An Evaluation of Role of Vitamin D in The Pathophysiology of Streptozotocin induced Type-II Diabetes Mellitus in Rats and its Impact on Oral Hypoglycemic/Antidiabetic Agents

Thesis submitted to
BLDE (Deemed to be University)
Vijayapur, Karnataka, India.

Faculty of Medicine
For the award of the degree of
Doctor of Philosophy in
Medical Pharmacology

By

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December-2022

DECLARATION BY THE CANDIDATE

I hereby declare that thesis entitled "**An Evaluation of Role of Vitamin D in The Pathophysiology of Streptozotocin induced Type-II Diabetes Mellitus in Rats and its Impact on Oral Hypoglycemic/Antidiabetic Agents"** is bonafide and genuine **research** work carried out by me under the supervision of **Dr. Akram A Naikawdi** (Guide) Professor and Head Dept of Pharmacology Shri B.M.Patil Medical College, Hospital & Research Centre, BLDE (Deemed to be University), Vijayapur, Karnataka, India. No part of this thesis has been formed the basis for the award any degree or fellowship previously.

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Dedication

To my Parents

Mr. & Mrs. Arun Rao Moharir and Rukmini Bai

and

To my wife Deepa and my children, Mr. Anup Moharir and Amogh Moharir Whose affection, love, and encouragement made me able to get such Success and Honor.

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LIST OF ABBREVIATIONS

%	**Percent**
µL	**Micro Litre**
µM	**Micro Meter**
µM/L	**Micro Moles Per Litre**
ADA	**American Diabetic Association**
ANOVA	**Analysis of Variance**
b. wt	**Body weight**
BG	**Blood glucose**
CKD	**Chronic Kidney Disease**
DBP	**Bound Vitamin D**
DM-	**Diabetes Mellitus**
ENOS	**Endothelial Nitric Oxide Synthase**
FBG	**fasting Blood Glucose**
GDM	**Gestational Diabetes Mellitus**
GLUT	**Glucose Transporter**
HbA1C	**Glycosylated Haemoglobin**
HDL	**High Density Lipoprotein**
HLA	**Humal Leucocyte Antigen**
IAEC	**Institutional Animal Ethics Committee**
IDDM	**Insulin Dependent Diabetic Mellitus**
IPF	**Insulin Promoter Factor**
LDL	**Low Density Lipoprotein**

mg	**milli gram**
mg/dl	**milligram per decilitre**
mg/kg	**milligram per kilogram**
MODY	**Maturity Onset Diabetes Mellitus**
NIDDM	**Non Insulin Dependentdiabetic Mellitus**
NOD	**NON OBESE DIABETES**
OGTT	**Oral Glucose Tolerance Test**
PTH	**Parathyroid Hormone**
SEM	**Standard Error Of Mean**
STZ	**Streptozotocin**
T2DM	**Type-2 Diebetes Mellitus**
TC	**Total Cholesterol**
TG	**Tryglycerides**
VDR	**Vitamin D Receptor**
VDRE	**Vitamin D Response Element**
VLDL	**Very Low Density Lipoprotein**

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1. INTRODUCTION

Diabetes mellitus is a metabolic illness with several underlying causes which is defined by chronic hyperglycemia and altered carbohydrate, lipid, and protein metabolism as a result of a problem with insulin secretion, action, or both. (1). There are currently four types of diabetes mellitus based on pathophysiology, type-1 insulin dependent diabetes mellitus; type-2, non-insulin dependent diabetes mellitus; type 3 other; type 4 gestational diabetes mellitus (Expert Committee 2019). However, type 1 and type 2 are the most prevalent. One of the biggest hazards to human health in the twenty-first century is diabetes mellitus (DM), which the World Health Organization (WHO) originally regarded as a condition of least importance (2). The number of people with diabetes mellitus has increased significantly during the past few years, particularly in emerging nations like India. In India, the prevalence of type 2 diabetes is currently 2.4% in rural areas and 11.4% in cities. Globally, more than 150 million individuals have diabetes, a number that is expected to rise to 300 million by 2025. Indians made up more than one fifth of them. India has been designated as the world's diabetic capital by the International Diabetes Federation. (3). This syndrome often manifests quickly in childhood and is brought on by T cell-mediated death of pancreatic beta cells, which results in total insulin insufficiency (5). 5–10% of persons with diabetes mellitus have type 1 illness (6). Contrarily, type 2 illness, sometimes known as adult-onset diabetes, is non insulin-dependent.diabetes mellitus (NIDDM); type 2 diabetes is becoming more common (7). Insulin resistance and reduced insulin production are features of type 2 illness (8), which can range from predominant insulin resistance with relative insulin deficiency to predominant secretory deficiency with insulin resistance. About 85–90% of diabetic patients have type 2 disease (9–11), which is particularly common in Asian people (12). Type 2 diabetes mellitus is largely

caused by insulin resistance, which is characterised as a state of diminished responsiveness to normal circulating levels of insulin. Insulin levels fall in the presence of insulin resistance. Insufficient signalling results in post-receptor abnormalities, such as decreased glucose transporter 4 translocation, due to an increase in insulin receptor number and insulin receptor kinase activity. The reduction of first-phase insulin secretion, an increase in proinsulin production, a deficiency in pulsatile insulin secretion, and the deposition of islet amyloid polypeptide are all indicators of impaired islet -cell function (13,14). Acute and chronic problems are both possible in diabetic patients. Ketoacidosis and ketoacidotic coma are examples of acute complications. Macrovascular and microvascular problems are two general categories for chronic complications. More than 70% of diabetic mortality is caused by macrovascular disorders, primarily myocardial ischaemia, congestive heart failure, and stroke. Stroke, a common cause of morbidity and mortality in diabetic patients, is also connected with diabetes and increases risk for the condition (15). Patients with type 1 or type 2 diabetes have a much higher risk of stroke when they have high morbidity.(16, 17) In the early stages of stroke, elevated blood sugar is typical, and a glucose level greater than 155 mg/dl within 48 hours of the beginning of stroke is linked to a high risk of mortality (18). One of the main causes of death among diabetic individuals is cardiovascular disease, especially myocardial infarction. Myocardium and coronary vasculature exhibit aberrant morphological and structural alterations in diabetic cardiomyopathy (19). The underlying mechanism involves the excessive production of highly reactive free radicals, mostly brought on by hyperglycemia, which subsequently leads to oxidative stress and worsens the onset, progression, and consequences of diabetes (20). Diabetic neuropathy, diabetic nephropathy, and diabetic retinopathy are examples of microvascular problems. The most prevalent

diabetic consequence is diabetic neuropathy, which affects up to 50% of people with type 1 or type 2 diabetes. (21) Progressive nerve fibre loss, together with both positive and negative clinical signs and symptoms like pain, paraesthesia, and loss of feeling, are the hallmarks of diabetic neuropathy. All retinal cell types experience functional and structural alterations as a result of the neurodegenerative illness known as diabetic retinopathy (22). In wealthy nations, this ailment continues to be the predominant contributor of blindness. Patients with type 1 diabetes are expected to develop sight-threatening retinopathy in 50% of cases and type 2 diabetes patients in 30% of cases (23). Diabetes-related vision loss is mostly brought about by diabetic macular oedema and consequences from aberrant retinal blood vessel development (angiogenesis). Increased retinal blood flow, which results from angiogenesis, contributes to the development of diabetic retinopathy(24). The majority of diabetic individuals (20–30%) will eventually develop some form of diabetic nephropathy, which can advance from microalbuminuria to overt nephropathy or macroalbuminuria to end-stage renal failure with a significant mortality rate (24). Approximately 20-30%of all diabetic patients will develop some form of diabetic nephropathy, which may progress from micro albuminuria to overt nephropathy or macro- albuminuria, to end stage renal failure with high mortality (25). Diabetic nephropathy is marked by an excessive build-up of extracellular matrix, thickening of the glomerular and tubular basement membranes, and an increase in the mesangial matrix. Glomerulosclerosis and tubulo-interstitial fibrosis eventually result from this condition (26). In diabetics, improved glycaemic management enhances lipid metabolism, lowering risk factors for numerous related disorders. Currently, the cornerstone of treatment is insulin and oral anti-diabetic medications, but they have limitations of their own. Cost and parenteral delivery of insulin, weight gain from sulfonylureas and meglitinides, hepatic

