnace Atomic Absorption (AA) Spectrophotometer equipped with an autosampler. The detector wavelength was set to 318.4 nm using a slit width of 0.40 nm. A vanadium lamp current of 10.0 mA was used. A deuterium lamp was used for background correction. Sample volumes of 20 μL were used followed by insertion into a graphite tube. The following temperature profile was used for vanadium analysis: two drying steps beginning at 80-120 °C ramped over 30 sec. with a hold time of 20 sec. and from 120-450 °C ramped over 25 sec. and held for 20 sec. This was followed by an ashing step starting at 900-1400 °C ramped for 25 sec. and held for 20 sec. and finally atomization at 2850 °C for 10 sec. A 10 sec. cleaning step at 3000 °C as well as a 5 sec. cool down were programmed for each run. The purge gas used in these analyses was argon used at a flow rate of 200 mL/min except during atomization at which time it was set to 40 mL/min. Vanadium concentrations were calculated from external standards based upon relative correlations in peak absorbance. Instrument performance and thus result validity was obtained through sample spikes, standard reruns and for the tissue samples, digested blanks and digested bovine liver 1577b (Standard Reference Material). The low-end detection limit of the instrument was 2.0 μg/L. All analyses were carried out in duplicate.

8. Statistical Analysis:

Statistical treatment of data was performed using a Students t-test. Results were reported as mean ± SE. Statistical significance was determined at a P level of < 0.05.

RESULTS

I. Vanadium Toxicity:

In our laboratory strong evidence of vanadium toxicity has been demonstrated in both Type 1 and Type 2 diabetic rats. STZ-induced diabetic Sprague Dawley rats treated with 2ml of a 20mg/ml sodium orthovanadate in water solution were effectively treated for hyperglycemia **(Figure 2)** but at the expense of severe toxicity. Over 70% of the diabetic rats orally gavaged with vanadate experienced diarrhea over an 11-week study and greater than 70% of these animals died during the course of the trial **(Figure 3)**. By comparison, non-diabetic control and diabetic animals gavaged with 2ml of water alone experienced none of these side effects. In a model of Type 2 diabetes, we found similar results. When treated with sodium orthovanadate the blood glucose levels of Zucker diabetic fatty rats were returned to near control levels **(Figure 4)**. The 15mg/ml vanadate/water solution gavaged to these animals produced diarrhea in over one half and resulted in the death of a single animal over the 4-month trial **(Figure 5)**.

II. Acute Effects of CT99:

The effect of both vanadate (vanadate/water solution) and CT99 on blood glucose control is shown in **Figure 6**. The initial blood glucose of the STZ-induced SD diabetic rats is approximately 22 mM and identical in both the CT99-D and vanadate-D treatment groups. This level is four fold higher than that of a non-diabetic (ND) Sprague Dawley rat. Upon treatment, blood glucose levels fell within the first hour to 17.4 ± 1.96 mM and

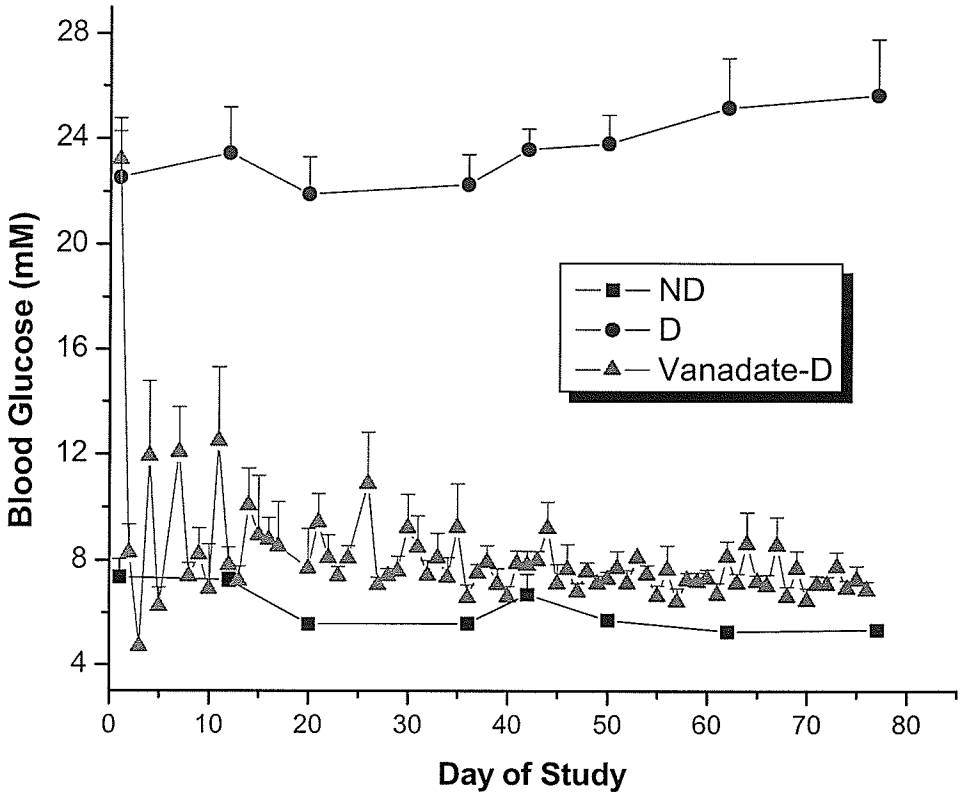


Figure 2. Blood glucose levels in non-diabetic (ND), diabetic (D) and water/vanadate-treated (Vanadate-D) Type 1 diabetic Sprague Dawley rats. All D data after day 4 significantly different from both vanadate-D and ND.

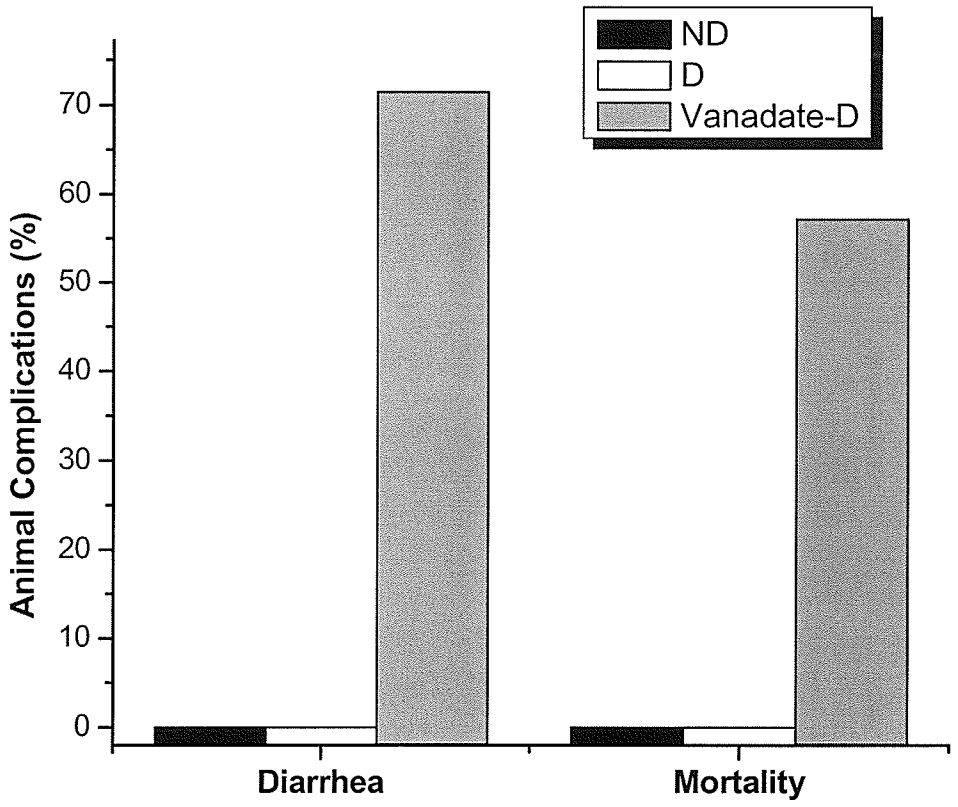


Figure 3. Incidence of diarrhea and death in non-diabetic (ND), diabetic (D) and water/vanadate-treated (Vanadate-D) Type 1 diabetic Sprague Dawley rats. Values represent % of animals experiencing these parameters; n = 5,5 and 7 in ND, D and Vanadate-D groups respectively.

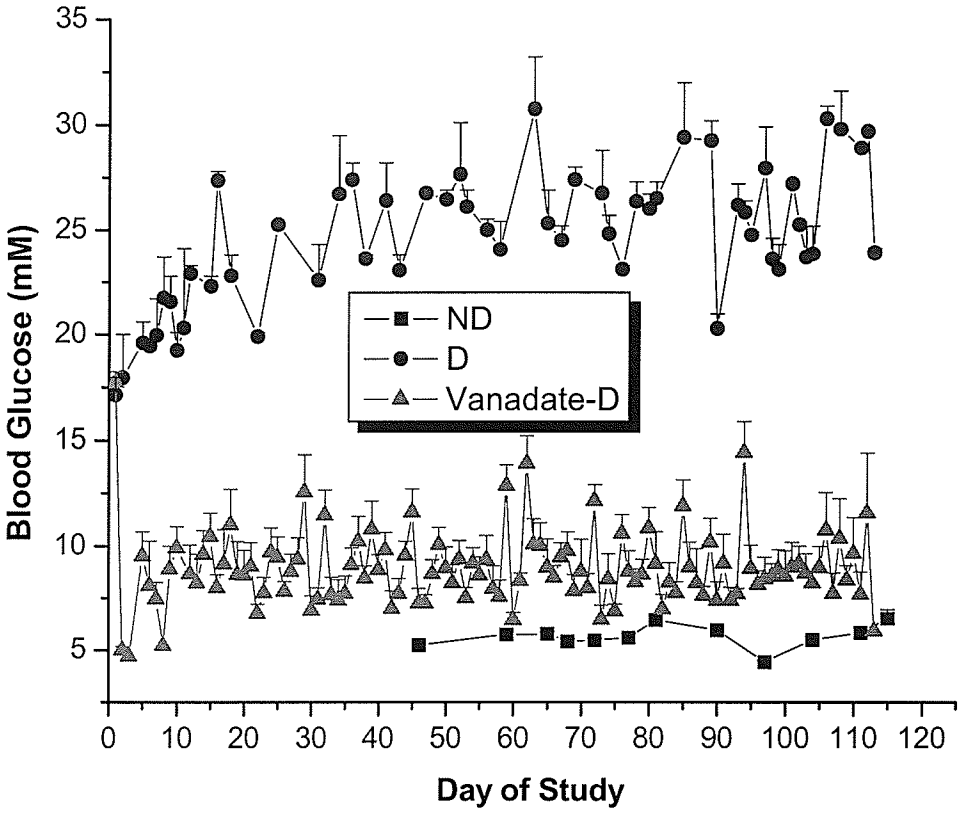


Figure 4. Blood glucose levels in non-diabetic (ND), diabetic (D) and water/vanadate-treated (Vanadate-D) Zucker diabetic fatty rats. All D data after day 4 significantly different from both vanadate-D and ND.

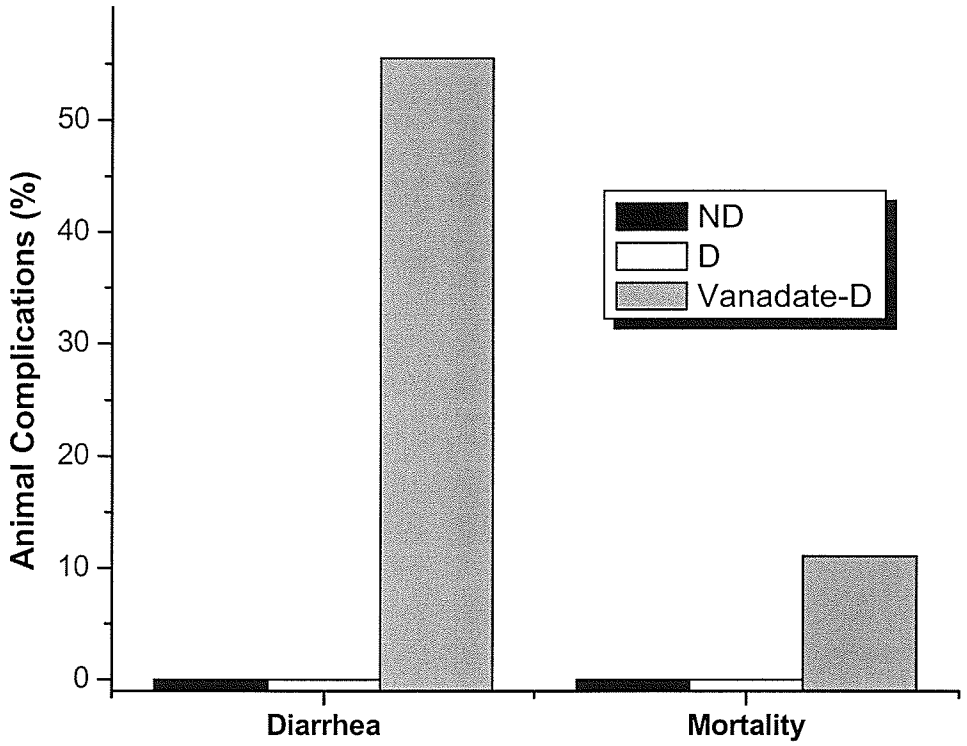


Figure 5. Incidence of diarrhea and death in non-diabetic (ND), diabetic (D) and water/vanadate-treated (vanadate-D) Zucker diabetic fatty rats. Values represent % of animals experiencing these parameters; n = 6, 6, 9 in ND, D and Vanadate-D groups respectively.

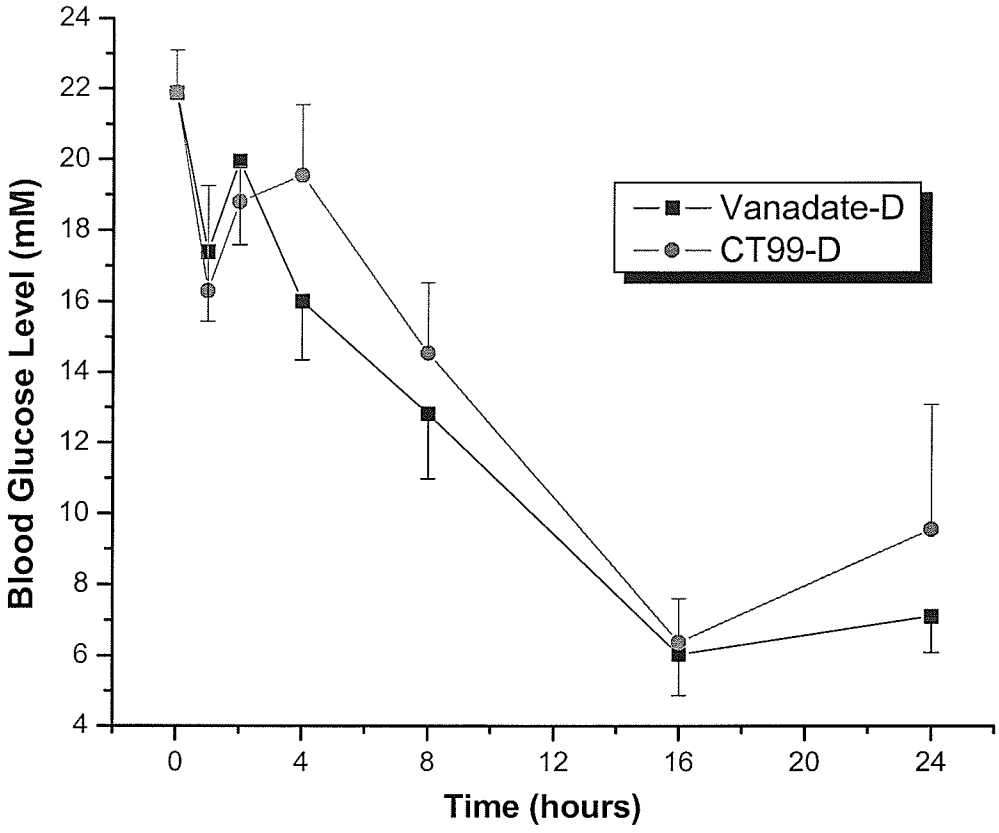


Figure 6. Acute blood glucose effects of Na-orthovanadate/ddH_2O (vanadate-D) and CT99-treatment (CT99-D) in type 1 diabetic rats. Each point represents mean ± SE of n = 6 in each treatment group.

16.3 ± 2.96 mM in the vanadate-D and CT99-D treatment groups respectively. During the second hour post-treatment blood glucose levels in both the vanadate-D and CT99-D treated animals rose slightly to 19.95 ± 2.36 mM and 18.8 ± 0.99 mM respectively. No statistically significant changes occurred over the next two hours and at the four hour time point blood glucose levels were 16.01 ± 1.67 mM (vanadate-D) and 19.55 ± 2.01 mM (CT99-D). Large drops in blood glucose levels occurred during the four to eight hour post-treatment interval. The vanadate-D treated animals displayed a glucose level of 12.83 ± 1.85 mM and the CT99-D animals showed blood glucose values of 14.55 ± 1.98 mM. This reduction in blood glucose level at the eight-hour time point was statistically significant in both groups ($P < 0.01$). An even more marked hypoglycemic effect was achieved over the following eight-hour interval. Sixteen hours after vanadate treatment, the vanadate-D and CT99-D rats' glucose levels were reduced to ND levels. The values at this point were 6.03 ± 1.16 mM and 6.38 ± 1.23 mM respectively, both significantly lower than the initial glucose levels ($P < 0.0001$). The final eight hours of glucose monitoring showed slight recovery in glycemic status to 7.11 ± 1.03 mM and 9.56 ± 3.53 in vanadate-D and CT99-D rats.

Vanadium levels were measured in several organs and plasma to compare the uptake and storage of vanadate when delivered in the H_2O and CT99 systems. Plasma vanadium levels are shown in **Figure 7**. All non-diabetic (ND) and diabetic (D) control animals had plasma vanadium levels lower than the detection limit of the AA spectrophotometer (2.00 μg/L). In contrast, the vanadium treated animals displayed detectable levels of vanadium at all time points. The highest levels of vanadium in plasma were found at the one and two hour time points (~ 2.5 mg/L) with decreasing

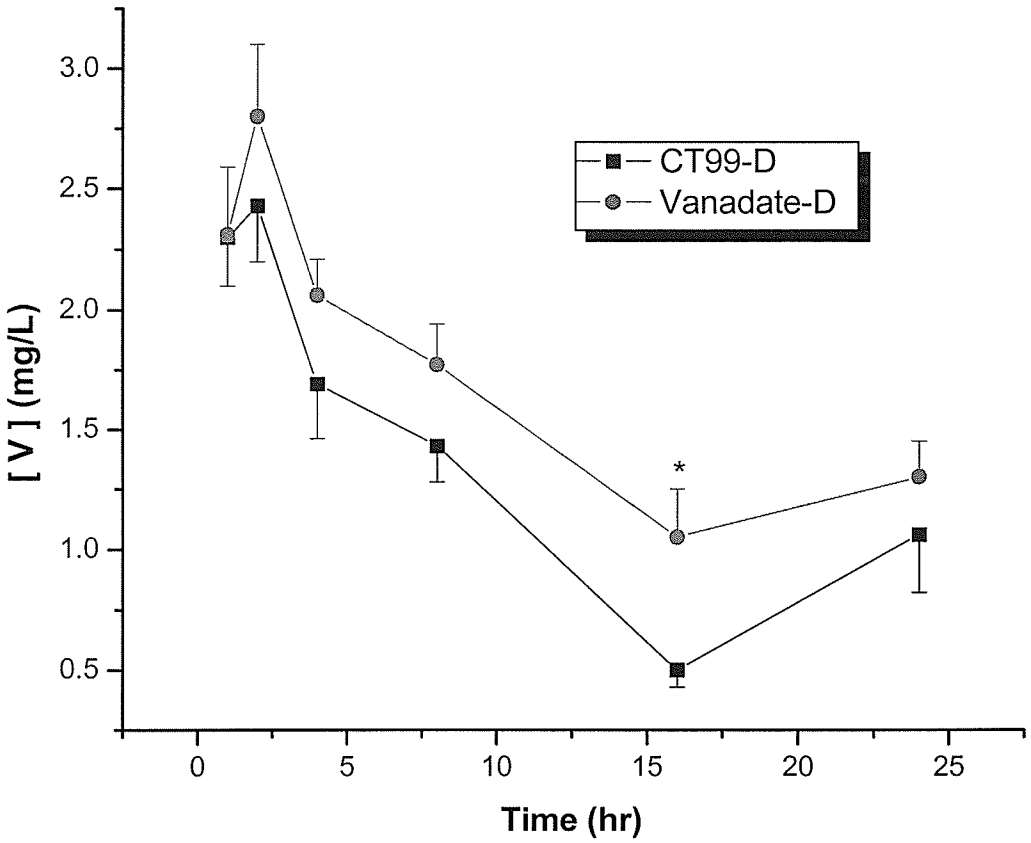


Figure 7. Plasma vanadium level changes in Type 1 diabetic rats treated with a single dose of Na-orthovanadate/ddH_2O (vanadate-D) or CT99. Each point represents mean ± SE of n = 6. * $P < 0.03$.

concentrations up to 16 hours post-treatment. CT99-D administration significantly reduced plasma vanadium concentration at the 16-hour time point compared to the vanadate-D treatment (0.50 ± 0.07 mg/L vs. 1.05 ± 0.20 mg/L).