impairment, increased risk of heart failure, increased risk of bone fractures from thiazolidinediones, and gastrointestinal problems from acarbose are only a few of the side effects (27). Allowing patients to live normal lives while achieving a normal metabolic state in order to slow down or prevent long-term effects of diabetes is the main objective of treating diabetes mellitus. The continual quest for novel, efficient, safer, and more affordable medications has been undertaken in order to meet these objectives. To date, a number of studies have been conducted to determine natural treatments for dominant diabetic disease and its complications. A fatsoluble vitamin, Vitamin D plays a key role in bone mineralization and calcium homeostasis. Rickets in infancy and osteomalacia in older age are both skeletal symptoms of Vitamin D deficiency, which is currently thought to be a pandemic. Wide-ranging extra-skeletal symptoms of Vitamin D insufficiency include cardiovascular, neuropsychiatric, endocrine, gastrointestinal, and renal impacts. (28). On the other hand, type 2 diabetes mellitus (T2DM) is one of the most illnesses that are common and have significant burdens and rates of complications. T2DM, in contrast to type 1 diabetes mellitus, is primarily characterised by decreased sensitivity to the insulin released by pancreatic beta cells that are still functional. Despite the extensive research done, increased insulin resistance is still not fully understood because of the intricate relationship between insulin receptors (IR), glucose transporters (GLUTs) in various tissues, fluidity of the plasma membrane, intracellular signalling, and transcriptional control of metabolism (29). Over the past ten years, mounting evidence has shown a connection between Vitamin D insufficiency and type 2 diabetes (30). The risk factors for Vitamin D insufficiency and type 2 diabetes (T2DM) include obesity, age, and a lack of physical activity (31). Based on research relating to the role of Vitamin D in glucose homeostasis, insulin secretion, and insulin sensitivity, reports have proposed a

causal relationship between Vitamin D deficiency and type 2 diabetes (T2DM) (32,33). The possible involvement of Vitamin D as a glucostatic and insulin secretagogue is becoming more and more popular (33). With varying results from research to study, the precise significance of Vitamin D in diabetes mellitus, particularly T2DM, is still up for debate. Additionally, the precise physiological and molecular mechanisms underlying the reported beneficial results are still not completely understood. In order to explore the impact on glycemic control and to highlight the underlying pathophysiological mechanisms, this study evaluated the role of Vitamin D in a T2DM-rat model produced by streptozotocin injection in combination with oral anti-diabetic medications.

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AIM AND OBJECTIVES

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AIM

"An Evaluation Of role of Vitamin D in the pathophysiology of streptozotocin induced type-II diabetes mellitus in rats and its impact on oral hypoglycemic/antidiabetic agents"

OBJECTIVES

1. To evaluate pathophysiological role of Vitamin D in type-2 diabetes mellitus and its impact on oral hypoglycaemic/antidiabetic agents in rats.
2. To evaluate role of Vitamin D on lipid metabolism in STZ induced type-II diabetes in rats.
3. To evaluate effect of Vitamin D on cardiovascular morbidity in STZ induced type-II diabetes in rats.

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

Diabetes mellitus is a complicated, chronic illness that necessitates ongoing medical attention along with risk-reduction measures that go beyond glycemic management. The prevention of acute problems and lowering the risk of long-term complications depend heavily on ongoing patient self-management education and assistance. There is substantial evidence to support a variety of therapies to enhance diabetes outcomes.(1)

History of Diabetes (2,3)

Clinical features similar to diabetes mellitus were described 3000 years ago by the ancient Egyptians. The term "diabetes" was first coined by Araetus of Cappodocia (81-133AD). Later, the word mellitus (honey sweet) was added by Thomas Willis (Britain) in 1675 after rediscovering the sweetness of urine and blood of patients (first noticed by the ancient Indians). It was only in 1776 that Dobson (Britain) firstly confirmed the presence of excess sugar in urine and blood as a cause of their sweetness. In modern time, the history of diabetes coincided with the emergence of experimental medicine. An important milestone in the history of diabetes is the establishment of the role of the liver in glycogenesis, and the concept that diabetes is due to excess glucose production Claude Bernard (France) in 1857. The role of the pancreas in pathogenesis of diabetes was discovered by Mering and Minkowski (Austria) 1889. Later, this discovery constituted the basis of insulin isolation and clinical use by Banting and Best (Canada) in 1921. Trials to prepare an orally administrated hypoglycemic agent ended successfully by first marketing of tolbutamide and carbutamide in 1955. This report will also discuss the history of dietary management and acute and chronic complications of diabetes.

(Canada) in 1921. Trials to prepare an orally administrated hypoglycemic agent ended successfully by first marketing of tolbutamide and carbutamide in 1955. This report will also discuss the history of dietary management and acute and chronic complications of diabetes.

1910- After studying the pancreatic, English physiologist Sir Edward Albert Sharpey-Schafer finds that non-diabetics regularly create chemical called insulin. The word "insula," which means "island" in Latin, refers to the pancreatic islets of Langerhans, which produce insulin.

1916- The first edition of The Treatment of Diabetes Mellitus is published by Elliott Joslin, MD. Joslin, a clinician and educator, is well-known throughout the world as one of the most important voices in the treatment of diabetes.

1921- Insulin is extracted from canine pancreases by Frederick Banting, MD, and Charles Best, MD, his then-student assistant. At the University of Toronto, Professor J.J.R. Macleod provided Banting and Best with a lab area where they were working. The dogs' blood sugar levels decrease after receiving the insulin injection and having their pancreases removed. In order to be utilised on people, James Collip purified the extract.. Despite the fact that all four men made significant contributions to the discovery of insulin, Banting and Macleod were given the 1923 Nobel Prize in Physiology or Medicine.

1923- Eli Lilly and Company starts producing insulin for use in industry.

1936- Protamine insulin, launched by Novo Nordisk in, was the first of many slower-acting insulin's that manufacturers developed in the next decades.

Priscilla White, MD, founds the Joslin Pregnancy Clinic in 1924, a year in which fewer than 50% of all infants born to mothers with diabetes survive. Dr. White achieves a 90% survival rate for children born to her patients fifty years later.

1940- The American Diabetes Association is founded to address the increasing incidence of diabetes and the complications that develop from the disease.

1949- Rachmiel Levine, MD, discovers that insulin works like a key, transporting glucose into cells. Becton Dickinson and Company begins production of a standardized insulin syringe designed and approved by the American Diabetes Association.

1950- The American Dietetic Association, and the U.S. Public Health Service devise a meal planner that divides foods into six groups, or "exchanges", based on the calories, carbohydrate, protein, and fat in each serving of food.

1952- The American Diabetes Association funds its first direct research grants.