Vanadium concentrations were analyzed in eight selected organs **(Figures 8-15)**. No vanadium was detectable in any organ of the ND or D animals with all levels lower than the detection ability of the AA spectrophotometer. In the CT99-D and vanadate-D treated animals the highest concentrations were found in bone > kidney > liver > pancreas > lung > heart > muscle > brain. Leg bone measurements display a gradual increase in vanadium concentration over the 24-hour period. Vanadate-D treated animals retained significantly more vanadium than their CT99-D counterparts at the 24-hour time point (5.91 ± 0.32 μg/g vs. 3.24 ± 0.79 μg/g respectively). The increased accumulation of vanadium occurred solely over the 16-24 hour period **(Figure 8)**. Kidney accumulation of vanadate was rapid and reached a maximum within the first two hours post-treatment. Levels remained stable from 4-24 hours with vanadate-D animals accumulating significantly more vanadium than the CT99-D rats over all time points **(Figure 9)**. Temporal deposition of vanadium in liver paralleled that of the kidney although levels of accumulation were lower **(Figure 10)**. Again at 8 and 16 hours vanadate-D rats retained significantly more vanadium than the CT99-D rats. Vanadium concentrations in pancreatic samples displayed the largest variability **(Figure 11)**. Notwithstanding, vanadate-D treated rats exhibited significantly higher levels of accumulation at two hours. All later time points showed no differences. Lung examination revealed similar levels of vanadium at all time points measured in the vanadate-D and CT99-D rats **(Figure 12)**. Levels peaked shortly after administration of the treatment solutions in both

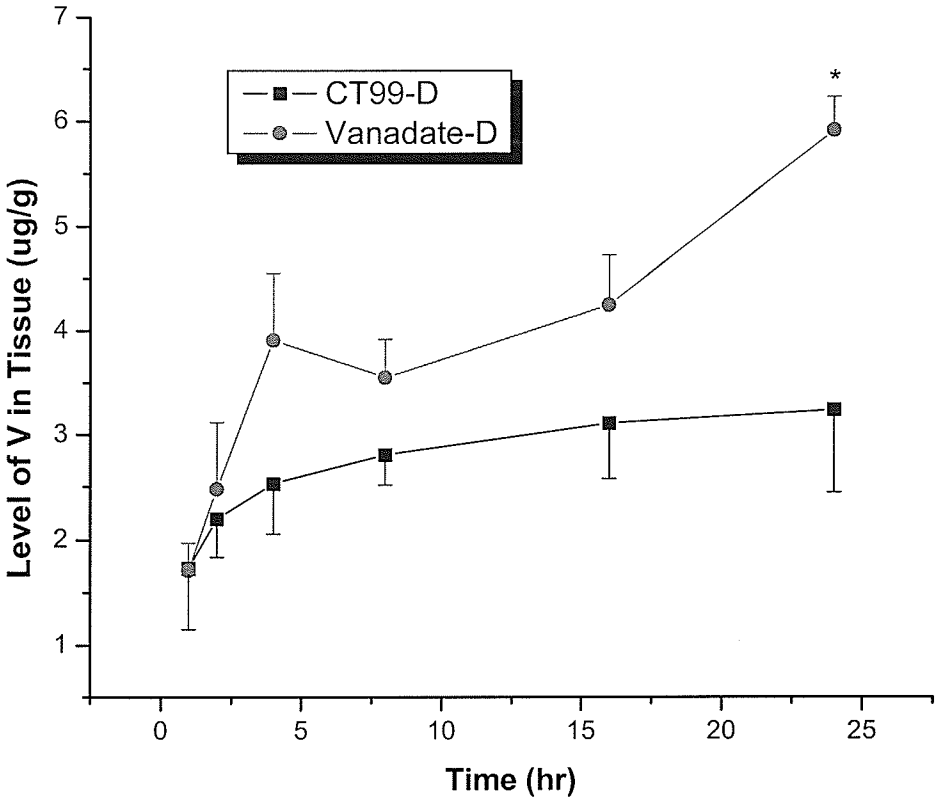


Figure 8. Vanadium level changes in bone of Type 1 diabetic rats treated with a single dose of Na-orthovanadate/ddH_2O (vanadate-D) or CT99. Each point represents mean ± SE of n = 6. * $P < 0.01$.

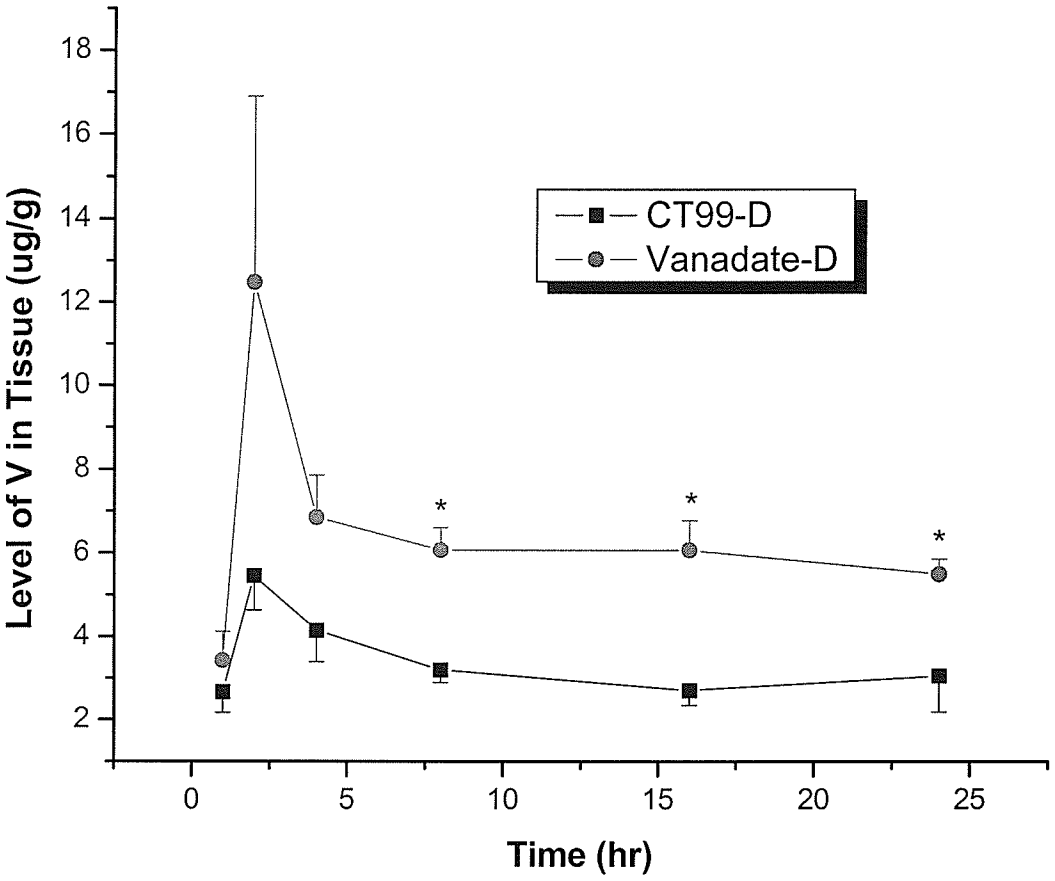


Figure 9. Vanadium level changes in kidney of Type 1 diabetic rats treated with a single dose of Na-orthovanadate/ddH_2O (vanadate-D) or CT99. Each point represents mean ± SE of n = 6. * $P < 0.03$.

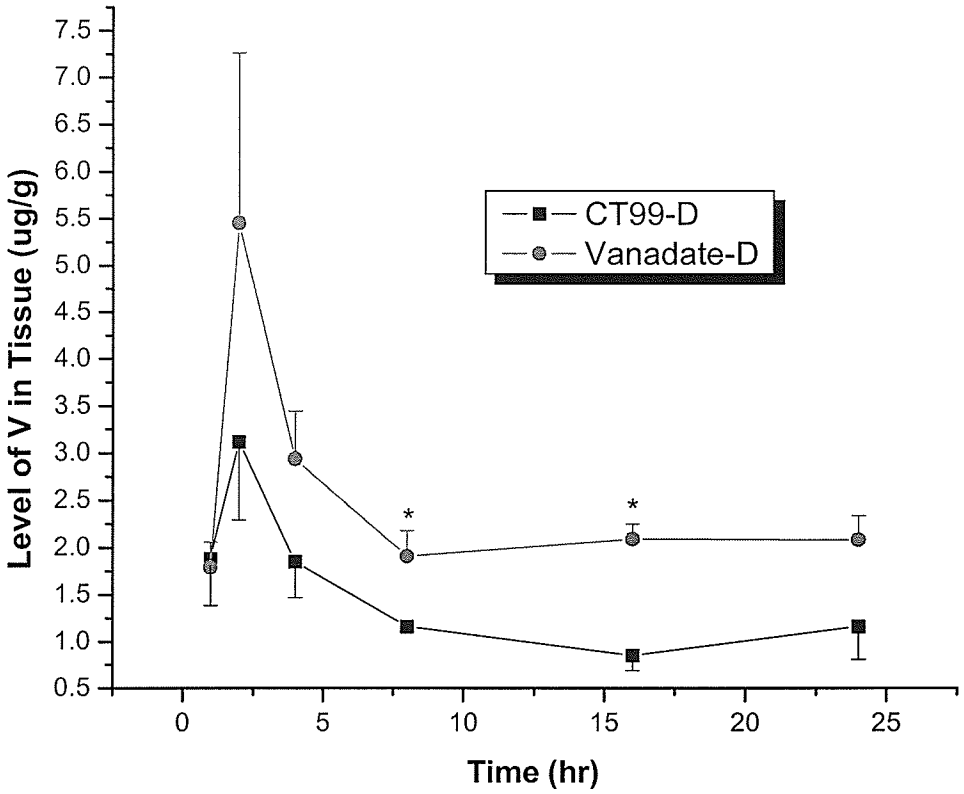


Figure 10. Vanadium level changes in liver of Type 1 diabetic rats treated with a single dose of Na-orthovanadate/ddH_2O (vanadate-D) or CT99. Each point represents mean ± SE of n = 6. * P < 0.03.

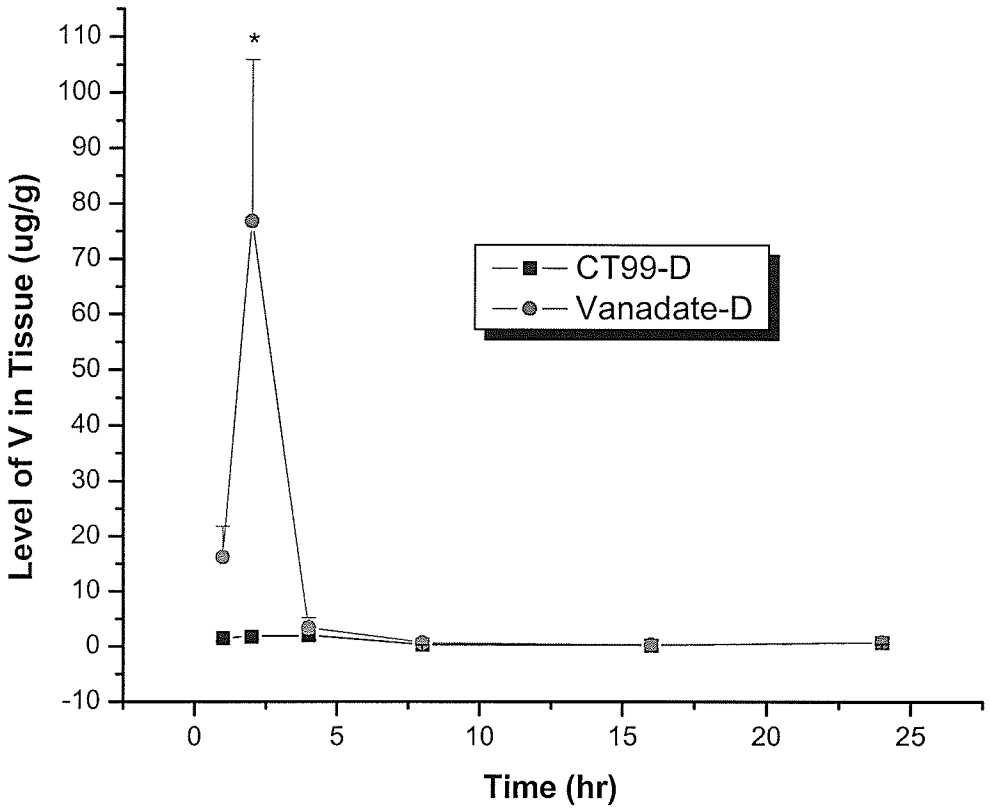


Figure 11. Vanadium level changes in pancreas of Type 1 diabetic rats treated with a single dose of Na-orthovanadate/ddH_2O (vanadate-D) or CT99. Each point represents mean ± SE of n = 6. * $P < 0.05$.

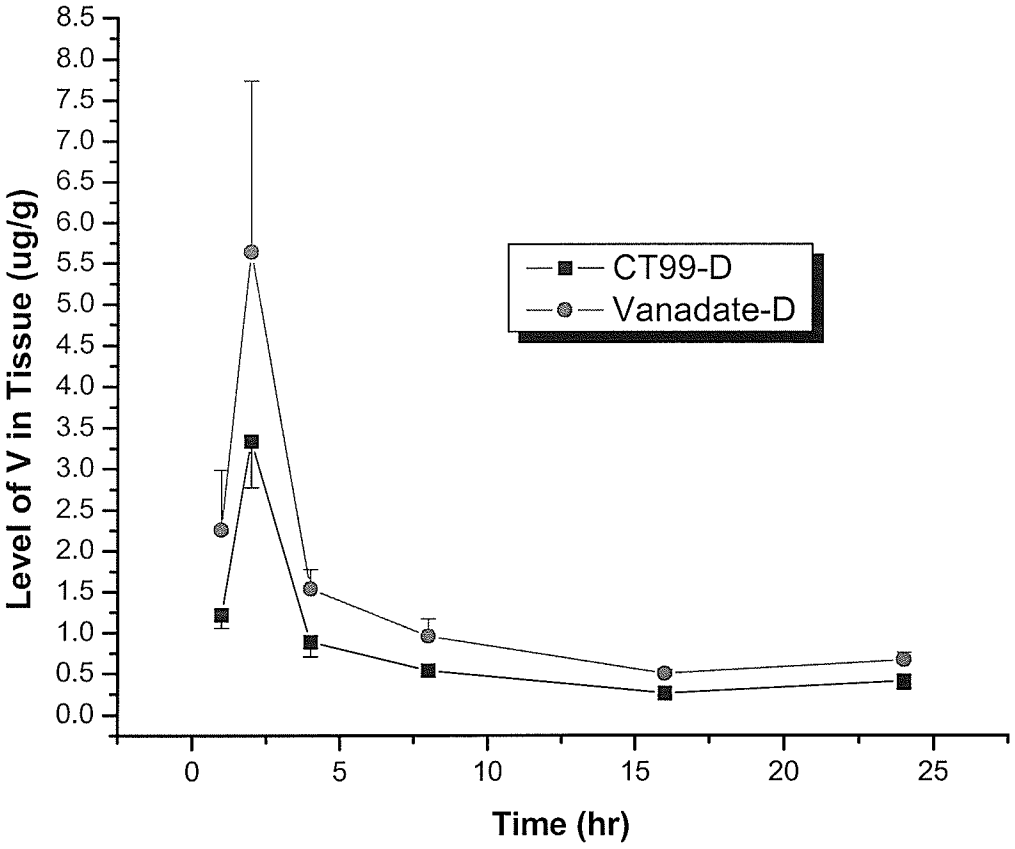


Figure 12. Vanadium level changes in lung of Type 1 diabetic rats treated with a single dose of Na-orthovanadate/ddH_2O (vanadate-D) or CT99. Each point represents mean ± SE of n = 6.

groups with two-hour values of 5.64 ± 2.10 ug/g and 3.34 ± 0.56 ug/g in vanadate-D and CT99-D respectively. By four hours and thereafter, levels remained < 1.0 ug/g. Significantly lower vanadium levels were found in hearts of rats receiving CT99 treatment than vanadate/water at both four and 16 hours **(Figure 13)**. CT99-D rat had levels of vanadium that plateaued at ~ 0.3 ug/g after four hours. Muscle sections from the lower leg revealed little differences between treatment groups and minimal accumulation by 24 hours **(Figure 14)**. Levels in both groups were less than 0.20 ug/g by this point. The lowest vanadium levels were found in brain (0.06 ug/g in vanadate-D and CT99-D) **(Figure 15)**. No differences at any time-point were noted.

III. Chronic Effects of CT99:

i) Type 1 Diabetes

The efficacy of CT99 as a chronic therapy in the treatment of diabetes was first examined in the STZ-induced Type 1 diabetic Sprague Dawley rat. The animals in this study were separated into 4 treatment groups **(Table 2).** The incidence of diarrhea and mortality for the vanadate-D and CT99-D animals are presented in **Figure 16**. Approximately 63% of the vanadate-D treated animals developed severe diarrhea and 37.5% of these animals died as a direct result of these complications. By comparison, CT99-D animals were completely free of both diarrhea and mortality. Because of the toxic effects of vanadate/water solution, further analyses of surviving D rats in this treatment group were limited to an analysis of its hypoglycemic effects.

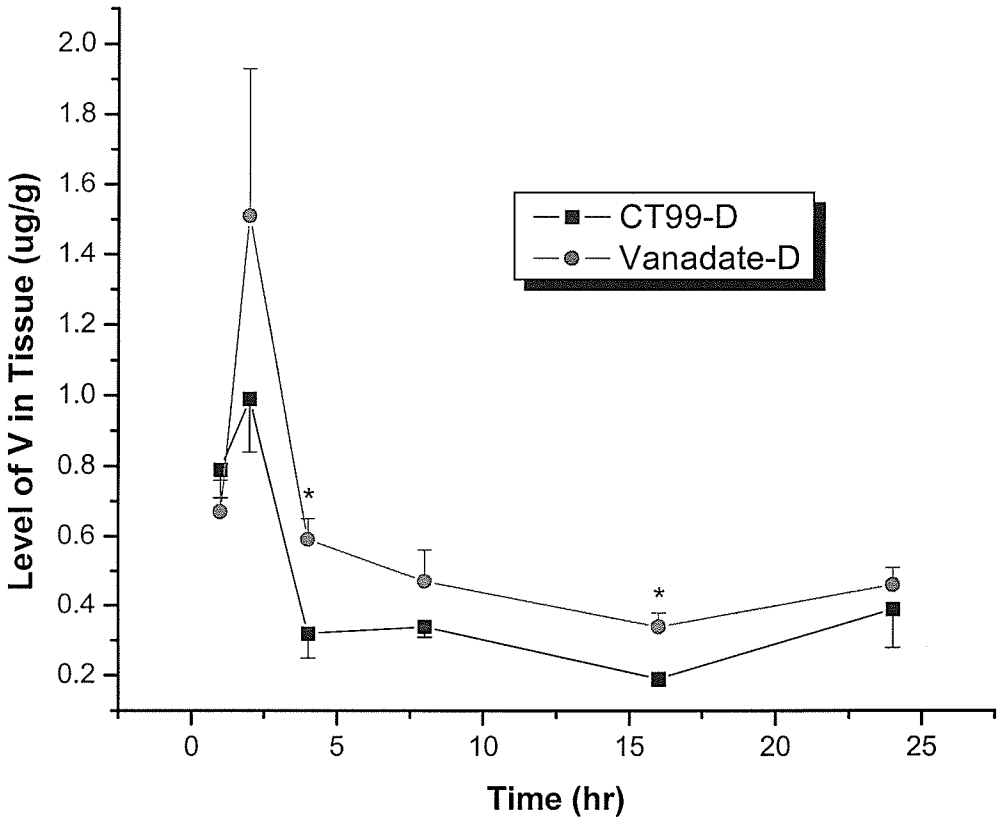


Figure 13. Vanadium level changes in heart of Type 1 diabetic rats treated with a single dose of Na-orthovanadate/ddH_2O (vanadate-D) or CT99. Each point represents mean ± SE of n = 6. * P < 0.02.

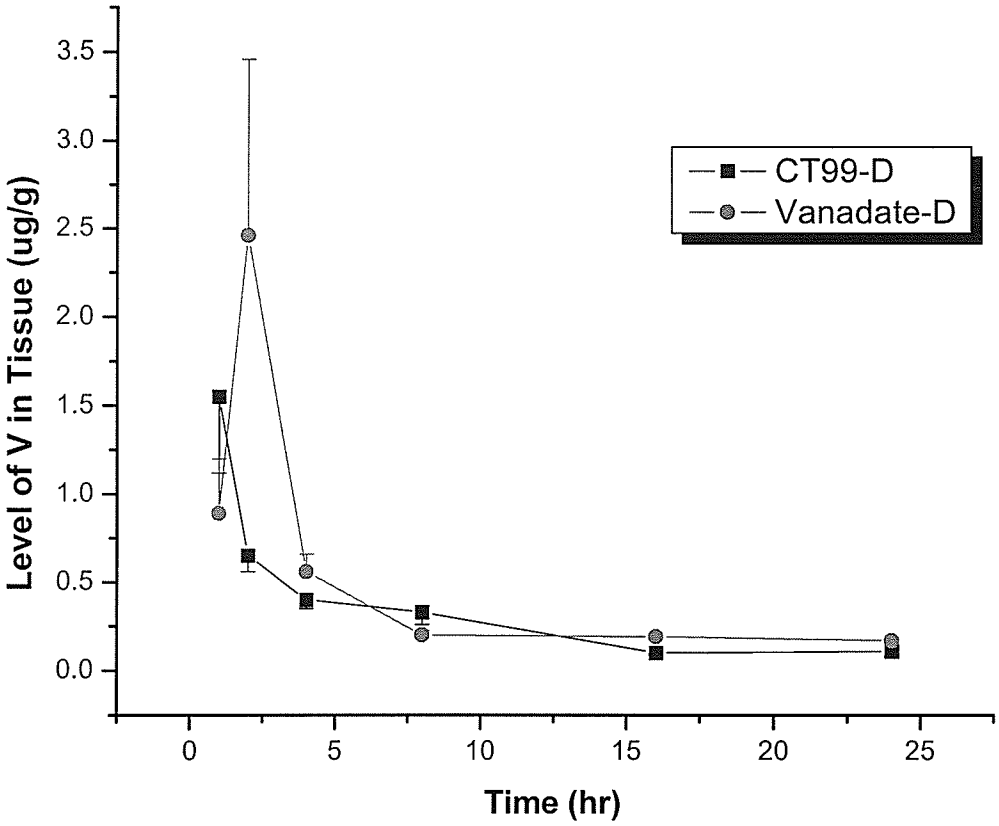


Figure 14. Vanadium level changes in muscle of Type 1 diabetic rats treated with a single dose of Na-orthovanadate/ddH_2O (vanadate-D) or CT99. Each point represents mean ± SE of n = 6.

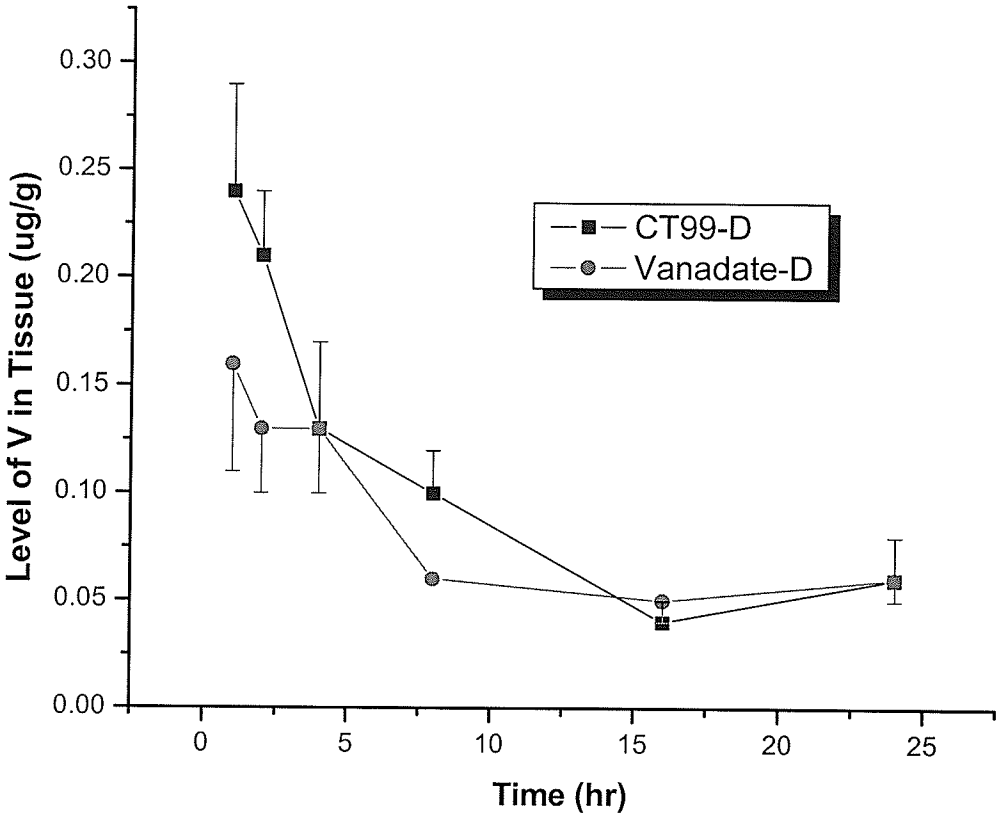


Figure 15. Vanadium level changes in brain of Type 1 diabetic rats treated with a single dose of Na-orthovanadate/ddH_2O (vanadate-D) or CT99. Each point represents mean ± SE of n = 6.

Table 2. Animal treatment groups for three-month trial of CT99 in Type 1 diabetes.

Group Title	Specifications	n
ND	Nutraceutical-treated non-diabetic SD rats	5
D	Nutraceutical treated diabetic SD rats	5
CT99-D	Diabetic SD rats treated with 20 mg vanadate/ ml nutraceutical	15
Vanadate-D	Diabetic SD rats treated with 20 mg vanadate/ ml water	8

ND = Non-diabetic, D = Diabetic, SD = Sprague Dawley rats

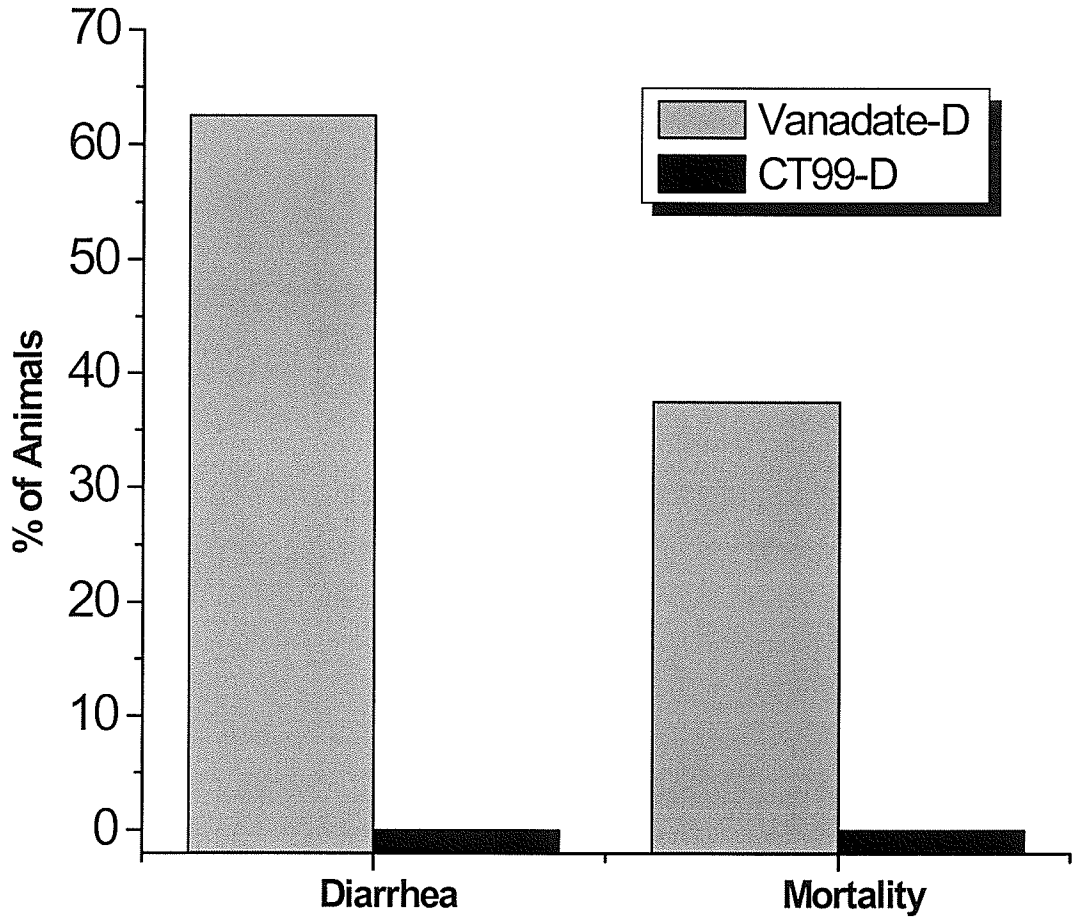


Figure 16. Diarrhea and mortality in diabetic SD rats treated with 40 mg vanadate in 2 ml water (vanadate-D) or 40 mg vanadate in 2 ml nutraceutical solution (CT99). Diarrhea and mortality are represented as the percentage of animals in each group.

Blood glucose levels were monitored throughout the study. The results are shown in **Figure 17.** Pre-treatment glucose levels in all of the diabetic animal groups were approximately 22 mM. D animals showed a gradual increase in blood glucose concentration rising from 22.5 mM to 25 mM over the 11-week experimental period. By comparison, ND animals had blood glucose levels of 5-7 mM throughout the study. The first treatment was administered on day 4 and this reduced blood glucose levels in the CT99-D animals to control levels (approximately 7.0 mM). This was found in both vanadate-D treated animals and CT99-D groups and was statistically different compared to the D rats. Glucose stabilization over the 11 week study was maintained at a level of ~ 8 mM in these groups. This closely resembled the ND blood glucose values. One of the most striking findings in this study was the long-term glycemic control of the CT99-D rats **(Figure 18).** These animals averaged 24 ± 5.6 days of consecutive normoglycemia *without treatment.* Therefore, for approximately 1/3 of the total study, glycemic control was achieved without further CT99 treatment. Chronic glycemic control in individual rats varied from 4-71 days of normoglycemia. Fifty percent of the CT99-treated diabetic animals exhibited glucose levels < 10 mM for greater than 15 consecutive days *without receiving further CT99 treatment.*

Another measure of the long-lasting hypoglycemic efficacy of CT99 is the frequency of treatments each rat received over the course of the study **(Figure 19).** On average, the CT99-D animals were treated only 17% of the days in this study. This amounts to one treatment every six days in order to maintain the daily blood glucose level of ~ 8 mM in the CT99-D animals.

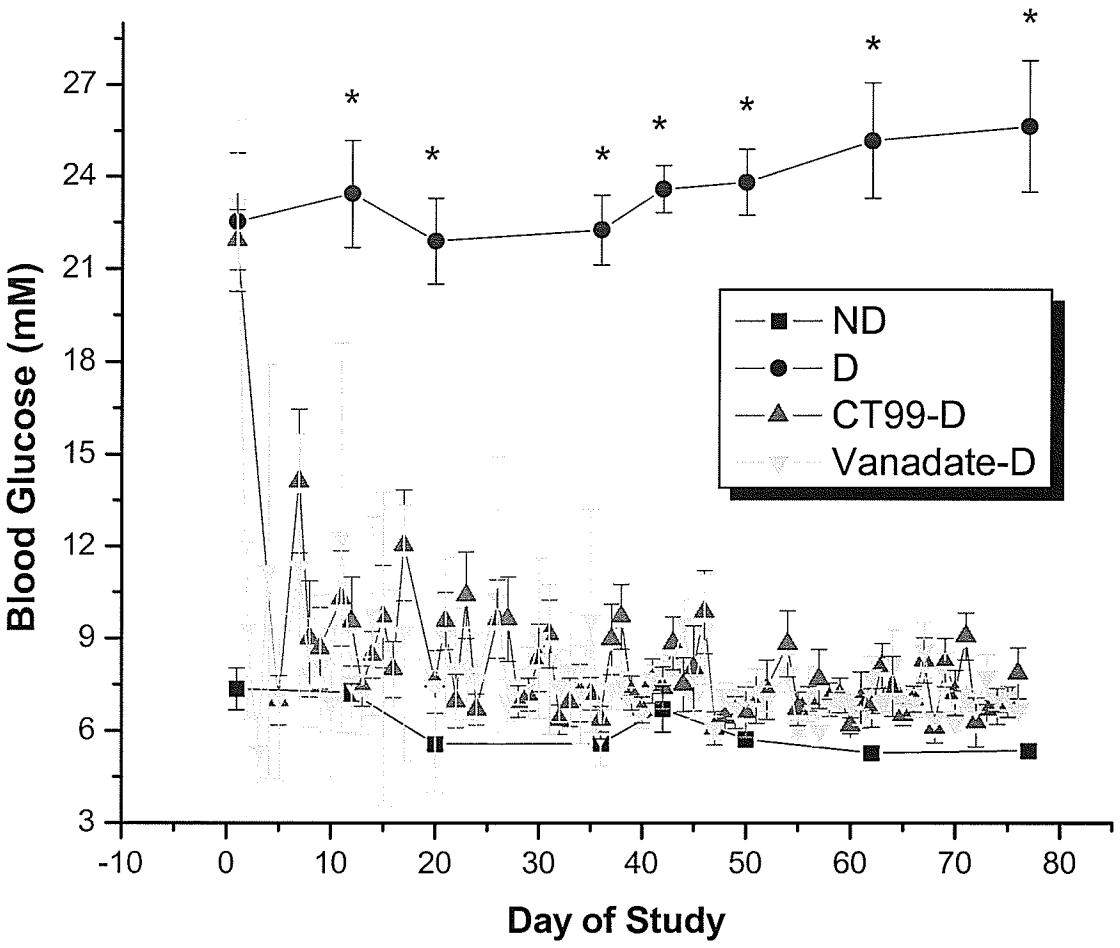


Figure 17. Blood glucose levels in non-diabetic (ND) control rats, diabetic (D) rats, CT99-treated diabetic (CT99-D) rats and water/vanadate-treated (vanadate-D) diabetic rats. Values represent mean ± SE of n = 5, 5, 15, 8, respectively, in each group. * $P < 0.0001$ vs. ND, CT99-D and vanadate-D.

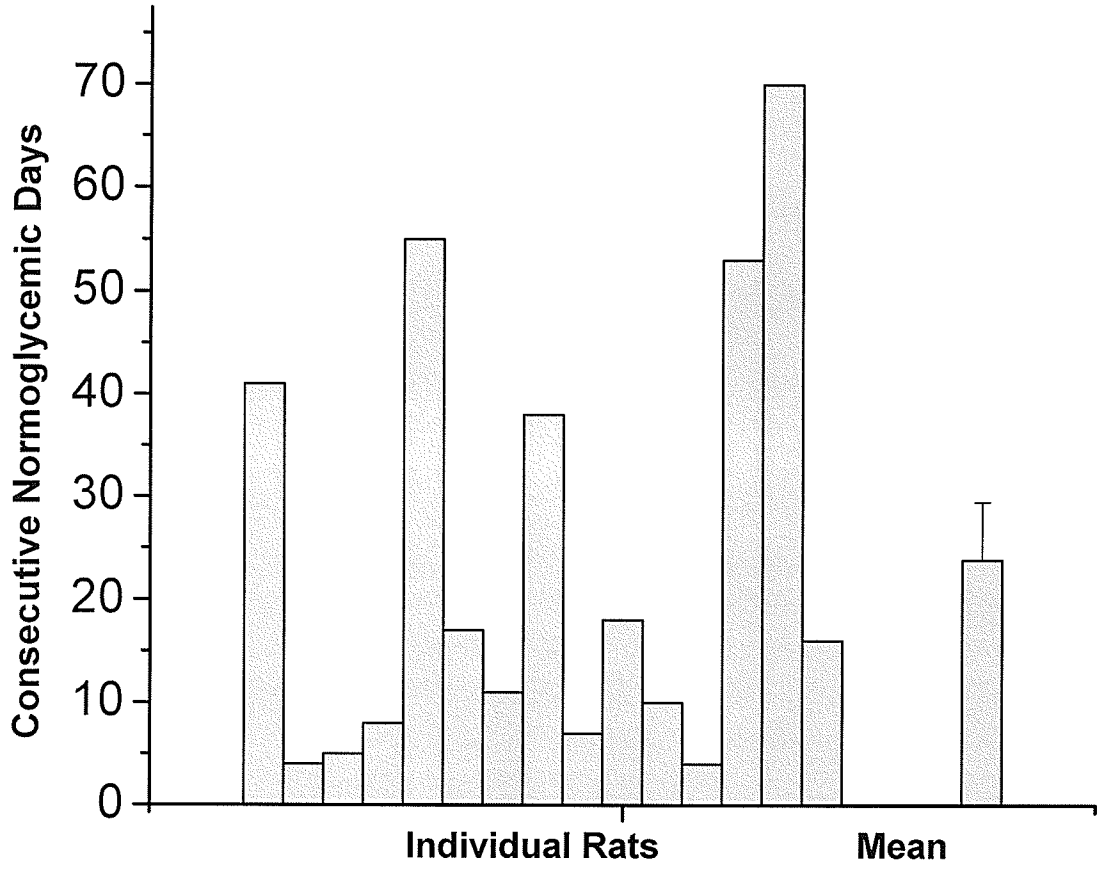


Figure 18. Persistent normoglycemic effects of CT99 treatment in diabetic rats. Blood glucose levels of CT99-treated diabetic rats were monitored daily. Values represent the consecutive days that blood glucose levels were < 10 mM in individual rats and as the mean of all animals, n = 15.

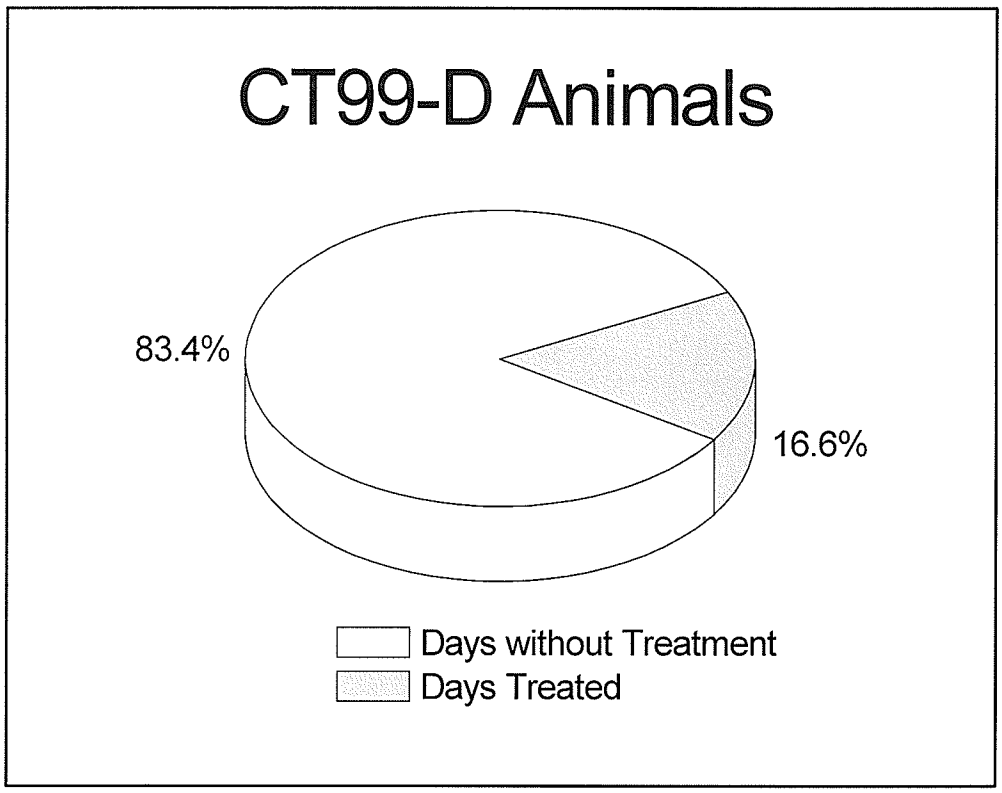


Figure 19. Frequency of CT99 treatment in diabetic rats. Values represent the mean number of days in the study that CT99-treated diabetic rats required treatment to return blood glucose levels to < 10mM, n = 15.

The potential for CT99 to have toxic side effects was investigated in greater detail. Sodium orthovanadate delivered in water has been shown to inhibit food and water intake [179]. It was possible therefore, that CT99 may affect these parameters as well. Both food and water intake were measured in the ND, D and CT99-D animals during the study **(Figure 20).** ND animals consumed approximately 67 ml of water and 25 g of rat chow daily. D rats consumed significantly more water and food (175 ml of water and 45 g of rat chow daily). CT99-treatment normalized water and food consumption. Water and food intake in CT99-D animals was 65 ml and 25 g, respectively.

As shown in **Figure 21**, D rats had significantly lower body weights during the course of this study than their ND counterparts. Although CT99 treatment did not normalize body weight, the rate of weight gain was greater in CT99-D than in D rats, as reflected by the slope of the lines in **Figure 21.** CT99-D animals (y = 3.08 g/day) gained weight more rapidly than the D rats (y = 1.94 g/day) but remained lower than the weight gain for the ND rats (y = 3.80 g/day).

Several additional parameters of toxicity and organ function were assessed **(Table 3)**. Liver toxicity was measured with plasma ALT enzyme assays. Plasma ALT levels in ND rats were 50 U/L whereas D animals had elevated ALT activity (64 U/L). ALT activity in CT99-D rats was normalized (51 U/L).

Kidney function was evaluated using a plasma creatinine assay, BUN analysis and measurement of urine specific gravity. Creatinine levels were significantly lower in the D animals in comparison to the ND rats. CT99-D rats had creatinine levels that were significantly improved vs. the D animals and not significantly different than ND values. BUN levels were not significantly different among the three groups. The specific gravity

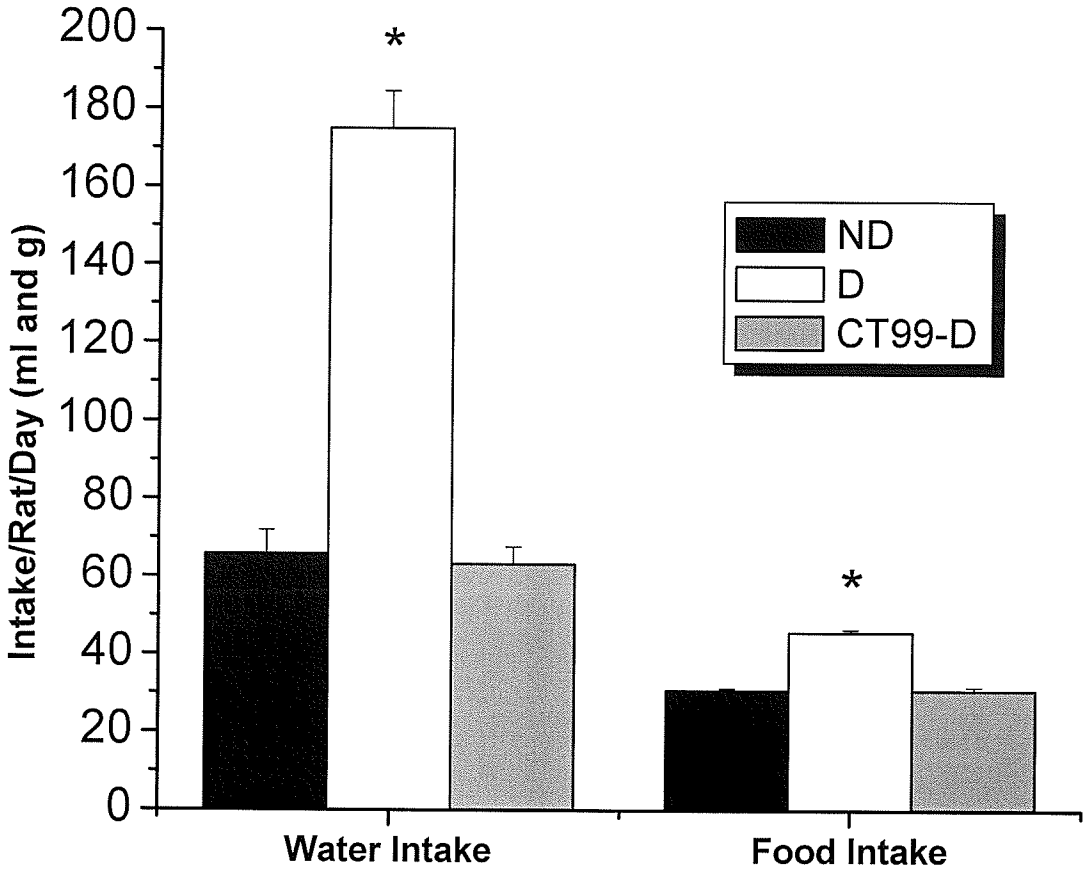


Figure 20. Water and food consumption in non-diabetic (ND) rats, diabetic (D) rats and CT99-treated (CT99-D) diabetic rats. Intake of water (ml) and rat chow (g) was measured over 24 hr periods on days 48-50. Data are mean ± SE of n = 5, 5, 15 respectively. * $P < 0.05$ vs. ND and CT99D.

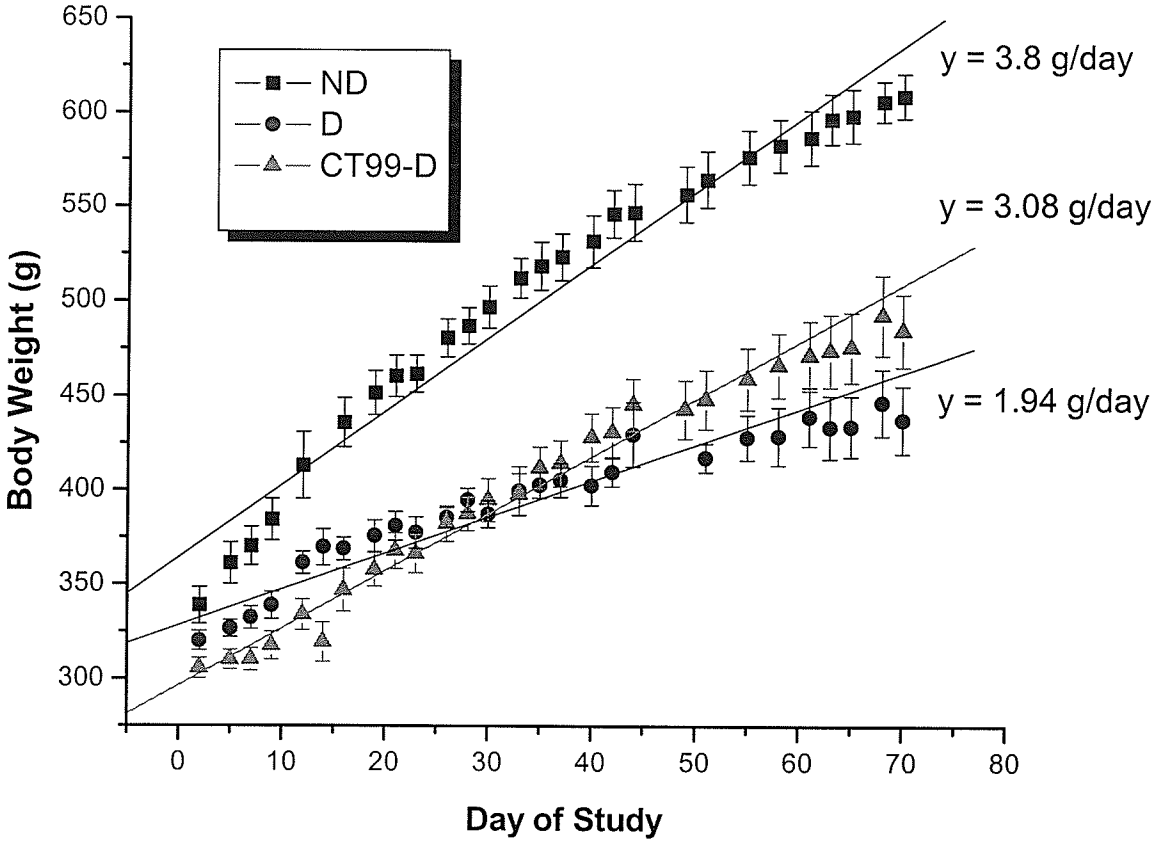


Figure 21. Body weights of non-diabetic (ND) rats, diabetic (D) rats and CT99-treated (CT99D) diabetic rats during the 11 week experimental period. Values represent mean ± SE of n = 5, 5, 15 respectively. Y = weight change as a function of time.