1953- Tablets for testing urine glucose become widely available, and urine test strips appear over the next few years. These options are simpler than using Benedict's solution, which must be mixed with urine and heated over boiling water.

1955- Sulfonylurea's, oral medications that stimulate the pancreas to release more insulin, are available. New, more potent forms of these drugs will become available later.

1959- Using radioimmunoassay technology, Solomon Berson, MD and Rosalyn Yalow, PhD develop a method for measuring insulin in the blood. They notice that some people with diabetes still make their own insulin, and they identify "insulin-dependent" (type 1) and "non-insulin-dependent" (type 2) diabetes.

1961- Glucagon, a hormone produced by the pancreas that raises glucose levels, is introduced by Eli Lilly and Company as a treatment for severe hypoglycaemia.

1964- The Ames Company introduces the first strips for testing blood glucose by colour code.

1966- The first successful pancreas transplant is performed at the University of Minnesota Hospital.

1970- The Ames Company introduces the first glucose meter.

1971- Insulin receptors are discovered on cell membranes. This discovery raises the possibility that missing or defective insulin receptors may prevent glucose from entering the cells, thus contributing to the insulin resistance of type 2 diabetes.

1972- The relationship between blood vessel disease and hyperglycaemia is reported. U100 insulin is introduced. With the availability of this single concentration and with insulin syringes marked with only a U100 scale, frequency of dosing errors could be reduced.

1974- Development of the Biostator enabled continuous glucose monitoring and closed loop insulin infusion. Human Leukocyte Antigens (HLAs) are discovered on cell surfaces. People with type 1 diabetes have specific patterns of HLA that are associated with varying levels of risk for diabetes.

1976- The first insulin pumps were invented.

1977- Rosalyn Yalow, PhD is awarded the Nobel Prize in Physiology and Medicine for her work in measuring insulin in the body. Boston researchers develop a test to measure glycosylated haemoglobin (A1C). A1C testing becomes the gold standard for measuring long-term diabetes control.

1978- Researchers at the City of Hope National Medical Centre in Duarte, California, and Genentech, Inc., in San Francisco, induce E. coli bacteria to produce insulin identical to human insulin. Portable insulin pumps are introduced and researchers achieve normal blood glucose levels in patients using them. But, due to their large size, they are impractical at this time. The National Diabetes Information Clearing

house is created by the federal government to gather and document all diabetes literature.

1979- The National Diabetes Data Group develops a new diabetes classification system:

1) insulin-dependent or type 1 diabetes

2) non-insulin-dependent or type 2 diabetes

3) gestational diabetes, and

4) diabetes associated with other syndromes or conditions. 2

1980- A new animal model of type 1 diabetes, the non-obese diabetic (NOD) strain of mouse is described in Japan. Introduction of the basal-bolus concept enabled "intensive insulin therapy" to be used in the clinic to effectively treat people with type 1 diabetes.

1982- The FDA approves human insulin produced by genetically altered bacteria. A 64K autoantibody is discovered and is found to be associated with type 1 diabetes.

1983- A link between hypoglycaemia and brain metabolism is established. Second-generation sulfonylurea's enter the market allowing patients to take smaller doses and with reduced side effects.

1984- The insulin molecule is identified to be a target of autoimmune response in individuals with type 1 diabetes.

1985- Scientists discover a relationship between pregnancy and the worsening of diabetic retinopathy.

1986- The National Diabetes Data Group reports that type 2 diabetes is more common among African Americans, Mexican Americans, and Native Americans than among Caucasians. Fifty percent of all Pima Indians in Arizona over the age of 35 have diabetes – the highest rate in the world.

1987- 64K autoantibody originally discovered in 1982 is found to be predictive of type 1 diabetes. Researchers determine that tight control of glucose levels during pregnancy is important for the health of the baby, and continue to study how diabetes increases the risk for birth defects.

1989- American Diabetes Association releases its first Standards of Care to guide physicians in the treatment of diabetes. Glucose is discovered to be distributed into muscle and fat cells via a transporter known as GLUT-4. Understanding how glucose is transported from the bloodstream into cells to be used as fuel is important to locating different drug targets that can improve insulin sensitivity.

1990- The 64K autoantibody associated with type 1 diabetes is identified. This protein, GAD, or glutamate decarboxylase, is an important enzyme involved in cellular communication in the brain and pancreas. The immune system's attack on GAD triggers a progressive autoimmune response that leads to diabetes.

1993- The Diabetes Control and Complications Trial (DCCT) showed that keeping blood glucose levels as close to normal as possible slows the onset and progression of eye, kidney, and nerve diseases caused by diabetes. In fact, it demonstrated that any sustained lowering of blood glucose helps, even if the person has a history of poor control.

1994- Captopril is FDA approved to treat end-stage renal disease. Leptin, the fat cell hormone that modulates feeding behaviour and hormone secretion, is cloned. The Scandinavian Simvistatin Survival Study (4S) showed that cholesterol lowering with statins markedly reduced the risk of myocardial infarction, stroke or death. The effect was greatest in individuals with diabetes.

Mid-1990s- The incretin hormone GLP-1 is discovered. Incretin hormones are secreted from the gut in response to food, and encourage the body to produce insulin.

Discovery of GLP-1 will later lead to a new class of diabetes drugs that can increase insulin secretion in response to glucose, and even increase the amount of beta cells in the pancreas.

1995- The drug metformin becomes available in the U.S. Metformin is a biguanide that prevents glucose production in the liver.

1996- The drug acarbose, brand name Precose (Bayer Corporation) becomes available in the U.S. Acarbose is an alpha-glycosidase inhibitor that slows digestion of some carbohydrates. Lispro (a lysine-proline analog) is introduced by Eli Lilly and Company as the world's fastest acting insulin.

1997- Troglitazone, brand name Rezulin (Parke-Davis), is approved by the FDA. It is the first in a class of drugs known as thiazolidinediones, and it improves insulin sensitivity in muscle cells. It is eventually removed from the market due to liver toxicity. Rosiglitazone and pioglitazone, also in this drug class, are later brought on to the market.

The terms "insulin-dependent diabetes" (IDDM) and "non-insulin-dependent diabetes" (NIDDM) had long been used to describe different groups of diabetes patients. The terms type 1 diabetes and type 2 diabetes are now accepted to define diabetes by cause rather than treatment. In addition, the fasting glucose level for diagnosing diabetes is lowered from 140 mg/dl to 126 mg/dl.

1998- Repaglinide, brand name Prandin (Novo Nordisk) is developed. Repaglinide belongs to a class of drugs known as meglitinides. They stimulate insulin secretion in the presence of glucose. The United Kingdom Prospective Diabetes Study (UKPDS) shows that people with type 2 diabetes who practice tight control of blood sugar levels and blood pressure levels reduce their risk of complications, similar to the results of

the DCCT in people with type 1 diabetes. Together these two studies transform the nature of diabetes care around the world.

2002- Treatment with the anti-CD3 monoclonal antibody, hOKT3gamma1(Ala-Ala), slows the deterioration of insulin production and improves metabolic control during the first year of type 1 diabetes in the majority of patients.

The American Diabetes Association defines prediabetes as impaired fasting glucose (IFG) and/or impaired glucose tolerance (IGT). IFG is defined as a fasting blood glucose of 100-125 mg/dl, and IGT is defined as a glucose level from 140 mg/dl – 199 mg/dl two hours after consuming a glucose-rich drink. Later, A1C levels of 5.7% to 6.4% are also used to identify individuals with prediabetes.

2005- Exenatide, brand name Byetta, is approved in the U.S. as a first-in-class incretin mimetic (GLP-1) drug to treat type 2 diabetes. An injectable drug, exenatide works by increasing insulin production in response to blood glucose levels. Pramlintide, brand name Symlin, is approved in the U.S. as an injectable adjunct treatment for people who use insulin at mealtimes but still fail to achieve desirable blood glucose levels.

2006- FDA approves JANUVIA (sitagliptin phosphate), the first in a new class of drugs known as DPP-4 inhibitors that enhance the body's ability to lower elevated blood sugar. DPP-4 is an enzyme that naturally blocks GLP-1 from working, so by inhibiting this enzyme, GLP-1 works in the gut to promote insulin secretion.

2008- The results of the ACCORD, ADVANCE and VADT studies are published and presented at the American Diabetes Association Scientific Sessions. All three studies fail to show a benefit of intensive glycaemic control on cardiovascular outcomes in people with type 2 diabetes who are at high cardiovascular risk. The results from these studies lead to clinical recommendations that call for a more individualized approach for setting glycaemic goals and treatment targets.

2013- FDA approves Invokana (Canagliflozin), the first in a new class of drugs known as the SGLT-2 inhibitors, for lowering elevated blood sugar in patients with type 2 diabetes. SGLT-2 inhibitors block the activity of sodium glucose transport proteins in the kidney, reducing glucose re-uptake and increasing secretion of glucose in the urine.

Global burden of Diabetes Mellitus

The diabetes epidemic is rising rapidly with the most dramatic documented increase being in low and middle-income countries. Diabetes is undoubtedly one of the largest health emergencies of the twenty-first century with a worldwide prevalence of 422 million (8.5% of adults aged 20–79), which is predicted to reach 642 million by 2040, i.e. one in every ten adults. The largest increases will take place in regions where economies are moving from low- to middle-income levels. About 75% of people with diabetes live in low- and middle-income countries. Furthermore, 318million people are estimated to have impaired glucose tolerance and 20.9 million live births are affected by some form of hyperglycaemia in pregnancy, of which 85.1% are due to gestational diabetes as per the IDF Atlas 2015.(8)

Both these conditions are associated with an increased risk of developing type 2 diabetes in later life. Currently, there are more people with diabetes mellitus in urban areas (269.7 million) than in rural areas (145.1 million). By 2040, the difference is expected to widen globally, with an estimated 477.9 million people with diabetes living in urban areas and 163.9 million in rural areas(1).

Half of the cases of diabetes are among people between 40 and 59 years of age. World Health Organization (WHO) estimates that globally, high blood glucose is the third leading risk factor for premature mortality after high blood pressure and tobacco use.

Burden of Diabetes Mellitus in India

In India, an estimated 7.8% of the population above 18 years of age has raised blood glucose levels or are on treatment for diabetes. This amounts to an estimated 60 million people with diabetes out of a population of over 1.3 billion. Awareness about their diabetes status varies from state to state and the proportion that is unaware of their diabetes status is very high in rural areas. Nearly 900 000 annual deaths are directly or indirectly attributed to diabetes

Type 1 diabetes is still not very common. However, of the 542 000 children aged up to 14 years with type 1 diabetes in 2015 globally, India had 70 200, the second largest number in the world after the USA. India also ranks highest in the list of top 10 countries with 36.5 million people with impaired glucose tolerance. Prevalence of diabetes varies from state to state in India. Partial results available from the Indian Council of Medical Research-India Diabetes (ICMR–INDIAB) Study reveal large inter-state variations in prevalence, ranging from 4.3% in Bihar to 13.6% in Chandigarh. The third repeat survey carried out by the National Nutrition Monitoring Bureau among the rural population in 2012 reported a prevalence of 8.2% and 6.8% among adult men and women for diabetes, respectively. The prevalence was reported to be high in the states of Kerala, Tamil Nadu and Gujarat (8.2–16.4%) among both genders. Analysis of secular trends revealed an increase in diabetes prevalence in the rural population at a rate of 2.02 per 1000 population per year.

Socioeconomic burden

Diabetes imposes enormous economic burden on individuals and families, national health systems and to society. Health spending on diabetes accounted for 10.8% of total health expenditure worldwide in 2013. The costs associated with diabetes also include productivity loss and disability, which can be a considerable burden to the

individual, families and society. When people have long-standing undiagnosed diabetes, the potential benefits of early diagnosis and treatment are lost. The costs related to undiagnosed diabetes are considerable.

One study from the USA found that undiagnosed diabetes was responsible for an additional US$ 18 billion in health-care costs in one year. If effective measures are not put in place, India stands to lose US$ 150 billion before2030 due to diabetes. India has one of the lowest public health expenditures as a proportion of total health expenditure. It is estimated that annually 60 million people are pushed into poverty due to catastrophic out-of-pocket (OOP) expenditure. Lack of quality health services in the public system often forces people to seek care from the private sector, even though the latter may not be affordable. Currently, expenditure on drugs constitutes about 67% of OOP expenditure on health care. In 2013, about 46% of private expenditure on health was paid out of pocket. As per the data from the National Sample Survey Organization (NSSO), the share of NCDs in OOP expenditure. (4)

Classification of Diabetes Mellitus

Diabetes Mellitus can be classified into the following general categories:(5)

1. Type 1 Diabetes Mellitus (due to β-cell destruction, usually leading to absolute insulin deficiency)

2. Type 2 Diabetes Mellitus (due to a progressive loss of insulin secretion on the background of insulin resistance)

3. Type 3 Diabetes Mellitus (Gestational diabetes mellitus (GDM) diabetes diagnosed in the second or third trimester of pregnancy that is not clearly overt diabetes)

4. Type 4 Diabetes Mellitus (Specific types of diabetes due to other causes, e.g., monogenic diabetes syndromes (such as neonatal diabetes and maturity-onset diabetes

of the young [MODY]), diseases of the exocrine pancreas (such as cystic fibrosis), and drug- or chemical-induced diabetes (such as with glucocorticoid use after organ transplantation, use of protease inhibitors in the treatment of HIV/AIDS).

Diabetes is a group of metabolic diseases characterized by hyperglycaemia resulting from defects in insulin secretion, insulin action, or both. The chronic hyperglycaemia of diabetes is associated with long-term damage, dysfunction, and failure of different organs, especially the eyes, kidneys, nerves, heart, and blood vessels. Several pathogenic processes are involved in the development of diabetes mellitus. These range from autoimmune destruction of the pancreatic β-cells with consequent insulin deficiency to abnormalities that result in resistance to insulin action. The basis of the abnormalities in carbohydrate, fat, and protein metabolism in diabetes is deficient action of insulin on target tissues. Deficient insulin action results from inadequate insulin secretion and/or diminished tissue responses to insulin at one or more points in the complex pathways of hormone action. Impairment of insulin secretion and defects in insulin action frequently coexist in the same patient, and it is often unclear which abnormality, if either alone, is the primary cause of the hyperglycaemia. Symptoms of marked hyperglycaemia include polyuria, polydipsia, weight loss, sometimes with polyphagia, and blurred vision. Impairment of growth and susceptibility to certain infections may also accompany chronic hyperglycaemia. Acute, life-threatening consequences of uncontrolled diabetes are hyperglycaemia with ketoacidosis or the nonketotic hyperosmolar syndrome.(6)

Long-term complications of diabetes mellitus include retinopathy with potential loss of vision; nephropathy leading to renal failure; peripheral neuropathy with risk of foot ulcers, amputations, and Charcot joints; and autonomic neuropathy causing gastrointestinal genitourinary, and cardiovascular symptoms and sexual dysfunction.