Table 3. Biochemical analysis in non-diabetic (ND), diabetic (D), and CT99-treated Type 1 diabetic (CT99-D) rats.

Assay	ND	D	CT99-D
Insulin (mM)	0.69 ± 0.17	0.29 ± 0.01*	0.54 ± 0.08
TG (mg/dl)	150 ± 15	250 ± 68	198 ± 22
Chol (mg/ml)	61.35 ± 5.30	82.75 ± 18.29	58.36 ± 4.59
Creat (mg/dl)	0.31 ± 0.02	0.23 ± 0.03*	0.31 ± 0.02
BUN (mg/dl)	16.55 ± 1.46	18.13 ± 1.00	17.17 ± 2.15
ALT (U/L)	49.66 ± 1.91	63.73 ± 4.35*	51.09 ± 4.57
Urine Gravity	1.01 ± 0.003	1.03 ± 0.005*	1.02 ± 0.005
Cataracts (%)	0	40*	0

TG = Triglyceride, Chol = Cholesterol, Creat = Creatinine, BUN = Blood Urea Nitrogen, ALT = Alanine Aminotransferase. Data is reported as mean ± SE of n = 5,5,15 in ND, D and CT99-D rats respectively. * $P < 0.05$ vs. ND animals.

of urine was significantly higher in D rats in comparison to ND rats. CT99-treatment returned this value to control levels.

Plasma cholesterol and triglycerides were also measured in the rats **(Table 3)**. Lipid levels tended to be higher in D rats when compared to ND values. However, this did not achieve statistical significance. CT99-treatment of D rats brought these indices closer to control values.

We also monitored the status of plasma insulin as a function of diabetes and CT99-treatment **(Table 3)**. Plasma insulin levels were very low in D rats as a result of the STZ administration (~ 42% of control). CT99-D rats had plasma insulin levels similar to the ND animals and significantly improved compared to the D rats.

During the course of the study, cataract formation was observed in the diabetic rats. Although this was not analyzed via conventional optic methods, the cataracts were easily visible as large areas of opaque cloudiness in the eye. Forty percent of the untreated diabetic rats had developed this lesion by the end of the study **(Table 3)**. None of the ND controls or the CT99-D rats exhibited any visual evidence of cataract formation **(Figure 22)**.

Finally, vanadium levels were measured in the ND, D and CT99-D animals following the study. All tissues measured (plasma, lungs, liver, bone, kidney, pancreas and heart) in the ND and D animals yielded concentrations of vanadium lower than the detection limit of the spectrophotometer ([V] < 5.00 ug/L). As expected, levels in both the vanadate-D and CT99-D animals were elevated. In all organs and plasma no significant differences were found between the two groups **(Figures 23, 24)**. Highest

Figure 22. Cataract complications of diabetes. Animal represents a diabetic (D) rat. Normal left eye similar to all CT99-D animals.

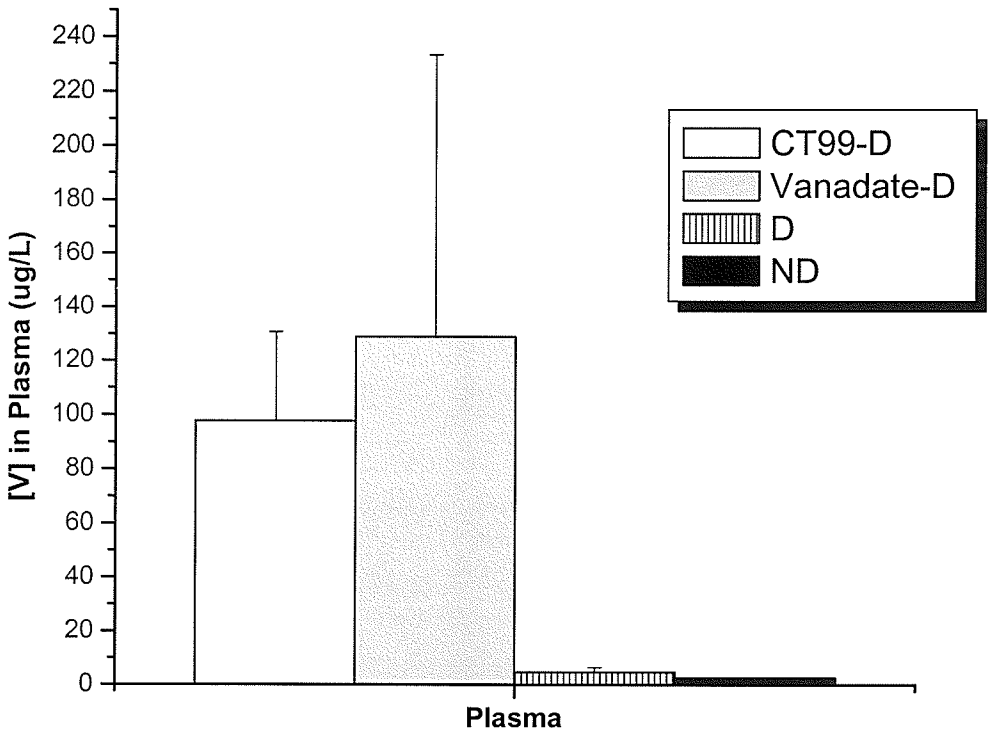


Figure 23. Plasma vanadium levels in CT99-treated diabetic (CT99-D), vanadate/water-treated diabetic (Vanadate-D), diabetic (D) and non-diabetic (ND) Sprague Dawley rats. Data represents mean ± SE of n = 8, 5, 5, 7 respectively.

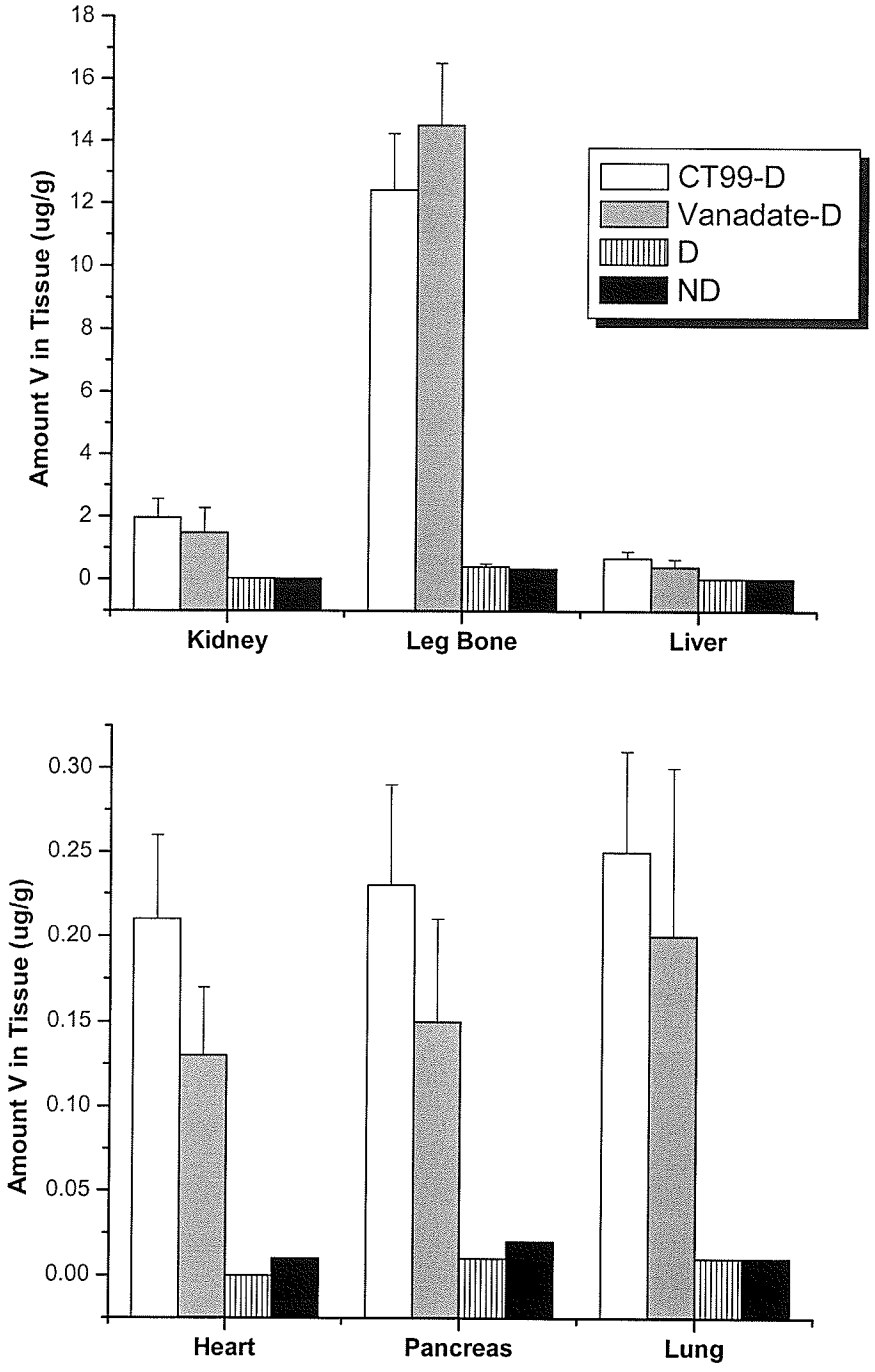


Figure 24. Organ vanadium levels in CT99-treated diabetic (CT99-D), vanadate/water-treated diabetic (Vanadate-D), diabetic (D) and non-diabetic (ND) Sprague Dawley rats. Data represents mean ± SE of n = 8, 5, 5, 7 respectively.

concentrations of vanadium were found in bone > kidney >liver > lung > pancreas > heart

As heart dysfunction is the leading cause of mortality in the diabetic population and diligent control of glycemic status reduces diabetic complications, the effect of CT99-treatment on cardiomyocyte function in diabetic SD rats was examined. **Figure 25** shows cardiomyocyte contractility in response to exposure to phophatidic acid (PA). Active cell shortening in ND animals is increased approximately 20% from basal levels with PA perfusion over 15 mins. of observation. Cardiomyocytes from D animals display an inhibition of active cell shortening at all time points. Contractility is reduced to 75% of pre-PA levels and this inhibition is statistically different from ND animals at all time points. Treatment of diabetic rats with CT99 completely normalized cardiomyocyte function while cardiomyocytes from insulin-treated diabetic rats, more closely resemble the D animals response. CT99-treated animals had statistically improved contractility vs. both D and insulin-D animals. Resting cell lengths before and after PA treatment of the same cardiomyocytes were also measured and this data is shown in **Figure 26.** Both ND and CT99-D resting cell lengths remain unchanged with treatment, while the cell lengths in insulin-D and D cells are reduced with PA treatment. This is statistically significant at 6 mins of treatment where insulin-D cells are a full micron shorter than ND cells.

Cardiac contractility is closely associated with Ca^{2+} homeostasis. As such we measured the effects of CT99-treatment on Ca^{2+} transients in response to PA perfusion **(Figure 27)**. Both ND and CT99-D cells responded to PA administration with an approximately 20% increase in intracellular Ca^{2+} as measured by Fura-2. Conversely,

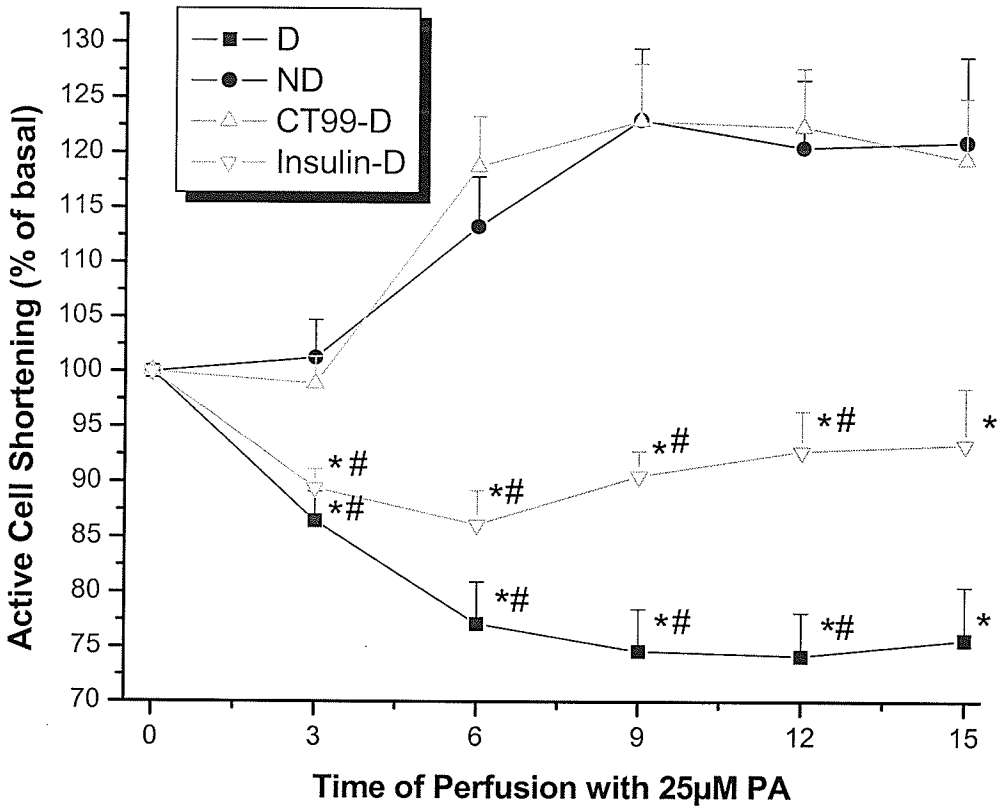


Figure 25. Effects of phosphatidic acid (PA) on cardiomyocyte contractility in non-diabetic (ND), diabetic (D), CT99-treated diabetic (CT99-D) and insulin-treated diabetic (insulin-D) Sprague Dawley rats. Data represents mean ± SE of n = 12, 10, 11 and 4 respectively. * $P < 0.05$ vs. ND and # $P < 0.05$ vs. CT99-D.

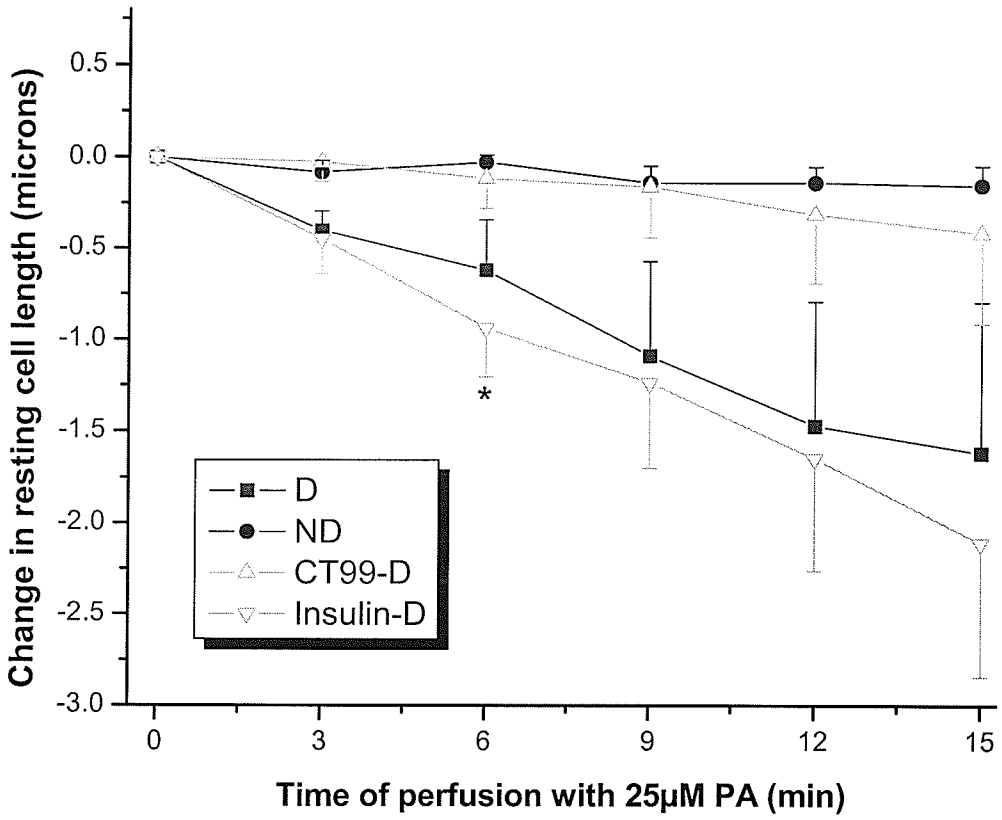


Figure 26. Effects of phosphatidic acid (PA) on cardiomyocyte resting cell length in non-diabetic (ND), diabetic (D), CT99-treated diabetic (CT99-D) and insulin-treated diabetic (insulin-D) Sprague Dawley rats. Data represents mean ± SE of n = 12, 10, 11 and 4 respectively. * $P < 0.05$ vs. ND.

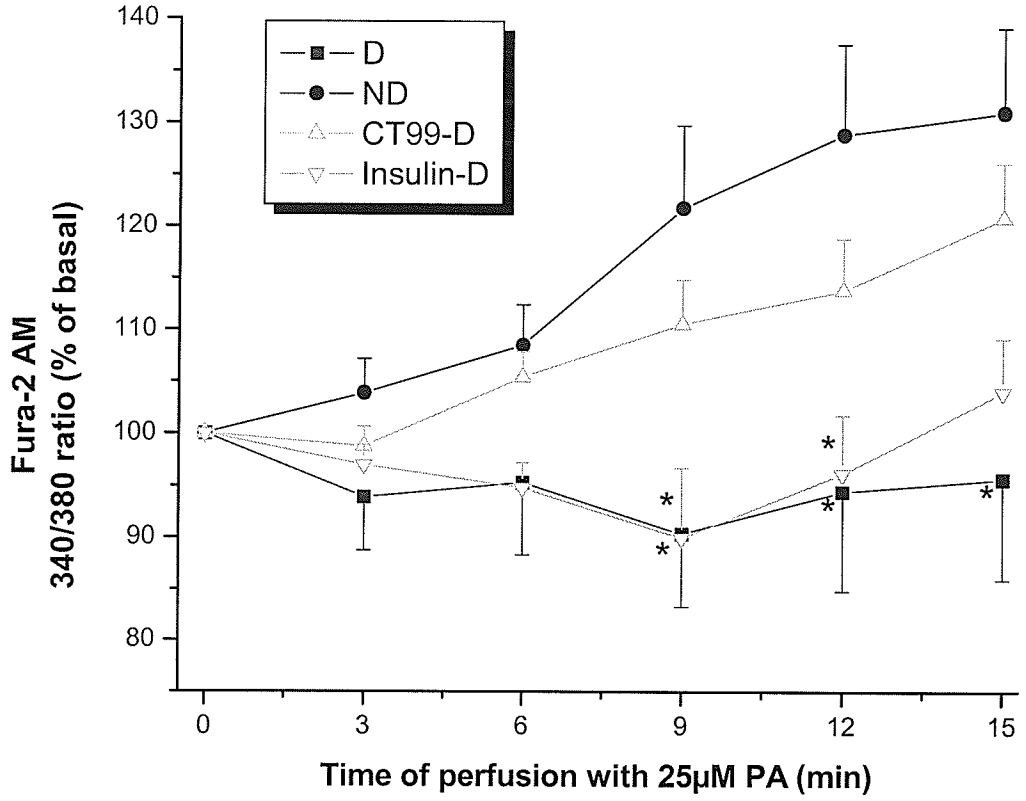


Figure 27. Effects of phosphatidic acid (PA) on cardiomyocyte intracellular Ca^{2+} levels in non-diabetic (ND), diabetic (D), CT99-treated diabetic (CT99-D) and insulin-treated diabetic (insulin-D) Sprague Dawley rats. Data represents mean ± SE of n = 13, 9, 6 and 15 respectively. * $P < 0.05$ vs ND.

both D and insulin-D cells show reduced intracellular Ca^{2+} and this was significantly depressed vs. ND animals at later time points.

A group of CT99-treated SD rats were used for an electron microscopic analysis of the effects of CT99 administration on pancreatic ultrastructure. As noted in the Materials and Methods, following the study the EM analysis was conducted in a blinded fashion to reduce bias (group1 = ND, group 2 = D, and group 3 = CT99-D). The pancreatic EM sections were analysed to examine the effects of both STZ and CT99-treatment on B-cell structure and therefore insulin production capability **(Figures 28-30).** ND sections reveal active B-cells with complete absence of necrotic tissue within the islets **(Figure 28)**. By contrast, D pancreatic sections reveal near complete necrosis of the islets with severe damage to most B-cells. Arrows indicate scarce numbers of remaining hyperactive B-cells **(Figure 29)**. CT99-D animals were made diabetic with intravenous STZ injections but subsequently treated with the CT99 compound. These pancreas images show characteristics of both hyperactive B-cells and necrotic tissue **(Figure 30)**. However, it appears an increased frequency of normal and hyperactive B-cells is apparent when compared to the D images.