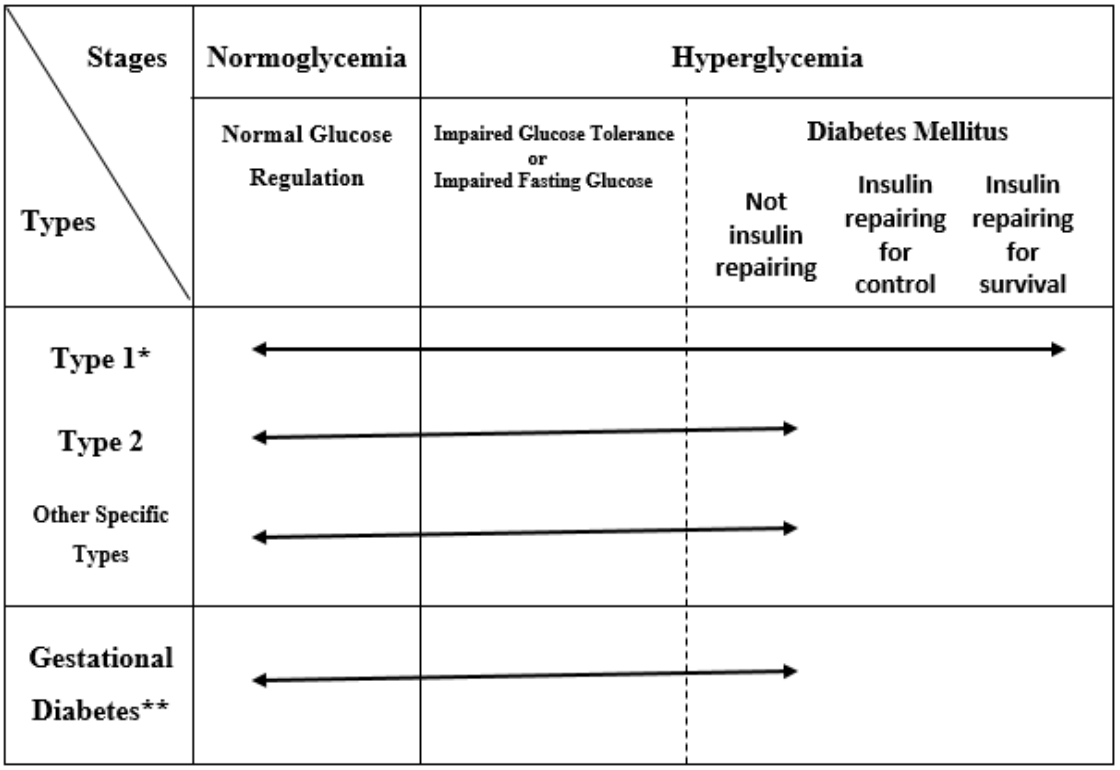
Patients with diabetes mellitus have an increased incidence of atherosclerotic cardiovascular, peripheral arterial, and cerebrovascular disease. Hypertension and abnormalities of lipoprotein metabolism are often found in people with diabetes.

The vast majority of cases of diabetes mellitus fall into two broad etiopathogenetic categories (discussed in greater detail below). In one category, type 1 diabetes mellitus, the cause is an absolute deficiency of insulin secretion. Individuals at increased risk of developing this type of diabetes mellitus can often be identified by serological evidence of an autoimmune pathologic process occurring in the pancreatic islets and by genetic markers. In the other, much more prevalent category, type 2 diabetes mellitus, the cause is a combination of resistance to insulin action and an inadequate compensatory insulin secretory response. In the latter category, a degree of hyperglycaemia sufficient to cause pathologic and functional changes in various target tissues, but without clinical symptoms, may be present for a long period of time before diabetes mellitus is detected. During this asymptomatic period, it is possible to demonstrate an abnormality in carbohydrate metabolism by measurement of plasma glucose in the fasting state or after a challenge with an oral glucose load or by HbA1c.

The degree of hyperglycaemia (if any) may change over time, depending on the extent of the underlying disease process (Table 1). A disease process may be present but may not have progressed far enough to cause hyperglycaemia. The same disease process can cause impaired fasting glucose (IFG) and/or impaired glucose tolerance (IGT) without fulfilling the criteria for the diagnosis of diabetes. In some individuals with diabetes mellitus, adequate glycaemic control can be achieved with weight reduction, exercise, and/or oral glucose-lowering agents. These individuals therefore do not require insulin. Other individuals who have some residual insulin secretion but require exogenous insulin for adequate glycaemic control can survive without it. Individuals

with extensive β-cell destruction and therefore no residual insulin secretion require insulin for survival. The severity of the metabolic abnormality can progress, regress, or stay the same. Thus, the degree of hyperglycaemia reflects the severity of the underlying metabolic process and its treatment more than the nature of the process itself.

Table 1. Classification of Diabetes



Assigning a type of diabetes mellitus to an individual often depends on the circumstances present at the time of diagnosis, and many diabetic individuals do not easily fit into a single class. For example, a person diagnosed with gestational diabetes mellitus (GDM) may continue to be hyperglycaemic after delivery and may be determined to have, in fact, type 2 diabetes mellitus. Alternatively, a person who acquires diabetes because of large doses of exogenous steroids may become normoglycemic once the glucocorticoids are discontinued, but then may develop diabetes mellitus many years later after recurrent episodes of pancreatitis. Another

example would be a person treated with thiazides who develops diabetes years later. Because thiazides in themselves seldom cause severe hyperglycaemia, such individuals probably have type 2 diabetes mellitus that is exacerbated by the drug. Thus, for the clinician and patient, it is less important to label the particular type of diabetes mellitus than it is to understand the pathogenesis of the hyperglycaemia and to treat it effectively.

Type 1 Diabetes Mellitus

This form, previously called “insulin dependent diabetes mellitus” or “juvenile-onset diabetes mellitus” accounts for 5–10% of diabetes and is due to cellular-mediated autoimmune destruction of the pancreatic β-cells. Autoimmune markers includeislet cell autoantibodies and autoantibodies to insulin, GAD (GAD65), the tyrosine phosphatises.

Type 1 diabetes mellitus is defined by one or more of these autoimmune markers. The disease has strong HLA associations, with linkage to the DQA and DQB genes. These HLA-DR/DQ alleles can be either predisposing or protective. The rate of b-cell destruction is quite variable, being rapid in some individuals (mainly infants and children) and slow in others (mainly adults). Children and adolescents may present with ketoacidosis as the first manifestation of the disease. Others have modest fasting hyperglycaemia that can rapidly change to severe hyperglycaemia and/or ketoacidosis with infection or other stress. Adults may retain sufficient b-cell function to prevent ketoacidosis for many years; such individuals eventually become dependent on insulin for survival and are at risk for ketoacidosis. At this latter stage of the disease, there is little or no insulin secretion, as manifested by low or undetectable levels of plasma C-peptide. Immune-mediated diabetes commonly occurs in childhood and adolescence, but it can occur at any age, even in the 8th and 9th decades of life. These patients are

also prone to other autoimmune disorders such as Hashimoto thyroiditis, celiac disease, Graves disease, Addison disease, vitiligo, autoimmune hepatitis, myasthenia gravis, and pernicious anaemia.

Idiopathic Type 1 Diabetes Mellitus

Some forms of type 1 diabetes mellitus have no known etiologies. These patients have permanent insulinopenia and are prone to ketoacidosis, but have no evidence of b-cell autoimmunity. Although only a minority of patients with type 1 diabetes fall into this category, of those who do, most are of African or Asian ancestry. Individuals with this form of diabetes suffer from episodic ketoacidosis and exhibit varying degrees of insulin deficiency between episodes. This form of diabetes is strongly inherited and is not HLA associated. An absolute requirement for insulin replacement therapy in affected patients may be intermittent.

Type 2 Diabetes Mellitus

Type 2 diabetes mellitus, previously referred to as "non–insulin-dependent diabetes mellitus" or "adult-onset diabetes mellitus," accounts for 90–95% of all diabetes mellitus. This form encompasses individuals who have insulin resistance and usually relative (rather than absolute) insulin deficiency. At least initially, and often throughout their lifetime, these individuals may not need insulin treatment to survive. There are various causes of type 2 diabetes mellitus. Although the specific aetiologies are not known, autoimmune destruction of b-cells does not occur, and patients do not have any of the other known causes of diabetes. Most, but not all, patients with type 2 diabetes mellitus are overweight or obese. Excess weight itself causes some degree of insulin resistance. Patients who are not obese or overweight by traditional weight criteria may have an increased percentage of body fat distributed predominantly in the abdominal region. Ketoacidosis seldom occurs spontaneously in type 2 diabetes

mellitus; when seen, it usually arises in association with the stress of another illness such as infection. Type 2 diabetes mellitus frequently goes undiagnosed for many years because hyperglycaemia develops gradually and, at earlier stages, is often not severe enough for the patient to notice the classic diabetes mellitus symptoms. Nevertheless, even undiagnosed patients are at increased risk of developing macrovascular and microvascular complications. Whereas patients with type 2 diabetes mellitus may have insulin levels that appear normal or elevated, the higher blood glucose levels in these patients would be expected to result in even higher insulin values had their β-cell function been normal. Thus, insulin secretion is defective in these patients and insufficient to compensate for insulin resistance. Insulin resistance may improve with weight reduction and/or pharmacological treatment of hyperglycaemia but is seldom restored to normal. The risk of developing type 2 diabetes mellitus increases with age, obesity, and lack of physical activity. It occurs more frequently in women with prior GDM, in those with hypertension or dyslipidaemia and in certain racial/ethnic subgroups (African American, American Indian, Hispanic/Latino, and Asian American). It is often associated with a strong genetic predisposition, more so than type 1 diabetes mellitus.

Type 3 Diabetes Mellitus (Gestational Diabetes Mellitus)

For many years, GDM was defined as any degree of glucose intolerance that was first recognized during pregnancy, regardless of whether the condition may have predated the pregnancy or persisted after the pregnancy. This definition facilitated a uniform strategy for detection and classification of GDM, but it was limited by imprecision. The ongoing epidemic of obesity and diabetes mellitus has led to more type 2 diabetes in women of childbearing age, resulting in an increase in the number of pregnant women with undiagnosed type 2 diabetes mellitus. Because of the number of pregnant

women with undiagnosed type 2 diabetes mellitus, it is reasonable to test women with risk factors for type 2 diabetes mellitus at their initial prenatal visit, using standard diagnostic criteria. Women with diabetes mellitus in the first trimester would be classified as having type 2 diabetes mellitus. GDM is diabetes mellitus diagnosed in the second or third trimester of pregnancy that is not clearly overt diabetes mellitus.

Type 4 Diabetes Mellitus

Monogenic Diabetes Syndromes

Monogenic defects that cause β-cell dysfunction, such as neonatal diabetes mellitus and MODY, represent a small fraction of patients with diabetes mellitus (5%). These forms of diabetes are frequently characterized by onset of hyperglycaemia at an early age (generally before age 25 years).

Neonatal Diabetes Mellitus

Diabetes diagnosed in the first 6 months of life has been shown not to be typical autoimmune type 1 diabetes mellitus. This so-called neonatal diabetes mellitus can either be transient or permanent. The most common genetic defect causing transient disease is a defect on ZAC/HYAMI imprinting, whereas permanent neonatal diabetes mellitus is most commonly a defect in the gene encoding the Kir6.2 subunit of the β-cell KATP channel. Diagnosing the latter has implications, since such children can be well managed with sulfonylureas.

Maturity- Onset Diabetes Mellitus of the Young

MODY is characterized by impaired insulin secretion with minimal or no defects in insulin action. It is inherited in an autosomal dominant pattern. Abnormalities at six genetic loci on different chromosomes have been identified to date. The most common form is associated with mutations on chromosome 12 in a hepatic transcription factor referred to as hepatocyte nuclear factor (HNF)-1a. A second form

is associated with mutations in the glucokinase gene on chromosome 7p and results in a defective glucokinase molecule. Glucokinase converts glucose to glucose-6-phosphate, the metabolism of which, in turn, stimulates insulin secretion by the β-cell. The less common forms of MODY result from mutations in other transcription factors, including HNF-4a, HNF-1b, insulin promoter factor (IPF)-1, and NeuroD1.(7)

Prevalence

The global prevalence of diabetes mellitus among adults over 18 years of age has risen from 4.7% in 1980 to 8.5% in 2014. Diabetes Mellitus prevalence has been rising more rapidly in middle- and low-income countries. The number of people with diabetes mellitus has risen from 108 million in 1980 to 422 million in 2014.In 2012, an estimated 1.5 million deaths were directly caused by diabetes mellitus and another 2.2 million deaths were attributable to high blood glucose. Almost half of all deaths attributable to high blood glucose occur before the age of 70 years. WHO projects that diabetes mellitus will be the 7th leading cause of death in 2030. Healthy diet, regular physical activity, maintaining a normal body weight and avoiding tobacco use are ways to prevent or delay the onset of type 2 diabetes mellitus. Diabetes mellitus can be treated and its consequences avoided or delayed with diet, physical activity, medication and regular screening and treatment for complications.(8)

There were 69.1 million cases of diabetes mellitus in India in 2015.Of these, it remained undiagnosed in more than 36 million people. The total mortality in adults due to diabetes mellitus was 1 million. The cost per person with diabetes mellitus was around 95$.

Since there is no global consensus on the diagnostic criteria for GDM, the prevalence of GDM ranged from 1.0 to 14.2 % of all pregnancies depending on diagnostic criteria and the study population (based on our literature search of Medline publications with

either population-based studies with sample size ≥500 or hospital-based studies with sample size ≥1000 and at least 70 % of population being screened for GDM) As shown in Table 2, the Southeast Asia region consistently reported the highest GDM prevalence with a median of 5.4 % (range of 3–14.2 %), followed by Eastern Mediterranean countries with a median of 4.75 % (range of 1.9–13.7 %). America, Africa, and Western Pacific appeared to have similar GDM prevalence with a median of 3.7 % across the three regions. Europe had the lowest GDM prevalence among all the WHO regions. The low prevalence was evident across all European countries (range 1.2–3.1 %) except for Italy. A population-based study in Sardinia, Italy, provided the largest GDM prevalence (22.3 %) of all the included studies. A second large population-based study from a mainland Italian city, Pisa, also reported a GDM prevalence of 8.7 %.(9)

Epidemiology

Type 1 diabetes mellitus strikes both children and adults at any age. It comes on suddenly, causes dependence on injected or pumped insulin for life, and carries the constant threat of devastating complications. Type 1 diabetes mellitus is a chronic illness characterized by the body's inability to produce insulin due to the autoimmune destruction of the beta cells in the pancreas. Onset most often occurs in childhood, but the disease can also develop in adults in their late 30s and early 40s.(10)