Although the results of our pilot study suggest CT99 is effective as an agent in the treatment of Type 1 diabetes, it is necessary to prove the absence of side effects after longer periods of treatment with the vanadium containing CT99 compound. Therefore we followed up this three month study with a year long trial of CT99 in STZ-induced diabetic SD rats. Both ND and CT99-D animals were monitored daily for a full year and again treated only if glucose values rose above 10 mM. Diabetic control (D) animals however, were followed daily for 10 weeks at which time these animals were sacrificed

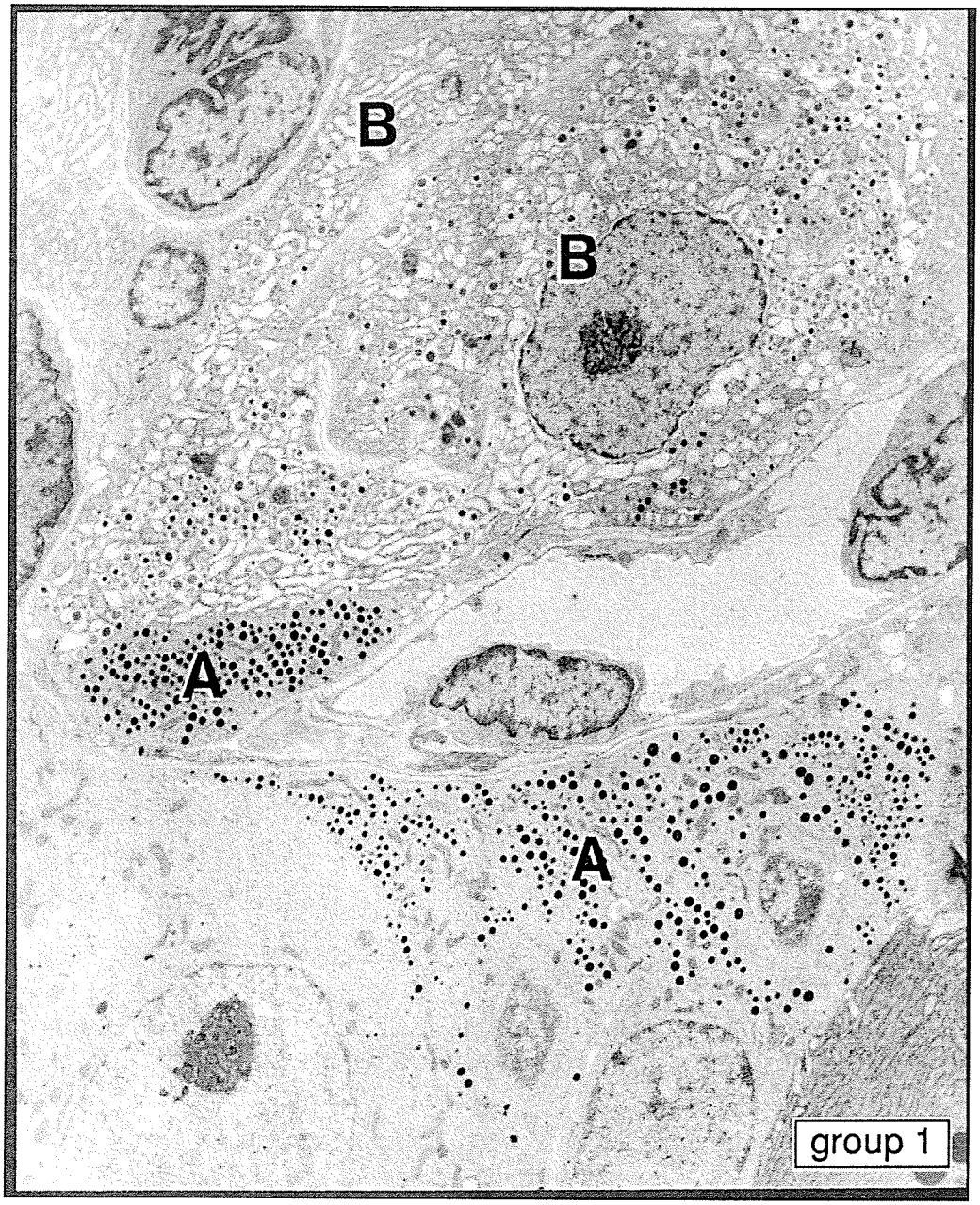


Figure 28. Electron microscopic image of pancreatic section from non-diabetic (ND) SD rat. B = ß-cell, A = α-cell.

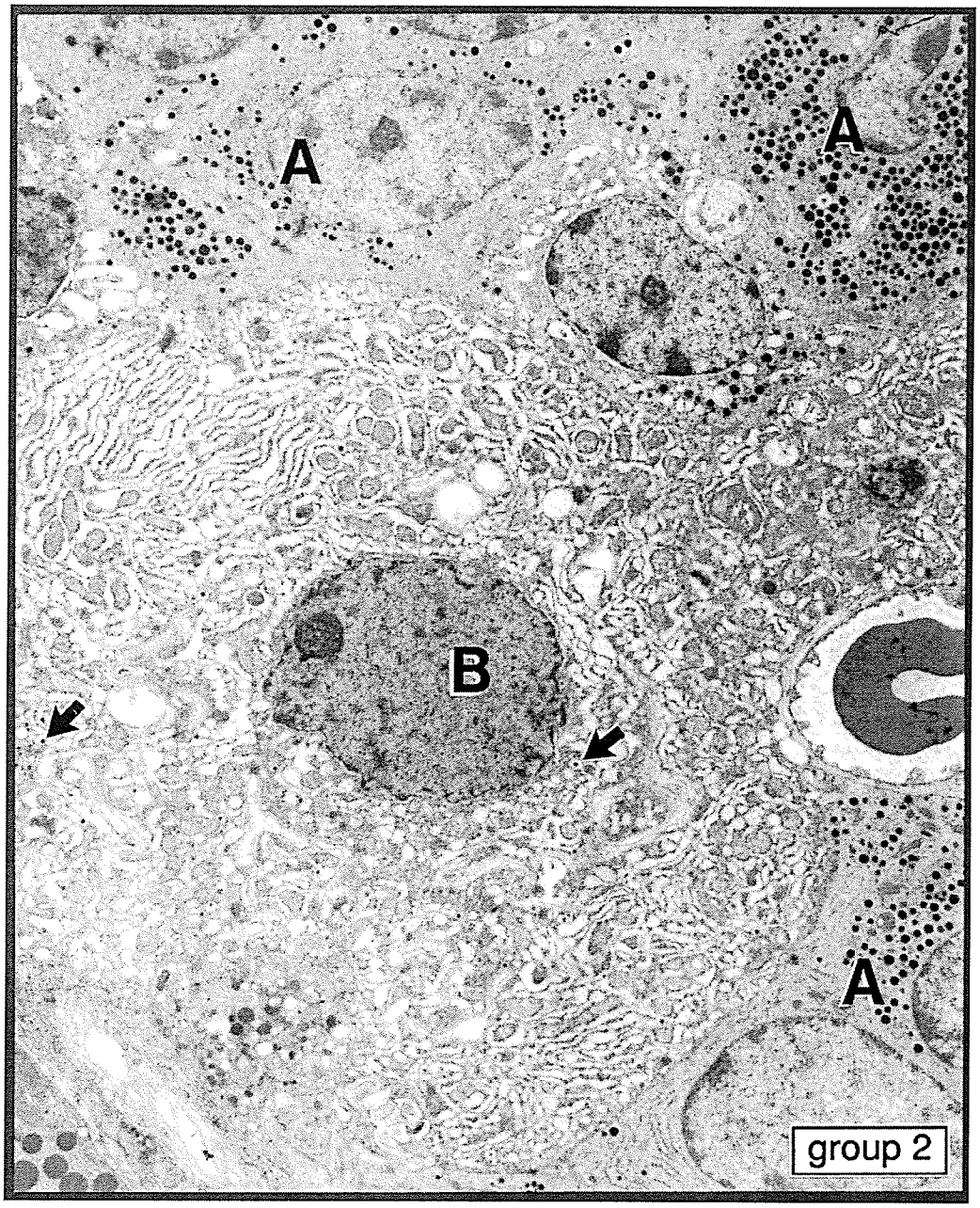


Figure 29. Electron microscopic image of pancreatic section from diabetic (D) SD rat. B = ß-cell, A = α-cell. Arrows display hyperactive ß-cells.

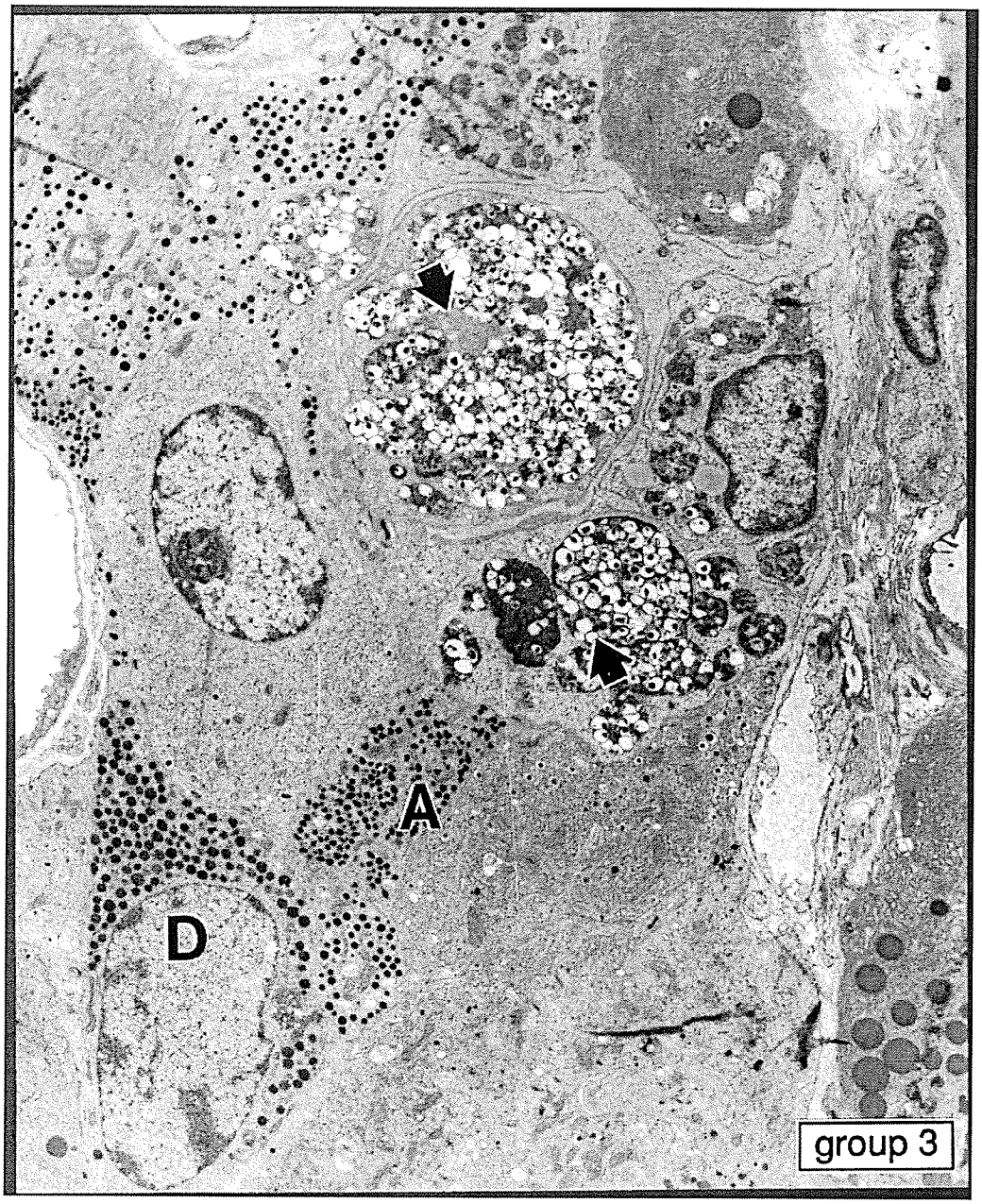


Figure 30. Electron microscopic image of pancreatic section from CT99-treated diabetic (CT99-D) SD rat. D = δ-cell, A = α-cell. Arrows display areas of hyperactive ß-cells.

because of deteriorating health as assessed by the animal care and veterinary staff in the animal care facility at St. Boniface Hospital Research Centre. A group of ND animals were also sacrificed at 10 weeks to monitor the severity of the diabetic state in the D animals at 10 weeks. These treatment groups are displayed in **Table 4**.

An account of the general health of all animals in the study is shown in **Figure 31**. All data is reported as the percentage of animals in each treatment group experiencing the health concern. Over the full year only 6.6% of ND animals experienced diarrhea while 75% of their D counterparts had diarrhea in only 10 weeks of monitoring. CT99-treatment reduced the occurrence of this toxicity to only 20%. Since ND animals at 10 weeks had no diarrhea, it might be expected that over a full year of monitoring rates of diarrhea in untreated D animals would only rise higher. Post-mortem examination of major organ systems found CT99-treatment did not increase the occurrence of internal tumor or cyst development. Both ND and CT99-D animals at one year were found to contain growths in approximately 45% of animals. At 10 weeks neither ND nor D animals showed evidence of growth development. External dermatological examination revealed evidence of lesions (erythema, dryness, scaliness, localized hair loss) in very few ND and CT99-D animals (6.6% and 4% respectively) at one year while 10-week-old animals in both the ND and D groups were free of these conditions. Visible cataract formation appeared in less than 10% of ND animals at one year while nearly 60% of D rats at 10 weeks showed cataracts. CT99-treatment completely eliminated the occurrence of cataract formation at one year. Finally, mortality rates were assessed as 13.3% and 16% in one year ND and CT99-D animals respectively. No premature deaths occurred in either of the 10-week rat groups.

Table 4. Animal treatment groups for 1 year trial of CT99 in Type 1 diabetes.

Group Title	Specifications	n
ND 10 weeks	Control non-diabetic SD rats periodically gavaged with water for 10 weeks	13
D 10 weeks	Control diabetic SD rats periodically gavaged with water for 10 weeks	12
ND 1 year	Control non-diabetic SD rats periodically gavaged with water for 1 year	15
CT99-D	20 mg/ml vanadate/nutraceutical treated diabetic SD rats for 1 year	27

ND = Non-diabetic, D = Diabetic, SD = Sprague Dawley rats

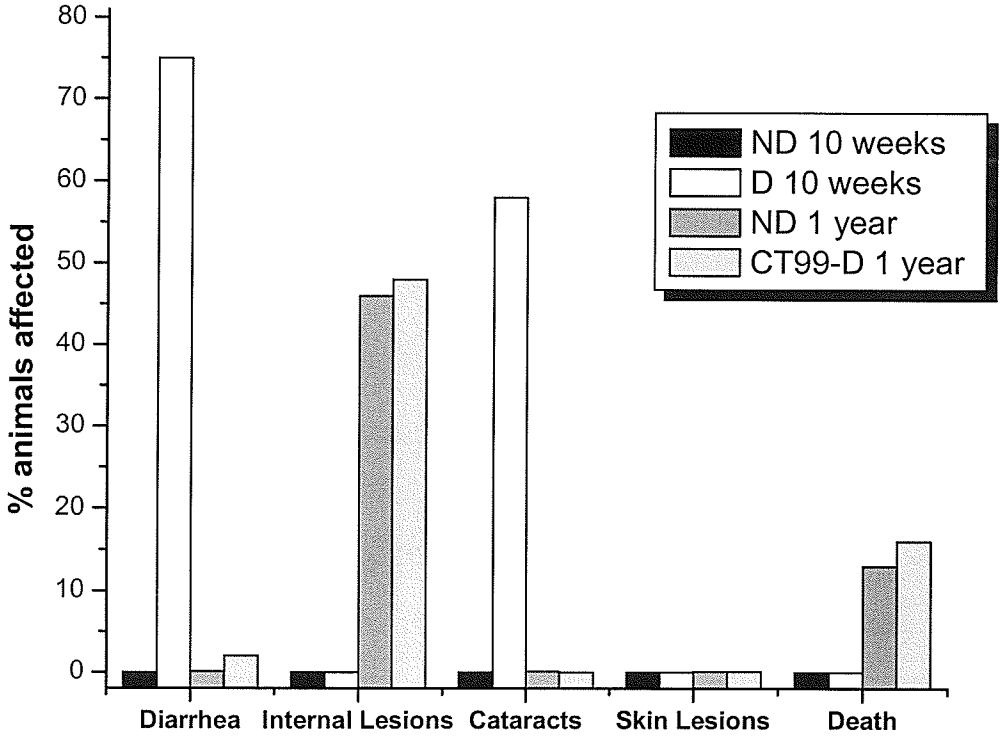


Figure 31. Health summary of animals in year long trial of CT99. Data represents % of non-diabetic (ND), diabetic (D) and CT99-treated diabetic (CT99-D) animals experiencing side effects over the term of each study. n = 13 (ND 10 weeks), 15 (ND 1 year), 12 (D) and 25 (CT99).

In this study three different sodium orthovanadate solids were used with the nutraceutical solution to prepare CT99 and these have been denoted CT99-Da, CT99-Db and CT99Dc. The three CT99 solutions differ only in the lot number of sodium orthovanadate supplied by Sigma Aldrich Canada Ltd. (Oakville ON). Thus CT99-Da was prepared from sodium orthovanadate developed by the supplier at different times from the CT99-Db and CT99-Dc solutions. The orthovanadate in these separate lot numbers was very different in terms of purity. The previous data **(Figure 31)** represented the overall data using all of these solutions. **Figure 32** shows the occurrence rates of diarrhea and death in each of these treatment groups individually. CT99-Da treated rats experienced no diarrhea or death, both of which are improvements upon the ND occurrence rates (6.6% and 13.3%). Conversely, CT99-Db and CT99-Dc treatment resulted in higher rates of diarrhea and death than found in ND rats. We have combined all of the data from the three CT99 solutions to make a fairer representation of the use of vanadium in CT99 in the treatment of these rats. Therefore, all further data will represent a combination of the three sodium orthovanadate treatment groups (a, b and c).

Glycemic status monitored daily for the full year is shown in **Figure 33**. As expected ND animals' blood glucose levels fluctuated only slightly daily and remained between 4 and 8 mM. Conversely, the D blood glucose values began at 21.9 mM and rose during the 10-week period to 27.7 mM. Following STZ treatment, CT99-D rats began the study in a similar glycemic range (21.7 mM). With CT99-treatment however, the glucose levels quickly normalized and remained around 8 mM for the first half of the study at which point an even further reduction to ND levels was achieved. For the final 150 days of the study, glycemic levels in ND and CT99-D rats were nearly identical (~ 5mM).

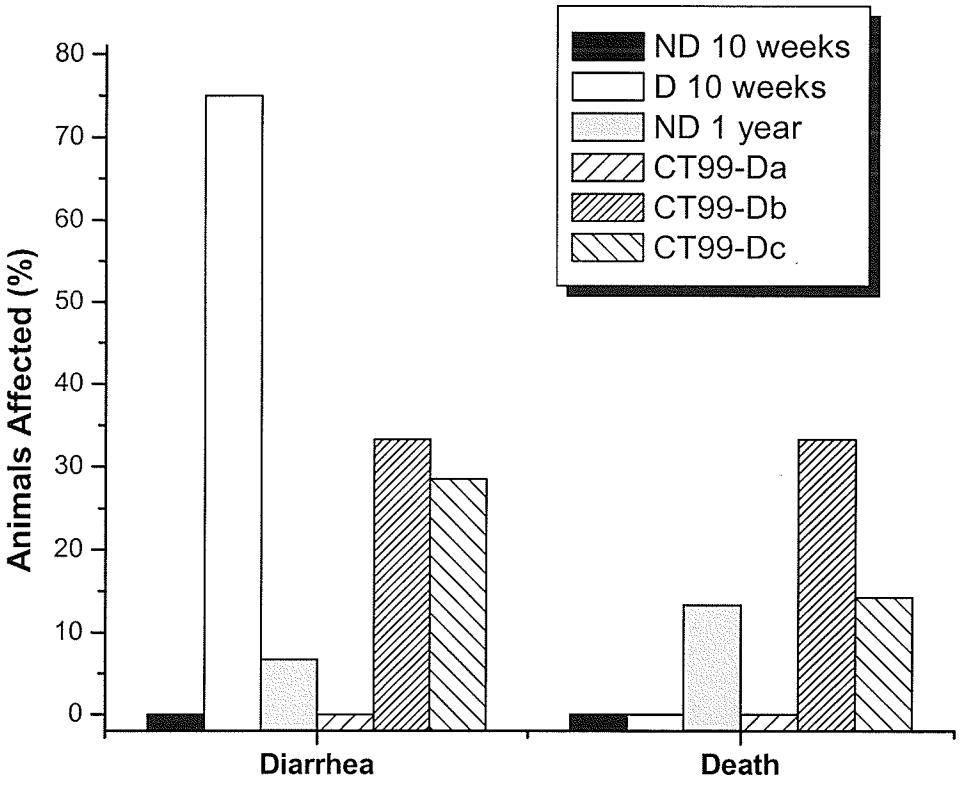


Figure 32. Health summary of diabetic SD rats treated with different CT99 solutions for 1 year. Data represents percent of animals affected in ND 1 year, D, ND 10 weeks, CT99-Da, CT99-Db, CT99-Dc with n = 13, 12, 15, 9, 9, 9 respectively.

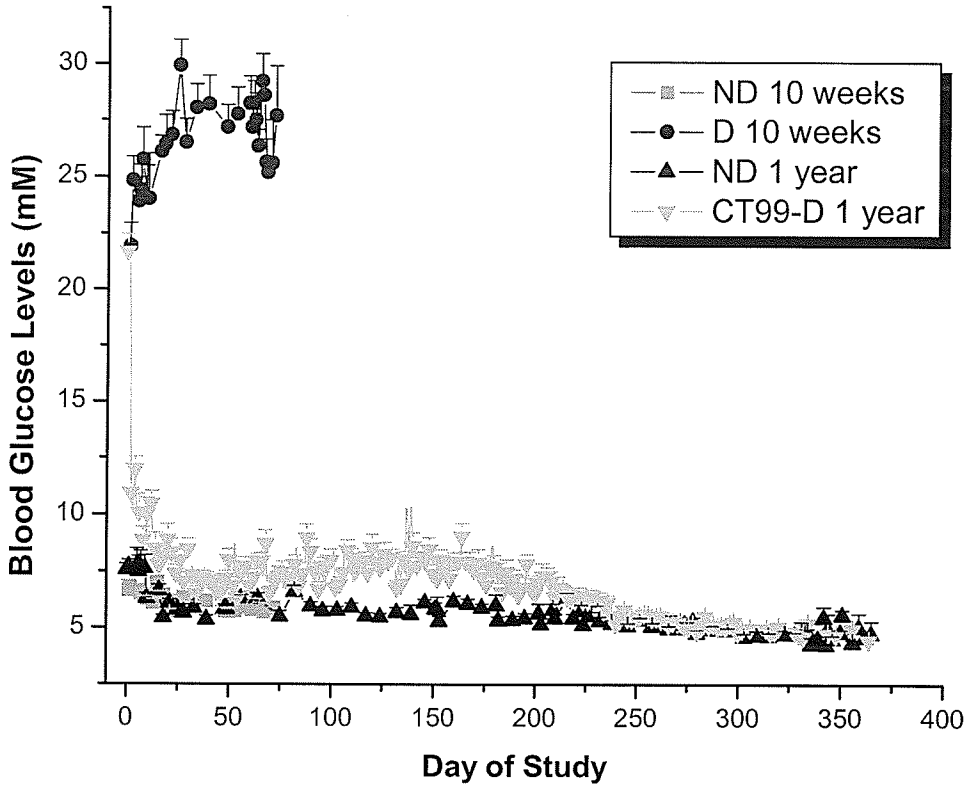


Figure 33. Effect of CT99 treatment on blood glucose in diabetic SD rats treated for one year. Values represent mean ± SE of n = 13, 12, 15, 25 in ND 1 year, D, ND 10 weeks and CT99-D respectively.

For long-term control of blood glucose levels, a more accurate measurement is glycated hemoglobin levels. These were also measured and are reported in **Figure 34.** Glycated hemoglobin in ND rats was 2.91 ± 0.10 %. In comparison, the percentage of hemoglobin that was glycated in D animals was significantly higher at 11.31 ± 0.31 %. CT99-treatment normalized this to 3.09 ± 0.09 %.

A final assessment of glucose tolerance is shown in **Figure 35**. In a glucose tolerance test the blood glucose level in response to a glucose load is measured over 6 hours. At all time points assessed, the blood glucose levels in D rats were significantly higher than both ND and CT99-D animals. Once again, CT99-treatment completely normalized the glucose tolerance of diabetic rats.

Further evidence of glycemic control in CT99-D animals is evidenced by the reduction in the number of treatments required to maintain glucose levels lower than 10 mM. As shown in **Figure 36** every animal required treatment at least once in the first month to lower blood glucose. However, by only the second month, less than half of all diabetic animals in the CT99-D group required a treatment to induce normoglycemia. Over the next several months, the percentage of rats needing a dose of CT99 fluctuated between 48 and 84 %. By the eight month of the study this number was reduced to 16 % and over the final 3 months of the study no animals required CT99 treatment to maintain their blood glucose levels at a normal level. In other words, all the CT99-treated diabetic rats maintained their blood glucose levels below 10 mM for the final three months of the study without receiving any treatment at all.