1.25 million Americans are living with T1D including about 200,000 youth (less than 20 years old) and over a million adults (20 years old and older). 40,000 people are diagnosed each year in the U.S. 5 million people in the U.S. are expected to have Type 1Diabetes Mellitus by 2050, including nearly 600,000 youth. Between 2001 and 2009 there was a 21% increase in the prevalence of Type 1Diabetes Mellitus in people under age 20. $14B Type 1Diabetes Mellitus-associated annual healthcare costs in the

U.S. Less than one-third of people with Type 1Diabetes Mellitus in the U.S. are achieving target blood glucose control levels. Type 1Diabetes Mellitus is associated with an estimated loss of life-expectancy of up to 13 years. (11) India accounts for most of the children with Type 1Diabetes Mellitus in South-East Asia. According to the 6th edition of the International Diabetes Federation diabetes atlas, India has 3 new cases of Type 1Diabetes Mellitus/100,000 children of 0-14 years. The prevalence of diabetes in India is variable, and three sets of data show 17.93 cases/100,000 children in Karnataka, 3.2 cases/100,000 children in Chennai, and 10.2 cases/100,000 children in Karnal (Haryana). The bottom line remains that Type 1 Diabetes Mellitus is quite prevalent and common.(12)

Type 2 diabetes mellitus is a common and increasingly prevalent illness that is largely preventable. In adults, type 2 diabetes accounts for about 90 to 95 percent of all diagnosed cases of diabetes mellitus; the remainder are adult-onset (or adult-diagnosed) type 1 diabetes mellitus, a form of diabetes mellitus for which the cause is unknown.(13) Many of the risk factors for type 2 diabetes mellitus include lifestyle decisions and can be eliminated or reduced with time and effort. Cases of diagnosed diabetes mellitus cost the United States an estimated $245 billion in 2012, a figure that is expected to rise with the increasing number of diagnosed individuals. Men are at slightly higher risk of developing diabetes mellitus than women, but age, excess weight (particularly around the waist), family history, physical inactivity, and poor diet are also significant risk factors for the illness. Although the exact prevalence is unknown, 4.6-9.2 percent of pregnancies may be affected by gestational diabetes, up to 10 percent of which result in a diagnosis of type 2 diabetes mellitus in the mother immediately following the pregnancy. Women who develop gestational diabetes mellitus during pregnancy have a 35 to 60 percent chance of developing type 2

diabetes mellitus within 10 to 20 years following the pregnancy. In general, a child has a 1 in 7 chance of developing diabetes mellitus if one parent was diagnosed before age 50. A child has a 1 in 13 chance if the parent was diagnosed after age 50. Some studies suggest that the child's risk of developing diabetes mellitus is greater if the mother has diabetes mellitus. If both parents have diabetes mellitus, the child's risk of developing it is approximately 50 percent. Research suggests that 1 out of 3 adults has pre-diabetes. Of this group, 9 out of 10 people don't know that they have pre-diabetes.

Type 2 diabetes mellitus risk increases with age. Although the number of children diagnosed with type 2 diabetes mellitus is increasing due to a growing number of overweight youth, it is considerably less common in children and young adults than in older individuals. Although men have a slightly increased risk of type 2 diabetes mellitus compared to women, this may be more significantly associated with lifestyle factors and body weight than innate gender differences. In 2004, high blood sugar as a result of diabetes mellitus led to an estimated 3.4 million deaths worldwide. More than eight of every 10 diabetes mellitus-related deaths occur in low- and middle-income countries. In developing nations, more than half of all diabetes mellitus cases go undiagnosed. WHO anticipates that worldwide deaths attributable to diabetes mellitus will double by 2030. Adults ages 40 to 59 comprise the world’s age group with the highest diabetes mellitus rates, although this is expected to shift to adults ages 60 to 79 by 2030.

Up to 85 percent of complications and morbidities among individuals with type 2 diabetes mellitus can be prevented, delayed, or effectively treated and minimized with regular visits to a health professional, appropriate monitoring and medication, and a healthy diet and lifestyle. Early identification of potential complications can provide

opportunities for intervention, education, and referral to a specialist when necessary.(13)

Currently, available evidence indicates that GDM presents a significant risk factor for development of type 2 diabetes mellitus and cardiovascular disease in women. Children whose mothers had diabetes mellitus during pregnancy are at increased risk of having obesity and type 2 diabetes mellitus at a young age. Future research will be needed to address how to determine whether better glucose control during pregnancy would prevent long-term consequences for both women and their children.(14)

Pathogenesis of Type 1 Diabetes Mellitus

It may be considered unusual to consider a period of three decades "historical." Yet, the evolution for our understanding of the natural history and pathogenesis of type 1 diabetes has been greatly advanced by a vast number of studies aimed at validating a model , proposed by the late Dr. George Eisenbarth in 1986 . As a result of this work, the majority of current conventional wisdom portrays type 1 diabetes mellitus as a T cell–mediated autoimmune disease involving the specific destruction of insulin-producing pancreatic β-cells.

In this model, persons destined to develop type 1 diabetes mellitus are assumed to begin life with a full cadre of β-cells. However, a "triggering" insult, likely initiates a process involving the recruitment of antigen-presenting cells. Antigen-presenting cells sequester self-antigens released by injured β-cells, followed by their transport to pancreatic lymph nodes where they are subsequently presented to autoreactive T cells. These T cells, rogue constituents brought to life due to genetically driven failures of thymic deletion (i.e., central tolerance) combined with defects in mechanisms designed to induce peripheral immune tolerance, come into play. This toxic duo, imparting lack-of-tolerance formation, again in the context of genetic susceptibility,

allows for migration of self-reactive T cells to islets, mediating β-cell killing and promoting further inflammation. When 85–90% of pancreatic β-cells meet their demise, symptoms of the disease occur. In the final stage of the model, the autoimmune process ends with the complete elimination of β-cells. (15)

Pathogenesis of Type 2 Diabetes Mellitus

The pathogenesis of type 2 diabetes mellitus is complex and involves the interaction of genetic and environmental factors. A number of environmental factors have been shown to play a critical role in the development of the disease, particularly excessive caloric intake leading to obesity and a sedentary lifestyle. The clinical presentation is also heterogeneous, with a wide range in age at onset, severity of associated hyperglycaemia, and degree of obesity. From a pathophysiologic stand point, persons with type 2 diabetes mellitus consistently demonstrate three cardinal abnormalities:

1. Resistance to the action of insulin in peripheral tissues,

particularly muscle and fat but also liver

2. Defective insulin secretion, particularly in response to a glucose stimulus

3. Increased glucose production by the liver

It has been suggested that the list of cardinal abnormalities in diabetes should be expanded to eight, adding accelerated lipolysis in the fat cell, incretin hormone deficiency and resistance, hyperglucagonemia, increased renal tubular reabsorption, and the role of the central nervous system (CNS) in metabolic regulation. Although the precise way in which genetic, environmental, and pathophysiologic factors interact to lead to the clinical onset of type 2 diabetes mellitus is not known, understanding of these processes has increased substantially. With the exception of specific monogenic forms of the disease that might result from defects largely confined to the pathways that regulate insulin action in muscle, liver, and fat or defects in insulin secretory

function in the pancreatic beta cell, it is currently believed that the common forms of type 2 diabetes mellitus are polygenic in nature and are caused by a combination of insulin resistance, abnormal insulin secretion, and other factors.