Another way of depicting the long-lasting effects of CT99 is also shown in **Figures 37-39**. Normoglycemia was maintained with fewer than 200 treatments in the

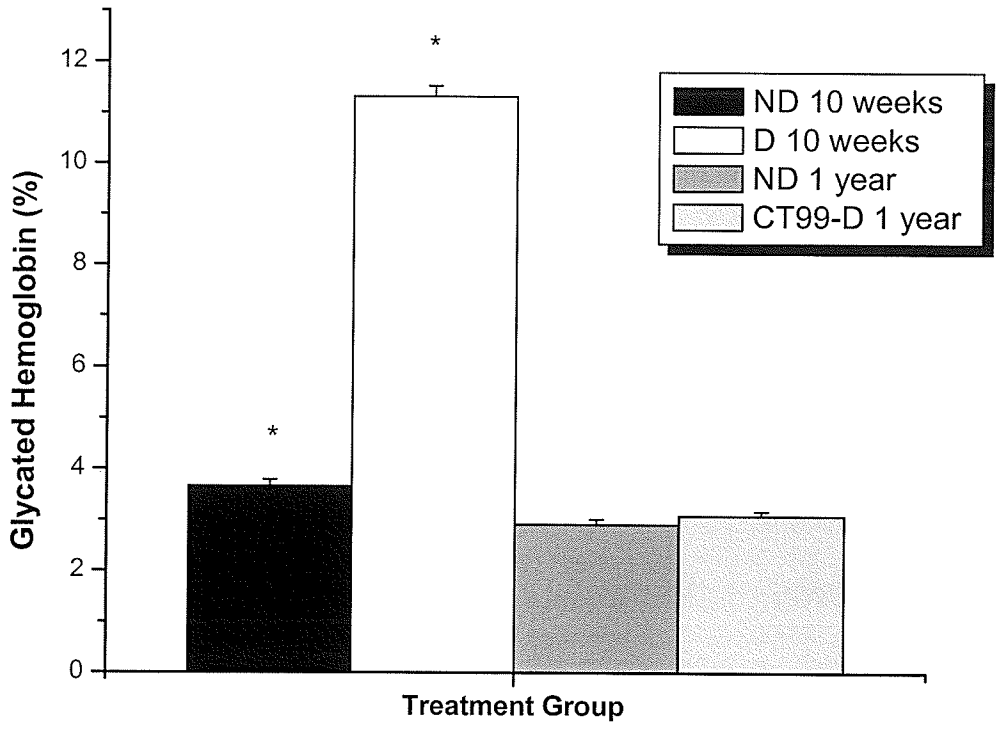


Figure 34. Effect of CT99 treatment on glycated hemoglobin in diabetic SD rats treated for 1 year. Data represents mean ± SE of n = 12, 10, 13, 17 in ND 1 year, D, ND 10 weeks and CT99-D respectively. * $P < 0.05$ vs. ND 1 year.

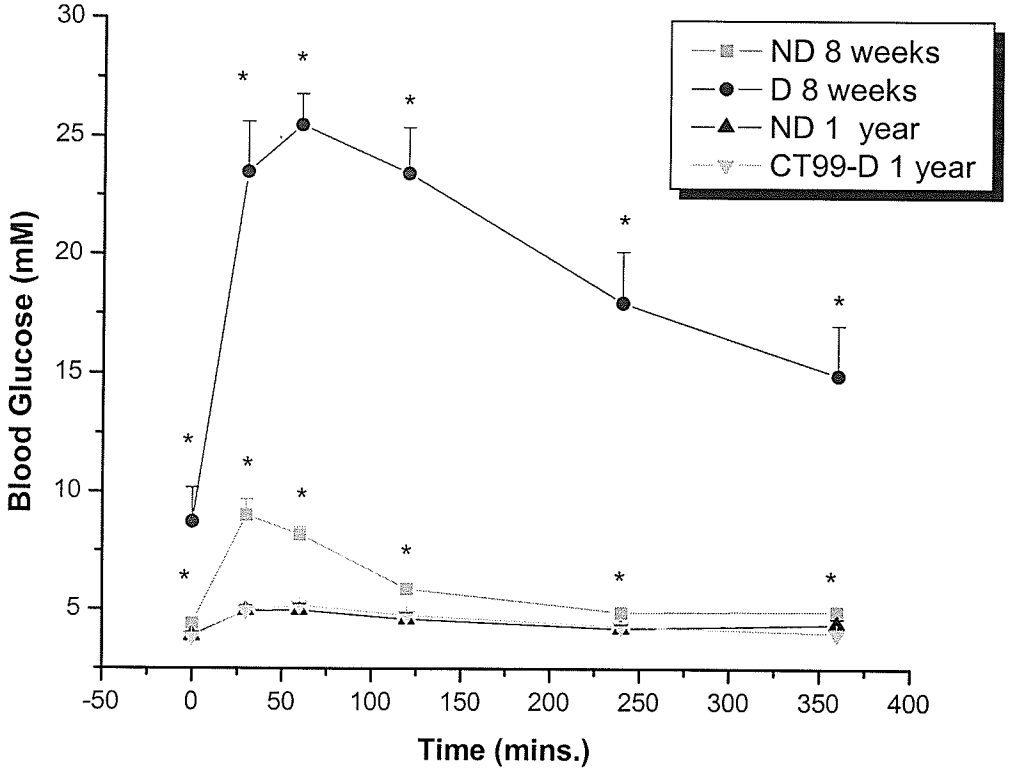


Figure 35. Effect of CT99 treatment on glucose tolerance in response to a _ load of glucose in diabetic SD rats treated for 1 year. Data represents mean ± SE of n = 8, 12, 12, 23 in ND 1 year, D, ND 10 weeks and CT99-D respectively. * $P < 0.05$ vs. ND.

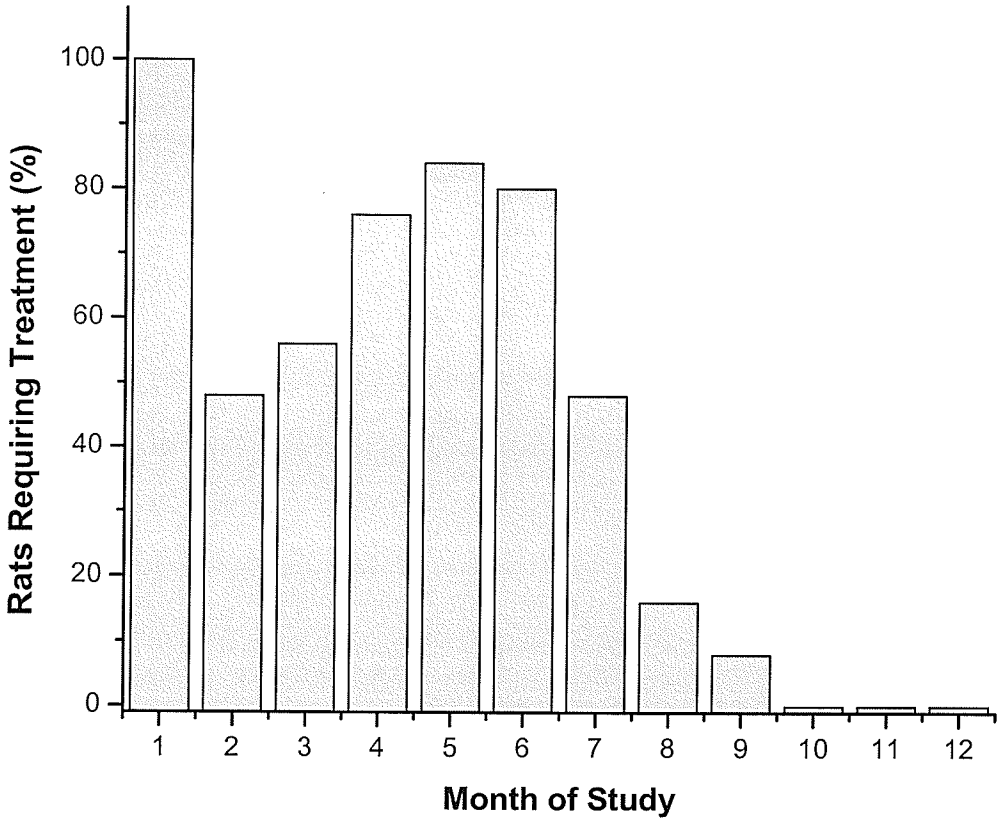


Figure 36. Percentage of diabetic SD rats requiring CT99-treatment monthly to maintain normoglycemia, n = 25.

first month of the study **(Figure 37)**. Recalling the n = 25, this amounts to less than eight doses of CT99 per rat per month to maintain blood glucose levels < 10 mM. Over the second to fourth months approximately 70 treatments were required per month and therefore less than three treatments with CT99 per month were required for normoglycemic status. Small fluctuations occured over the fifth to seventh months but from the eighth month onward, treatment with CT99 was almost unnecessary to control blood sugar status. Only 8 treatments were required in the ninth month and these were the last doses of CT99 administered.

The glycemic control of individual animals is expressed in **Figure 38**. The total number of treatments administered over one year ranged from three to 65 per rat. The mean was 26.48 ± 3.99 treatments. Therefore, animals were on average gavaged twice a month to control diabetic hyperglycemia. This data does not incorporate the improved glucose control over time. As such, **Figure 39** shows the average percentage of days that animals required CT99 during the first and second halves of the one-year trial. Over the initial six months, CT99 was administered only 14 % of the days to control blood sugar. However, the long-term efficacy of CT99 in diabetes therapy is accentuated over the second half of the study. Diabetic animals only required treatment 1.1 % of the days during the second six months of the study.

Both food and water intake were monitored throughout the experiment **(Figures 40 and 41)**. The first assessment of water intake was performed at 15 weeks. ND intake measured 99.75 ± 4.55 ml/day **(Figure 40)**. By comparison water intake in D animals was significantly elevated even at the 6-week time-point (175.00 ± 9.45 ml/day). With significantly lower body weights at this interval, the D increase is even more notable.

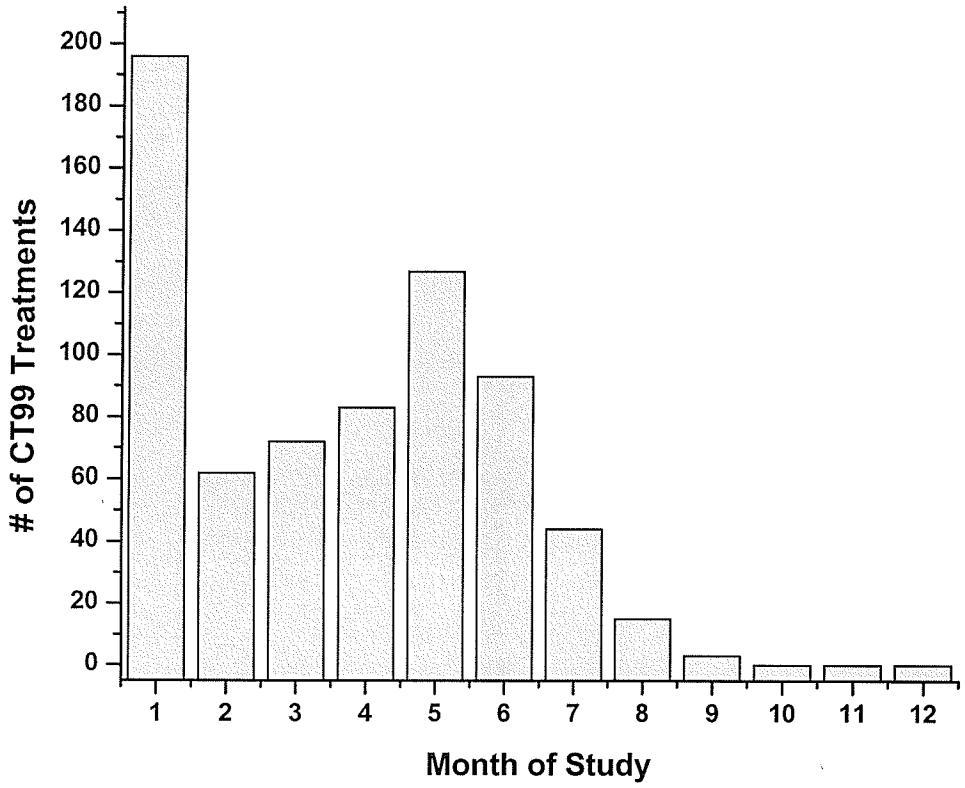


Figure 37. Total number of CT99-treatments required per month to maintain normoglycemia in the 25 diabetic SD rats.

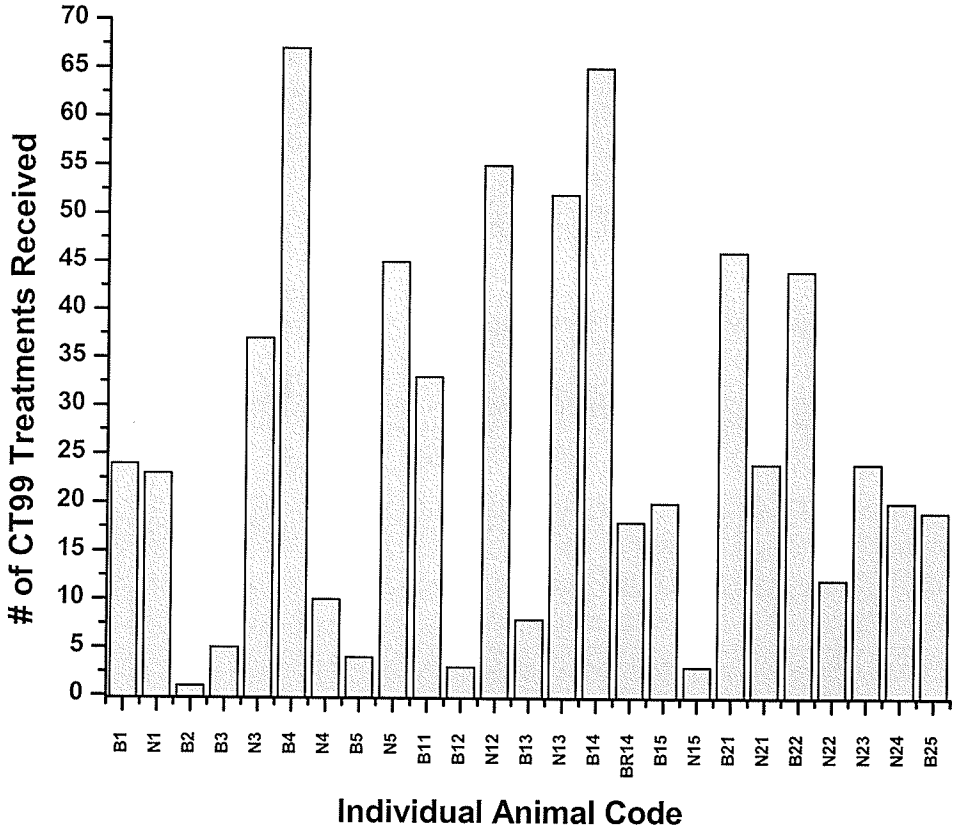


Figure 38. Total number of CT99-treatments received by each SD rat over 1 year of study, n = 25.

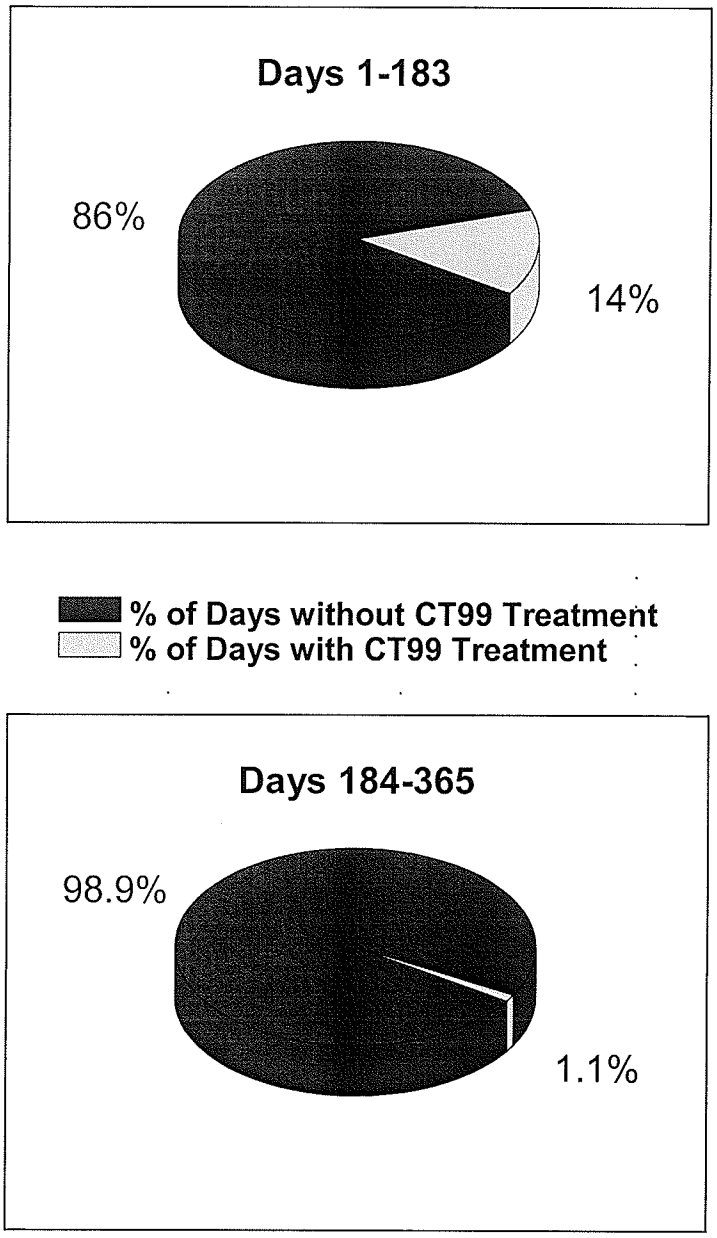


Figure 39. Days requiring treatment for diabetic SD rats treated with CT99. Data represents mean of n = 25 animals for first and last half of study. Diabetic animals were only administered CT99 if their blood glucose concentration was > 10 mM.

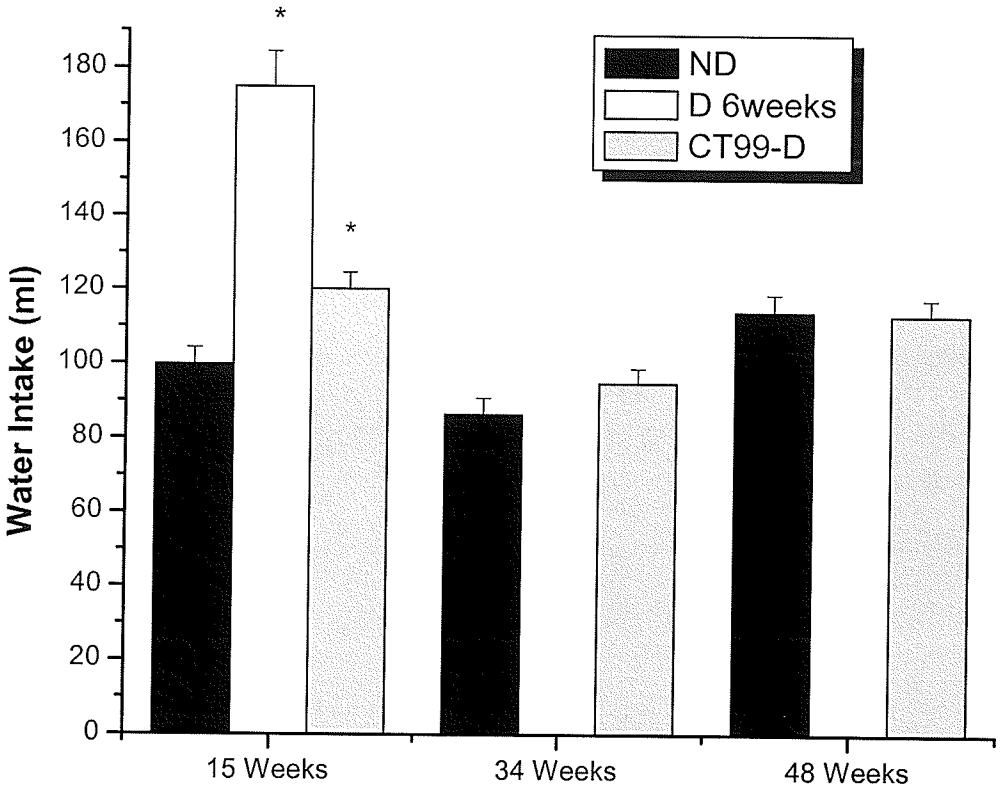


Figure 40. Effect of CT99 treatment on water intake in diabetic SD rats treated for 1 year. Data represents mean ± SE of n = 16, 5, 25 in ND, D and CT99-D respectively. * $P < 0.05$ vs ND.

CT99-D water intake was reduced significantly from the D intake but at 15 weeks remained higher than the ND animals. However, water intake differences between ND and CT99-D groups were not significantly different at either the 34 or 48-week measurements.

Food intake was similarly different amongst the groups **(Figure 41).** Once again, the 6-week D rats (45.58 ± 0.66 g/day) showed significantly elevated food intake vs. the ND rats at 15 weeks (59.19 ± 2.42 g/day) and although CT99-D food intake (58.87 ± 1.01 g/day) was elevated vs. ND, it was significantly lower than the D rats. However at both 34 and 48 weeks the differences between ND and CT99-D food intake was absent. Thus, both water and food intakes were normalized in CT99-D animals over the course of the study.

To augment the food and water intake data, we also monitored the weights of all animals during the study **(Figure 42).** All animals began the study at the same weight, approximately 260 g. However, during the first 60 days the ND animals gained weight at a rate of 4.26 g/day while the D animals only gained 1.10 g/day although eating and drinking significantly more. CT99-treatment of D rats restored a more normal metabolic profile and during this same time frame these animals increased body weight at a rate of 3.44 g/day. Throughout the majority of the study the ND and CT99-D rats paralleled each other with respect to weight gain. As expected, the diabetic animals (CT99-D) had significantly lower body weights. The trend toward CT99-D animals' weights approaching the ND rats continued throughout the study and by year end the two groups had statistically similar weights (815.11 ± 19.92 g and 859.3 ± 28.51 g respectively).

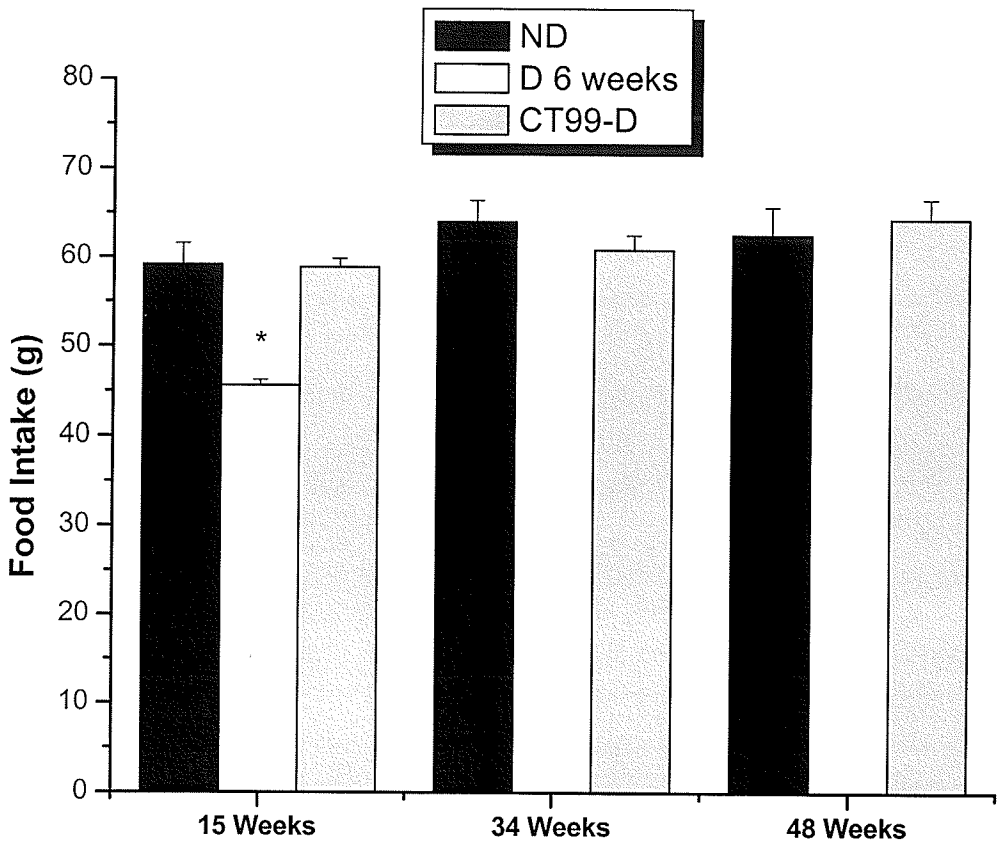


Figure 41. Effect of CT99 treatment on food intake in diabetic SD rats treated for 1 year. Data represents mean ± SE of n = 16, 5, 25 in ND, D and CT99-D respectively. * $P < 0.05$ vs ND.

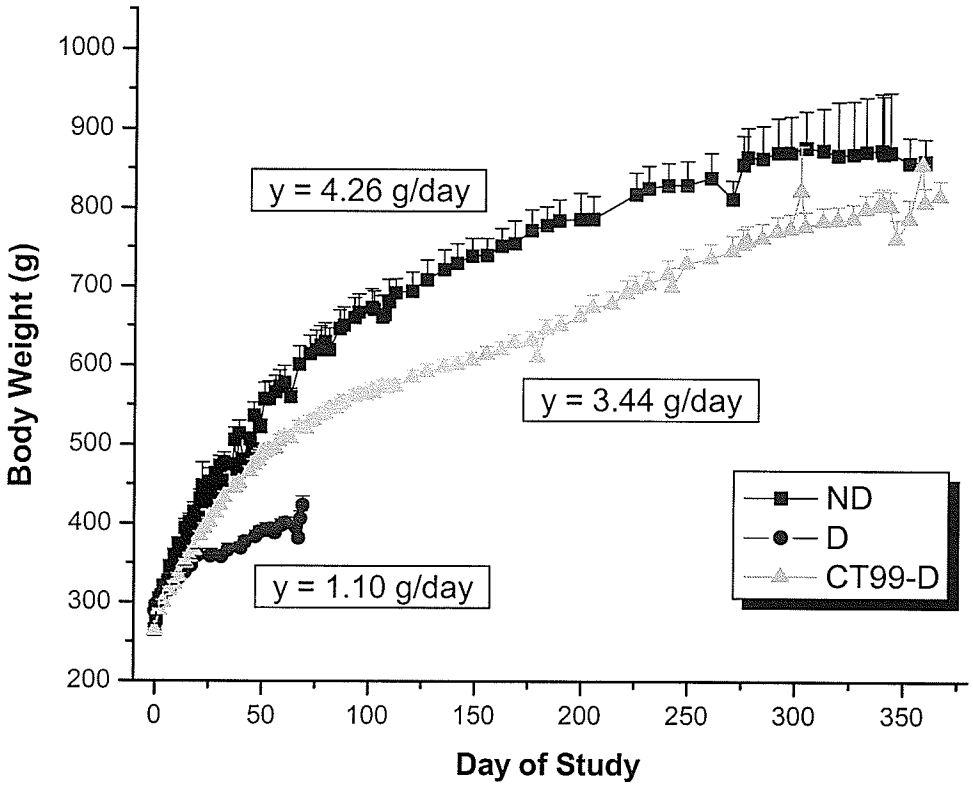


Figure 42. Effect of CT99 treatment on body weight in diabetic SD rats treated for 1 year. Data represents mean ± SE of n = 15, 12, 25 in ND, D and CT99-D respectively while y = rate of weight gain from day 7 to 67.

CT99-treated animals were analyzed for several biochemical parameters to assess potential toxicity **(Table 5)**. Neither D nor CT99-D rat serum albumin levels differed significantly from ND animals. Serum alkaline phosphatase levels were not different between ND and CT99-D rats (119.08 ± 12.44 and 121.38 ± 11.16 U/L) but D levels were significantly elevated (574.67 ± 102.80 U/L). For alanine aminotransferase (ALT), untreated diabetic (D) rats had significantly higher levels than either ND or CT99-D animals. The treatment of D animals with CT99 reduced ALT levels vs. ND as well. Diabetic animals (D) also had statistically elevated levels of both serum amylase and aspartate aminotransferase (AST) while CT99-treatment normalized these enzymes. Other significantly altered biochemical parameters in the D rats included blood urea nitrogen (BUN), cholesterol, triglycerides, uric acid, and urine specific gravity while CT99-D animals all had values restored to those of the ND animals. The only parameter measured in which CT99-D animals differed significantly from their ND counterparts was total bilirubin (0.42 ± 0.02 mg/dL vs. 0.35 ± 0.02 mg/dL). Creatinine levels were unchanged amongst the three groups. Serum globulin and total protein levels were elevated in CT99-D vs. D rats but there were no changes vs. ND animals.

CT99-treatment of diabetic animals did result in significant changes to the circulating insulin pool **(Figure 43)**. ND animals at one year had plasma insulin levels of 2.30 ± 0.65 ng/ml. STZ injection resulted in a significant reduction in this level as D animals at just 10 weeks of age revealed insulin levels of 0.38 ± 0.12 ng/ml. CT99-D animals at one year showed insulin levels significantly higher (1.34 ± 0.22) than their D counterparts and not significantly altered vs. the ND animals.

Table 5. Biochemical analysis in 1 year non-diabetic (ND), 10 week diabetic (D), and 1 year CT99-treated Type 1 diabetic (CT99-D) rats.

Assay	ND	D	CT99-D
Albumin (g/L)	2.45 ± 0.08	2.29 ± 0.13	2.59 ± 0.06 #
Alk Phos (U/L)	119.08 ± 12.44	574.67 ± 102.80 *	121.38 ± 11.16
ALT (U/L)	113.67 ± 13.03	291.89 ± 74.42 *	82.24 ± 5.89 *#
Amylase (U/L)	1734.42 ± 80.82	1367.11 ± 113.90 *	1739.81 ± 65.80
AST (U/L)	160.50 ± 14.30	450.89 ± 92.66 *	159.81 ± 9.63
BUN (mg/dL)	19.23 ± 0.94	28.49 ± 1.51 *	20.10 ± 0.98
Chol (mg/dL)	88.60 ± 7.47	61.79 ± 5.43 *	105.94 ± 6.09
Creat (mg/dL)	0.54 ± 0.03	0.43 ± 0.05	0.50 ± 0.03
Globulin (g/dL)	3.30 ± 0.10	3.01 ± 0.16	3.50 ± 0.06 #
Tot Bili (g/dL)	0.35 ± 0.02	0.38 ± 0.03	0.42 ± 0.02 *
Protein (g/dL)	5.75 ± 0.15	5.30 ± 0.28	6.09 ± 0.11 #
TG (mg/dL)	117.03 ± 23.01	32.5 ± 3.01 *	114.59 ± 12.17
U Acid (mg/dL)	1.09 ± 0.20	1.81 ± 0.29 *	1.24 ± 0.22
Urine Sp Grav	1.01 ± 0.001	1.03 ± 0.003 *	1.01 ± 0.002

Alk Phos = alkaline phosphatase, ALT = alanine aminotransferase, AST = aspartate aminotransferase, BUN = blood urea nitrogen, Chol = cholesterol, Creat = creatinine, Tot Bili = total bilirubin, TG = triglyceride, U Acid = uric acid, U Spec Grav = urine specific gravity. Data is reported as mean ± SE of n = 12, 9, 21 in ND, D and CT99-D rats respectively. * $P < 0.05$ vs. ND animals. # $P < 0.05$ vs. D.

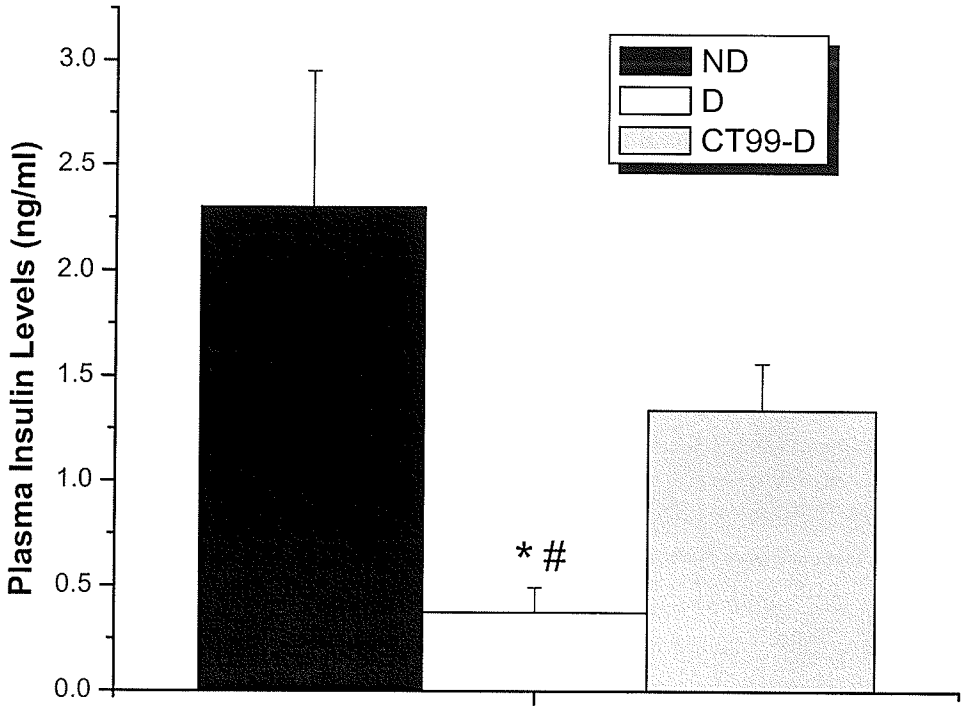


Figure 43. Effect of CT99-treatment on plasma insulin levels in diabetic SD rats treated for 1 year. Data represents mean ± SE of n = 12, 5, 20 in ND, D and CT99-D animals respectively. * $P < 0.05$ vs ND, # $P < 0.05$ vs CT99-D.

ii) Type 2 Diabetes

Greater than 85% of all diabetics are of the Type 2 variety [3]. Therefore, CT99 was tested in a model of this form of diabetes, the Zucker diabetic fatty rat (ZDF). Six animal groups were established as shown in **Table 6.** All animals were monitored for overt signs of vanadium toxicity and the results are shown in **Figure 44**. ND and D control animals not receiving vanadium were free of gastrointestinal (GI) toxicity. Vanadate-D30 treated rats responded to the first vanadium dosage with significant GI toxicity. Fifty % of these animals developed diarrhea within the first 12 hours post-treatment. Comparatively, when administered as CT99, the same 30 mg Na-orthovanadate dose resulted in no apparent toxicity (CT99-D30 rats). The larger 40 mg dose of vanadium is shown to have a dose dependant effect as 100% of vanadate-D40 animals developed diarrhea. CT99 partially alleviated the toxicity with 25% of these animals remaining unaffected (CT99-D40). Mortality rates for all groups are shown in the same figure. No animals in either of the ND or D control groups died during the course of the study. Mortality rates rose significantly when vanadium was administered conventionally in ddH_20. It is shown that the 40 mg dose caused 75 % mortality in a water vehicle. This was lowered to 42 % with CT99 treatment. More significantly, the 30 mg dosage of vanadium administered as CT99 resulted in zero mortality while the vanadate-D30 method of delivery caused a 10% death rate. Due to the toxicity and mortality resulting from vanadate-D40, vanadate-D30 and CT99-D40, further analysis of these treatment groups was discontinued. Further analysis here would have been biased by sample differences.

Table 6. Animal treatment groups for use of CT99 in Type 2 diabetes.

Group Title	Specifications	n
ND	Nutraceutical-treated non-diabetic Zucker rats	4
D	Nutraceutical treated ZDF rats	6
CT99-D40	2 ml of 20 mg vanadate/ ml nutraceutical treated ZDF rats	12
Vanadate-D40	2 ml of 20 mg vanadate/ ml water treated ZDF rats	12
CT99-D30	2 ml of 15 mg vanadate/ ml nutraceutical treated ZDF rats	10
Vanadate-D30	2 ml of 15 mg vanadate/ ml water treated ZDF rats	10

ND = Non-diabetic, D = Diabetic, ZDF = Zucker diabetic fatty rat, n = sample size.

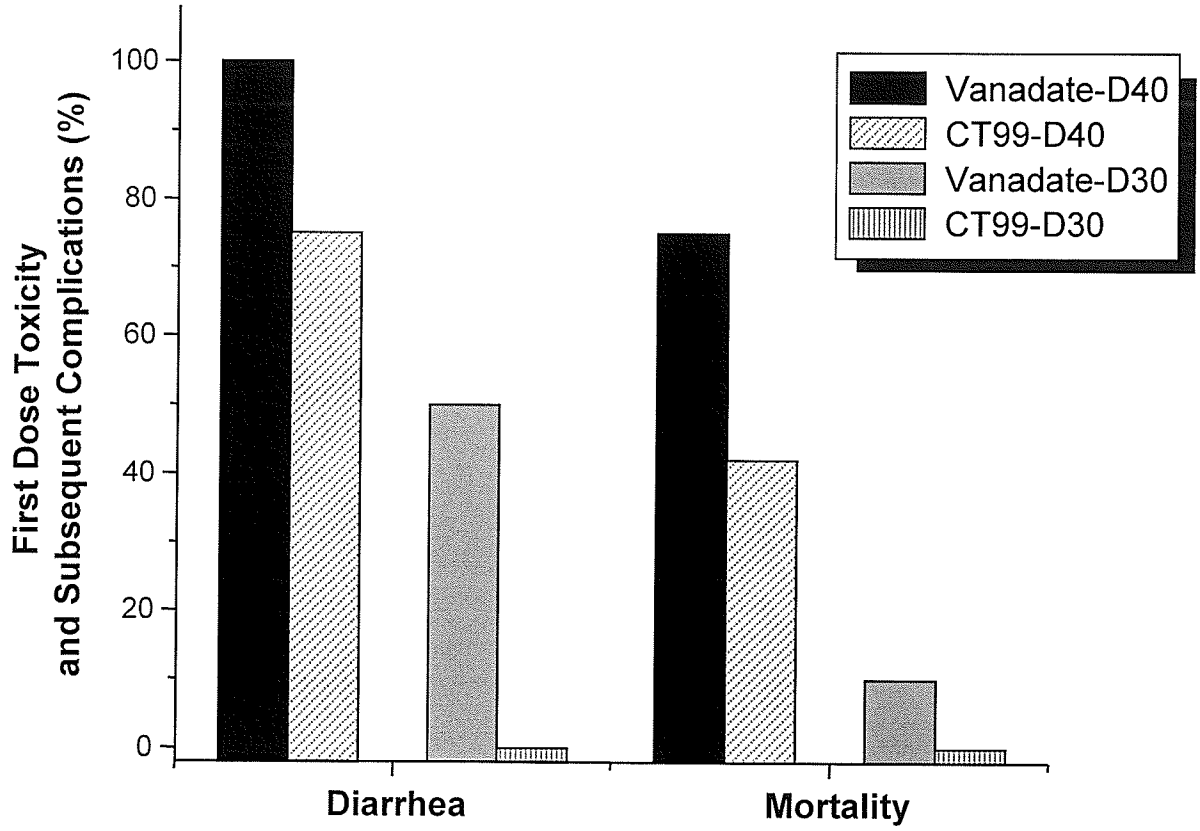


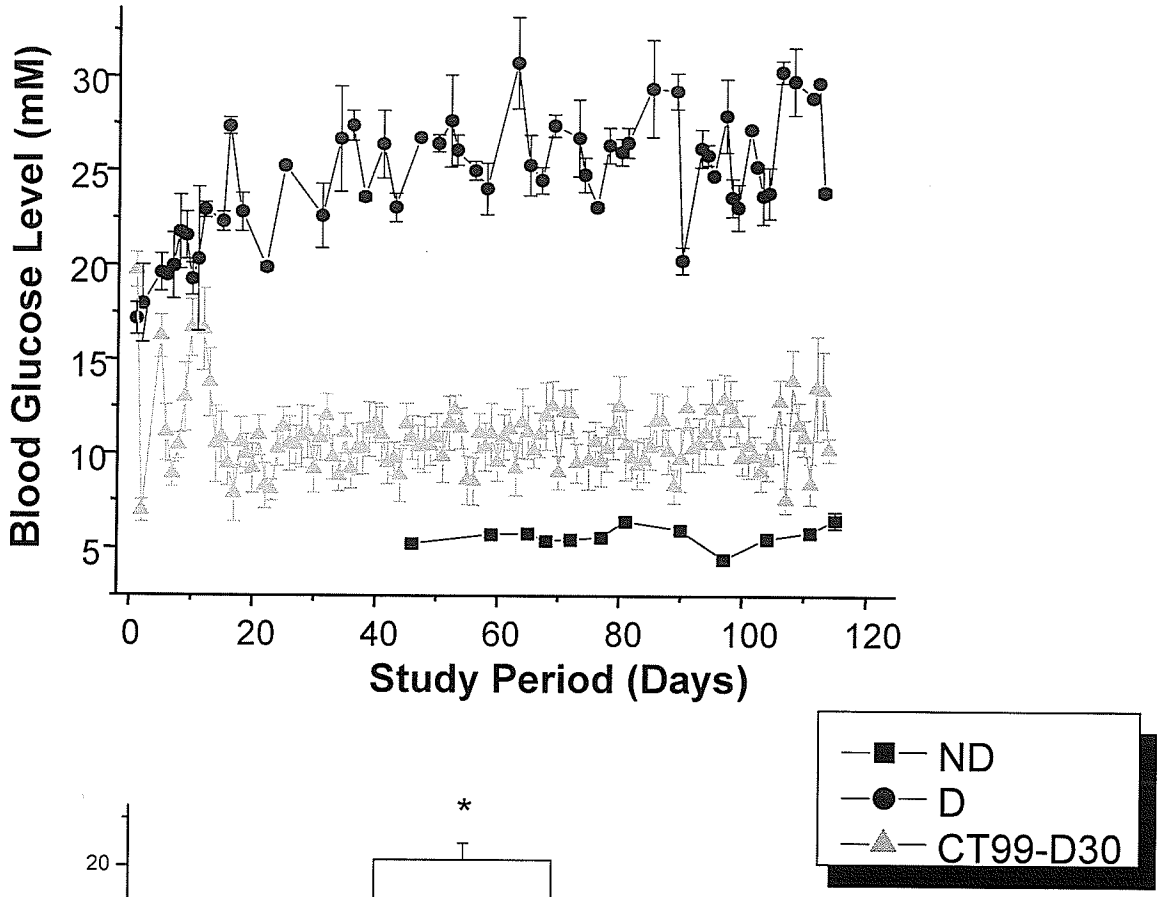
Figure 44. Toxicity and mortality in Zucker diabetic fatty rats orally administered sodium orthovanadate either conventionally in water (Vanadate-D) or as CT99 (CT99-D). Bars represent percent of animals affected in each group at 30 mg and 40 mg vanadium doses.

The effect of CT99-D30 on blood glucose control in ZDF rats is shown in **Figure 45a**. ND Zucker rats were normoglycemic throughout the course of the study with average blood glucose levels of ~ 6 mM. It must be noted that these animals were only available for order at an age equivalent to the ZDF's in our study at 45 days. Therefore these controls were followed from time of arrival (day 45) to the end of the 4 month trial (day 117). D rats not treated with vanadium had significantly higher glucose levels. Blood glucose rose from an initial level of ~ 18 mM to > 25 mM by the end of the study. Prior to treatment, the CT99-D30 animals had an average glucose level of ~ 20 mM. Following the first dose of CT99, the glycemic level was reduced to near control levels of 7 mM and long-term stabilization was maintained over the 4-month study at a glucose level of ~ 10 mM.

Clinically, fasting blood glucose levels are commonly used to assess glycemic control in diabetes. **Figure 45b** shows 12-hour fasting blood glucose in the ND rats equalled 4.42 ± 0.06 mM, while the D animals exceeded 20 mM (20.28 ± 0.70 mM). CT99-30 treatment of D animals significantly lowered fasting glucose levels (6.34 ± 0.25 mM) compared to that of their D complement.

Body weights were monitored throughout the study to assess the effect of vanadium on weight gain **(Figure 46)**. ND animals gained weight at a rate of 1.24 g/day. D animals, not unexpectedly, showed significantly reduced weight gain (0.083 g/day) and remained nearly stagnant over the final 100 days of the study. CT99-D30 treated animals regained a normal anabolic profile with weight gains equalling that of the ND rats (1.22 g/day).

a)



b)

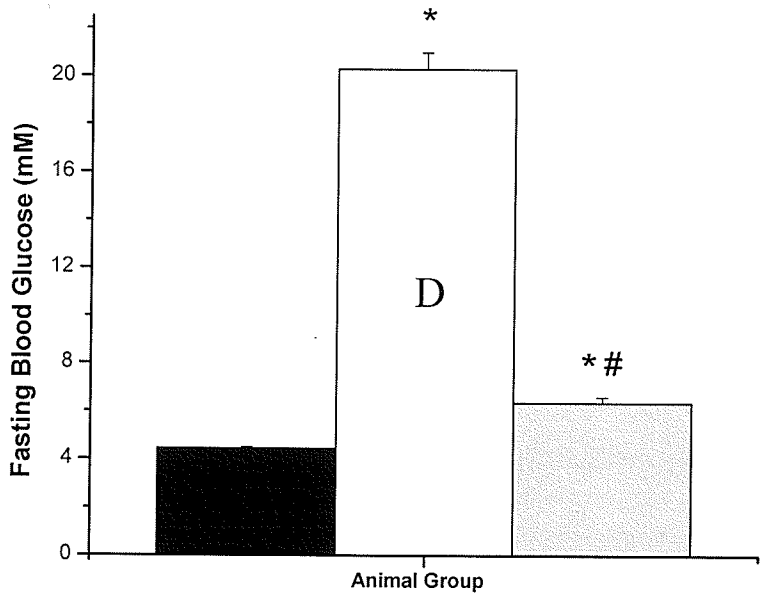


Figure 45. Effect of CT99 on blood glucose in Zucker Diabetic Fatty rats. Daily blood glucose levels (a) and fasting blood glucose levels (b) of non-diabetic (ND), diabetic (D) and CT99-D30 Zucker rats. Values are mean ± SE of n = 4, 6 and 10 animals respectively. * $P < 0.05$ vs. ND. # $P < 0.05$ vs. D.

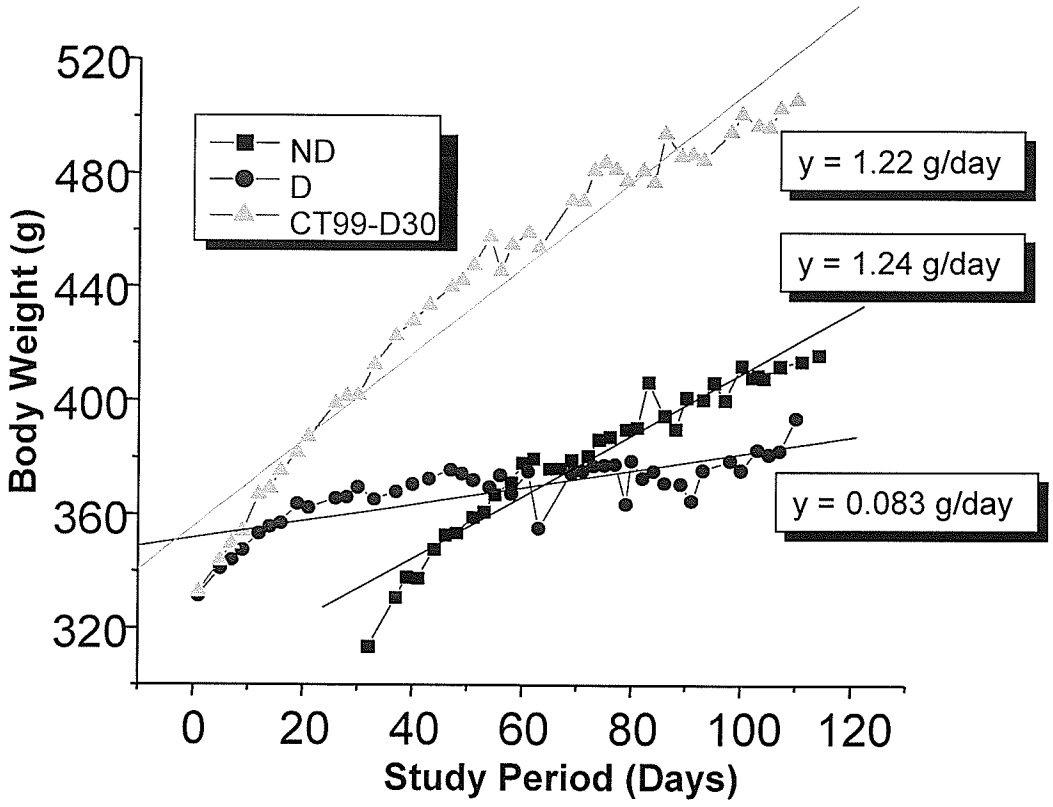


Figure 46. Effects of CT99 treatment on body weight of Zucker Diabetic Fatty rats. Values represent mean ± SE of n = 4, 6 and 10 animals in the ND, D and CT99-D30 animals respectively.

Similarly, both food and water intake were measured during the study **(Figure 47)**. Individual ND rats ingested ~ 24 g of food and 30 ml of water/day. By comparison, D rats consumed ~ 36 g and 130 ml of food and water respectively, both significantly larger than the ND animals. Normalization of food intake occurred with CT99-D30 treatment (~ 20 g). Water intake in these animals was also significantly reduced to ~ 52 ml/day.

Several biochemical indicators of organ toxicity were assessed and the results are shown in **Table 7**. Aspartate aminotransferase (AST) is used as a measure of potential liver toxicity. Diabetic control rats (D) show significantly depressed AST levels (71.60 ± 3.73 U/L) compared to the ND control rats (106.08 ± 19.76 U/L). This was restored with CT99-D30 treatment (92.29 ± 6.53 U/L). Creatinine and blood urea nitrogen (BUN) levels are used as biochemical indicators of renal function/dysfunction. Again, D animals had statistically different levels than their ND counterpart with respect to creatinine levels (0.27 ± 0.01 mg/dl vs. 0.35 ± 0.02 mg/dl respectively). CT99-D30 brought the creatinine levels back to ND values (0.35 ± 0.02 mg/dl). No significant changes were seen in the three groups when BUN was assessed.

Plasma insulin levels in all Zucker animals were also measured **(Table 7)**. ND animals reveal circulating plasma insulin levels of 0.384 ± 0.12 ng/ml while their D counterparts have substantially lower levels (0.172 ± 0.03 ng/ml). The CT99-D30 rats (1.26 ± 0.20 ng/ml) express plasma insulin levels more than 4 times higher than the ND animals and more than 7 times greater than their D complement.

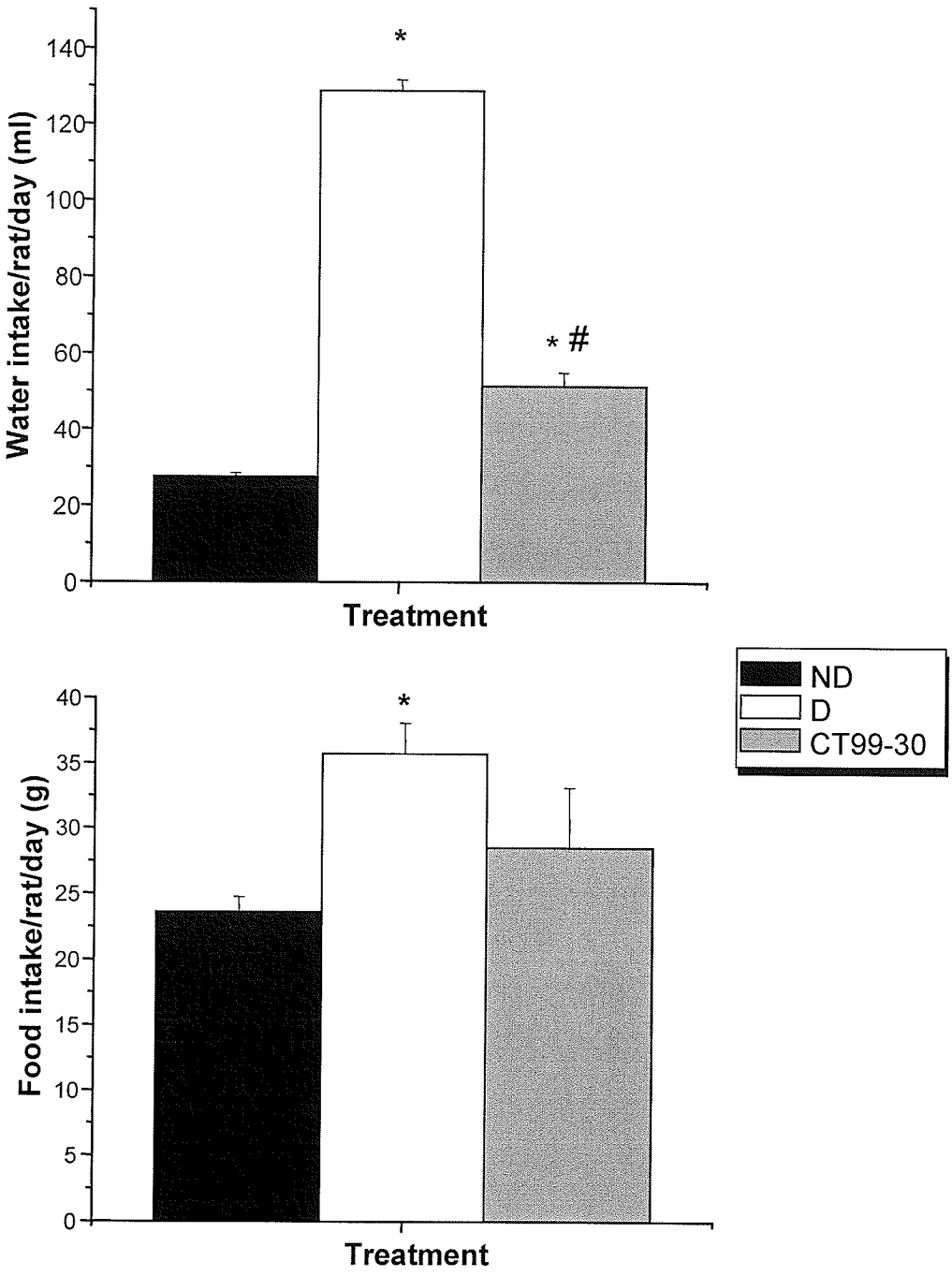


Figure 47. Consequences of CT99 treatment on food and water intake in Zucker diabetic fatty rats. Values represent mean ± SE of n = 4, 6 and 10 of ND, D and CT99-D30 animals respectively.

Table 7. Biochemical analysis in plasma samples from non-diabetic (ND), diabetic (D), and CT99-treated Type 2 diabetic (CT99-30D) rats at 4-month endpoint.

Assay	ND	D	CT99-30D
AST (U/L)	106.08 ± 19.76	71.60 ± 3.73*	92.29 ± 6.53
Creat (mg/dL)	0.35 ± 0.02	0.27 ±0.01*	0.35 ±0.02
BUN (mg/dL)	16.9 ± 1.32	18.35 ± 1.04	20.09 ± 1.08
Insulin (mM)	0.384 ± 0.12	0.172 ± 0.03*	1.26 ± 0.20*

AST = Aspartate aminotransferase, Creat = Creatinine, BUN = Blood Urea Nitrogen. Data is reported as mean ± SE of n = 4, 6 and 10 in ND, D and CT99-30D rats respectively. * $P < 0.05$ vs. ND animals.

DISCUSSION

The ability of vanadium compounds to reduce hyperglycemia in animal models of diabetes was shown 17 years ago by John McNeill [129]. Most laboratories have since attempted to administer vanadium compounds to diabetic animals and humans by suspending these agents in water. The concentration of vanadium necessary to reduce hyperglycemia however is associated with some severe side effects, namely gastrointestinal toxicity and high mortality rates [129,169-177]. Therefore, rather than attempting to chemically modify the vanadium compound itself, we have suspended sodium orthovanadate in a nutraceutical solution known to have anti-diarrheal and beneficial gastrointestinal properties. The resulting compound was named CT99.

First, we wanted to prove that the nutraceutical component of CT99 did not detract from vanadium's potent anti-diabetic hypoglycemic effect. In **Figures 6 and 17**, it is shown that 40 mg of vanadium dissolved in CT99 nutraceutical has an equivalent hypoglycemic action as the vanadate/water solution. Over the entire 24-hour time course the CT99-D rats mimic the glucose level of their vanadate-D treated counterparts **(Figure 6)**. Most importantly, at 16 hours post treatment the CT99 solution was capable of reducing the glycemic level of the diabetic animals to that of a non-diabetic rat (~ 6 mM).

It was hypothesized that CT99 would be able to control glucose without side effects by reducing the amount of vanadium able to cross the gastrointestinal tract lining during passage from the stomach to the sigmoid colon. Measurement of vanadium in blood was therefore assessed and is shown in **Figure 7**. Plasma vanadium levels immediately after administration are similar with both CT99 and vanadate/water

solutions. However, by the 16-hour time point CT99 reduced the uptake of vanadium into blood. Important to note is that this time point corresponds to the that of maximal hypoglycemic action and may therefore play a role in the anti-diarrhea and anti-diabetic effects of CT99.

Although vanadium is known to be an essential element for higher animals its biologic effects are still unknown [91,92]. Normal levels of vanadium in animals are very low (10-100 uM) and therefore the dose of vanadium used in this study is a potential toxicity concern to different organ systems. **Figures 8-15** present the levels of vanadium in these selected organs acutely after one dose of CT99 and vanadate/water. CT99-D treated rats had reduced vanadium levels in five of eight selected organs (bone, kidney, liver, pancreas and heart) compared to vanadate-D treatment, while equal levels were found in the other three organs. The potential for CT99 to reduce vanadium uptake over longer periods of treatment may therefore be a mechanism to reduce toxicity. Dai and colleagues have shown that over a one-year treatment period with vanadium in drinking water, accumulation occurred in a similar fashion to that in this study (bone > kidney > liver...). It must be mentioned that McNeill's group found no organ dysfunction with these vanadium levels [176, 260]. Therefore, since CT99 significantly reduced vanadium levels in five of eight organs vs. vanadate-D after one treatment, the potential benefits of CT99 treatment used long-term may be staggering.

Vanadium usage as a therapeutic clinical agent has been limited to date because of its deleterious side effects. The elimination of both diarrhea and mortality in CT99-D animals at three months suggests that the antidiarrhetic properties of the nutraceutical work synergistically with the hypoglycemic action of sodium orthovanadate **(Figure 16)**.

Over a full year CT99-treatment was asscociated with an extremely low incidence of diarrhea **(Figure 31)** and a mortality rate equivalent to non-diabetic animals. The mechanism by which the CT99 nutraceutical reduces vanadium toxicity is undetermined. However, two of the possibilities include reducing vanadium uptake or altering the direct effects by which vanadate causes diarrhea. The former option is probably less likely. **Figures 8-15** show the acute accumulation of vanadium upon administration of CT99. At most time points examined vanadium levels in plasma and the organ tissues were not significantly different from those associated with vanadium administered in water. It has been observed throughout our studies that most cases of diarrhea occur just following the first dose of vanadium and this is supported by other groups [261]. As such, the importance of the nutraceutical in inhibiting vanadate absorption acutely across the gut wall appears minimal. However, over the long-term these small differences may accumulate and lower total body vanadium accumulation. This may reduce peripheral tissue toxicity. Without a vanadate-D group maintained during the full year trial this is speculation as these levels would need to be contrasted with CT99-D animals. A more likely postulate concerning the ability of CT99 to protect animals from both diarrhea and mortality is that this nutraceutical synergistically combines with vanadate to reduce direct vanadium toxicity. Diarrhea normally results from dysfunction involving osmotic imbalance, increased fluid secretion, inflammation, or a reduction in gut motility [262]. Vanadate may, therefore, promote diarrhea by any of these mechanisms. However, since acute diarrheal episodes are so common with vanadium treatment this would make the chances of an inflammatory process being involved in the vanadium effects less probable. Alternatively, vanadium may act osmotically to draw fluid (especially water) from the

cells in the gut wall to the intestinal lumen. However, osmotic diarrhea is produced by poorly absorbed solutes and **Figures 8-15** show vanadium to be taken up into plasma and organs within hours of oral administration. Instead, diarrhea may be a result of a toxic mechanism altering ion channel activity within the brush border of the GI tract, increasing active secretion of water into the lumen. The activities of several transporters are crucial to normal fluid absorption and secretion. These include the Na^+/Cl^- transporter and the Cl^- channel. Both pathways are dependant on phosphorylation for activation. Therefore vanadate, a known phosphatase inhibitor, may inhibit the deactivation of G proteins and cAMP in a manner similar to the cholera bacteria. This would inhibit the Na^+/Cl^- transporter reducing Na^+ uptake while activating the Cl^- channel promoting a major loss of the Cl^- ion. Both of these effects would lead to a severe loss of water and electrolytes from the intestine. Although the exact mechanism of vanadium-induced diarrhea is unknown, it remains clear that CT99 may avoid any of these processes by combining with and altering vanadate's actions. Extensive examination of the stool contents and GI pathology is a crucial next step in the determination of the diarrhoeal mechanism. We have identified that vanadium purity is essential in the CT99 compound. Three different sodium orthovanadate derivatives were assessed and significant differences existed amongst these with respect to their effects on both diarrhea and mortality **(Figure 32)**. These Sigma® preparations of sodium orthovanadate varied from 95-99.9% pure and the differences may be involved in the differing toxicities, although this is difficult to prove as the exact products are not reproducible by the parent company.

The type of diabetes had an influence on the sensitivity of the rat to the anti-diarrhetic action of CT99. In the Type 2 diabetic rats, dose dependant toxicity was

evident. When administered 40 mg of vanadium in CT99 ZDF animals exhibited high rates of both diarrhea and death, however the nutraceutical solution still reduced toxicity vs. its vanadium/water counterpart **(Figure 44)**. Reducing the dosage of vanadium to 30 mg resulted in the complete elimination of diarrhea and death while a vanadate/water solution still yielded greater than 50 % diarrhea and 10 % mortality. Clearly, CT99 still provided substantial protection against the most common side effects of vanadium treatment in both Type 1 and Type 2 diabetes. It is also uncertain if the poorer protection afforded by CT99 in the ZDF rats was due to the type of diabetes present or the strain of the rat. An additional study of the effects of CT99 in another model of Type 2 diabetes (i.e. the db/db mouse) would resolve this issue.

In human diabetic patients, constantly fluctuating blood glucose levels result in many of the complications of the disease [263,264]. Presently, the conventional pharmacologic treatment for Type 1 diabetes is insulin therapy. Although intramuscular injections of insulin are effective in acutely reducing blood glucose levels, an oral delivery method for controlling blood glucose levels would minimize patient discomfort, eliminate needle costs and disposal concerns, and reduce chances of infection. Additionally, the fluctuating blood sugar levels that can be associated with insulin therapy necessitate monitoring and treatment more than once per day.

The ability of CT99 to reduce blood glucose levels not only acutely but chronically as well is apparent from the data shown in **Figures 6, 17, 33, 34, 35 and 45**. With a single dose of CT99, glucose levels dropped in diabetic animals to non-diabetic control levels. More importantly, this glycemic control is sustainable over weeks and months at a time without further treatment requirements. The commonly used marker for

effective glycemic control over long periods of time is glycated hemoglobin (GHb). With high circulating glucose levels in plasma, the hemoglobin protein is modified with the addition of carbohydrate residues. In non-diabetic or well-controlled diabetic patients, GHb levels should be lower than 7%. As **Figure 34**, indicates CT99-treatment normalized GHb levels in diabetic rats. GHb was reduced from over 11% in diabetic animals to approximately 3% in CT99-D animals. Therefore, in addition to its mode of delivery, the capacity of CT99 to regulate blood glucose levels over extended periods of time represents another exciting advantage of this therapy over the conventional insulin injection method. This characteristic appears more powerful in the Type 1 diabetic rats. Type 2 rats required more diligent treatment to maintain normoglycemia **(Figure 45)**. It must be emphasized that the initial pre-treatment glucose levels of both CT99-D and D animals were very high (22.5mM). Thus, even extreme hyperglycemia is controlled well with CT99.

Another risk associated with injected insulin in Type 1 diabetes is hypoglycemic shock. Continuous monitoring of drug dosage and blood glucose levels is mandatory. In these studies, 40mg of CT99 given to D animals was effective in controlling glucose levels long-term and was not associated with a single hypoglycemic event (reducing blood glucose < 3 mM). Notwithstanding, doses in excess 40 mg may result in hypoglycaemic episodes. Therefore, although monitoring of glycemic levels with CT99-treatment may require less diligence than conventional insulin therapy, the dosage of CT99 should still be closely monitored. Additionally, during both month long and year long trials, the CT99 dose of 20 mg vanadate/ ml nutraceutical did not have to be

increased despite large gains in animal body mass. This may certainly be important when the goal of eliminating side effects is as essential as it is with vanadium.

The reduced frequency of CT99-treatment required to maintain normoglycemia attests to blood glucose stability. As such one might speculate that secondary complications common with insulin therapy may be minimized with CT99 treatment. Indeed, by the end of the study 40% of D rats had developed severe cataracts, a common complication of uncontrolled hyperglycemia **(Table 3)**. However, none of the CT99-treated D rats exhibited cataracts. This further substantiates the strong hypoglycemic effect of the CT99 compound. The most devastating complication of diabetes however, remains cardiovascular disease and dysfunction [265]. **Figures 25-27** provide evidence that CT99 is cardioprotective via its ability to control hyperglycemia. CT99-treatment of diabetic animals restored cardiac cell shortening ability and intracellular Ca^{2+} release in response to phosphatidic acid. These indices were both returned to non-diabetic control levels with the use of CT99. The strongest evidence of CT99 efficacy remains the ability of the compound to control diabetes for greater than 3 months consecutively without further treatment **(Figures 36, 37, 39)**.

The efficacy of CT99 to reduce tissue toxicity was substantiated by biochemical analyses **(Tables 3, 5, 7)**. Significant improvements in plasma creatinine urine specific gravity and normal ALT levels in CT99-D animals suggest metabolic normalization, in addition to extended glycemic control. After extended periods of treatment with CT99, alkaline phosphatase, amylase, AST, BUN and uric acid were also restored to normal levels.

The mechanism by which vanadate lowers blood glucose levels is still not completely understood [137,180,266,267]. A mechanism proposed by Malabu et al suggested that appetite suppression alone produces hypoglycemia [179]. However, McNeill and colleagues contested this postulate [268]. In our studies strong evidence of a return to a normal metabolic profile existed. After a period of one year D rats treated with CT99 regained normal body mass. Both water and food intake was also normalized with CT99-treatment. These results support conclusions drawn from McNeill's group. A more plausible possibility for the hypoglycaemic action of vanadate concerns its effect on plasma insulin concentration. The significantly increased plasma insulin levels found in the CT99-D rats versus D animals provide evidence for either regeneration of ß-cells within the pancreatic islets or functional stimulation of those ß-cells not destroyed by STZ. The latter may be more plausible because we observed significantly higher plasma [insulin] in ZDF rats treated with CT99 as well. Furthermore, electron microscopic analysis of pancreatic islets confirmed necrosis in ß-insulin secretory cells in both CT99-D and D rats. However, the quantity of hyperactive B-cells found in the CT99-D animals was significantly greater than the D rats. This would argue against ß-cell regeneration as a potential mechanism. Thus, it appears possible that in the CT99-D rats, the remaining functional ß-cells are more active in order to control blood glucose. Evidence exists both to support and refute this mechanism of action for vanadate [269-271].

It is also possible that vanadate may have an intracellular site of action that is independentof its potential effects on ß-cell function. The intracellular phosphorylation process critical for glucose transporter (GLUT 4) movement to the cell surface to induce glucose transport would be stimulated by phosphatase inhibition. Vanadate is a known

potent phosphatase inhibitor [108,109]. A clear understanding of the specific phosphatase that vanadate may target to alter glucose homeostasis would represent critical information. Thus, CT99 may work through insulin receptor independent and dependent mechanisms to control blood sugar levels.

Regardless of the mechanism of action of the CT99 compound, it is clear that it is a potent hypoglycemic agent effective both acutely and, more importantly, chronically in both Type 1 and Type 2 diabetes. Notably, this compound acts in the absence of any detectable side effects. The benefits offered by the oral route of administration cannot be underestimated considering the millions of insulin-dependent diabetic patients that currently require intramuscular injections on a daily basis.

CONCLUSIONS

1. CT99 provides a vehicle through which vanadium salts can be administered orally.

2. CT99 eliminates evidence of the overt toxicity associated with vanadate in conventional water solutions.

3. Metabolic profiles, namely body weight and food/water intakes, of diabetic animals treated with CT99 return towards their non-diabetic counterparts.

4. Biochemical parameters assessed in diabetic animals treated with CT99 are improved vs. the non-CT99 treated diabetic animals.

5. The effects of CT99 last for extended periods of time. In fact, some diabetic animals initially treated with CT99 show no signs of diabetes and require no further treatment to remain normoglycemic.

6. CT99 is effective in the treatment of both Type 1 and Type 2 diabetes in rat models.

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