From a pathophysiologic standpoint, it is the inability of the pancreatic β-cell to adapt to the reductions in insulin sensitivity that occur over a lifetime that precipitates the onset of type 2 diabetes mellitus. The most common factors that place an increased secretory burden on the β-cell are puberty, pregnancy, a sedentary lifestyle, and overeating leading to weight gain. An underlying genetic predisposition appears to be a critical factor in determining the frequency with which beta cell failure occurs.

Genetically, type 2 diabetes mellitus consists of monogenic and polygenic forms. The monogenic forms, although relatively uncommon, are nevertheless important, and a number of the genes involved have been identified and characterized. The genes involved in the common polygenic forms of the disorder have been far more difficult to identify and characterize.

Insulin Basics

- There are different types of insulin depending on how quickly they work, when they peak, and how long they last.
- Insulin is available in different strengths; the most common is U-100.
- All insulin available in the United States is manufactured in a laboratory, but animal insulin can still be imported for personal use. Inside the pancreas, beta cells make the hormone insulin. With each meal, beta cells release insulin to help the body use or store the blood glucose it gets from food.

In people with **type 1 diabetes**, the pancreas no longer makes insulin. The beta cells have been destroyed and they need insulin shots to use glucose from meals. People with **type 2 diabetes** make insulin, but their bodies don't respond well to it. Some

people with type 2 diabetes need diabetes pills or insulin shots to help their bodies use glucose for energy.

Insulin cannot be taken as a pill because it would be broken down during digestion just like the protein in food. It must be injected into the tissue under your skin for it to get into your blood. In some rare cases insulin can lead to an allergic reaction at the injection site. Talk to your doctor if you believe you may be experiencing a reaction.

Types of Insulin

- **Rapid-acting insulin**, begins to work about 15 minutes after injection, peaks in about 1 hour, and continues to work for 2 to 4 hours. *Types: Insulin glulisine (Apidra), insulin lispro (Humalog), and insulin aspart (NovoLog)*
- **Regular or Short-acting insulin** usually reaches the bloodstream within 30 minutes after injection, peaks anywhere from 2 to 3 hours after injection, and is effective for approximately 3 to 6 hours. *Types: Humulin R, Novolin R*
- **Intermediate-acting insulin** generally reaches the bloodstream about 2 to 4 hours after injection, peaks 4 to 12 hours later, and is effective for about 12 to 18 hours. *Types: NPH (Humulin N, Novolin N)*
- **Long-acting insulin** reaches the bloodstream several hours after injection and tends to lower glucose levels fairly evenly over a 24-hour period. *Types: Insulin detemir (Levemir) and insulin glargine (Lantus)*

Premixed insulin can be helpful for people who have trouble drawing up insulin out of two bottles and reading the correct directions and dosages. It is also useful for those who have poor eyesight or dexterity and is convenient for people whose diabetes has been stabilized on this combination. In 2015 an inhaled insulin product, Afrezza, became available in the U.S. Afrezza is a rapid-acting inhaled insulin that is administered at the beginning of each meal and can be used by adults with type 1 or

type 2 diabetes. Afrezza is not a substitute for long-acting insulin. Afrezza must be used in combination with injectable long-acting insulin in patients with type 1 diabetes and in type 2 patients who use long-acting insulin.

 Inhaled insulin begins working within 12 to 15 minutes, peaks by 30 minutes, and is out of your system in 180 minutes. *Types: Technosphere insulin-inhalation system (Afrezza)*

Characteristics of Insulin

Insulin has 3 characteristics:

- **Onset** is the length of time before insulin reaches the bloodstream and begins lowering blood glucose.
- **Peaktime** is the time during which insulin is at maximum strength in terms of lowering blood glucose.
- **Duration** is how long insulin continues to lower blood glucose.

Insulin Strength

- All insulins come dissolved or suspended in liquids. The standard and most commonly used strength in the United States today is U-100, which means it has 100 units of insulin per milliliter of fluid, though U-500 insulin is available for patients who are extremely insulin resistant.
- U-40, which has 40 units of insulin per milliliter of fluid, has generally been phased out around the world, but it is possible that it could still be found in some places (and U-40 insulin is still used in veterinary care).
- If you're traveling outside of the U.S., be certain to match your insulin strength with the correct size syringe.

Monogenic Forms of Diabetes Mellitus

In the monogenic forms of diabetes mellitus, the gene involved is both necessary and sufficient to cause disease. In other words, environmental factors play little or no role in determining whether a genetically predisposed person develops clinical diabetes mellitus. The monogenic forms of diabetes mellitus usually are diagnosed in younger patients, often in the first 2 to 3 decades of life; however, if only mild, asymptomatic elevations in blood glucose occur, the diagnosis may be missed until later in life.

Monogenic Forms of Diabetes Mellitus Associated with Insulin Resistance

Mutations in the Insulin Receptor.

Numerous mutations have been identified in the insulin receptor gene in various insulin-resistant patients. At least three clinical syndromes are caused by mutations in the insulin receptor gene. Type A insulin resistance is defined by the presence of insulin resistance, acanthosis nigricans, and hyperandrogenism. Patients with leprechaunism have multiple abnormalities, including intrauterine growth retardation, fasting hypoglycaemia, and death within the first 1 to 2 years of life. The Rabson-Mendenhall syndrome is associated with short stature, protuberant abdomen, and abnormalities of teeth and nails; pineal hyperplasia was a characteristic in the original description of this syndrome.

These mutations impair receptor function by a number of different mechanisms, including decreasing the number of receptors expressed on the cell surface, such as by decreasing the rate of receptor biosynthesis (class 1), accelerating the rate of receptor degradation (class 5), or inhibiting the transport of receptors to the plasma membrane (class 2). The intrinsic function of the receptor may be abnormal if the affinity of insulin binding is reduced (class 3) or if receptor tyrosine kinase is inactivated (class 4). The insulin resistance that is associated with insulin receptor mutations can be

severe, manifesting in the neonatal period (e.g., leprechaunism and Rabson-Mendenhall syndrome), or it can occur in a milder form in adulthood, leading to insulin-resistant diabetes with marked hyperinsulinemia, acanthosis nigricans, and hyperandrogenism.

Monogenic Forms of Diabetes Mellitus Associated with Defects in Insulin Secretion (5)

Mutant Insulin Syndromes.

The first syndrome associated with diabetes mellitus to be characterized in terms of the clinical picture, genetic mechanisms, and clinical pathophysiology was that associated with mutant insulin or proinsulin. Persons with this disorder present clinically with a mild, non–insulin-dependent form of diabetes mellitus. Affected persons characteristically have marked hyperinsulinemia on routine insulin assays. Increases in the concentration of insulin in association with diabetes mellitus usually indicate insulin resistance, but in this syndrome, insulin resistance can be easily excluded because the patients respond normally to administration of exogenous insulin. Characterization of the insulin by high-performance liquid chromatography (HPLC) reveals that the hyperinsulinemia results from the presence of the abnormal insulin or proinsulin and related breakdown products. The increased concentrations of insulin appear to be related to the presence of mutations in regions of the insulin molecule that are important for receptor binding, particularly the carboxy-terminus of the insulin B chain.

Table 2. 2016 - 2017 ADA guidelines for diagnosing diabetes (17)

FPG ≥126 mg/dL (7.0 mmol/L). Fasting is defined as no caloric intake for at least 8 h.*
OR
2-h PG ≥200 mg/dL (11.1 mmol/L) during OGTT. The test should be performed as described by the WHO, using a glucose load containing the equivalent of 75 g anhydrous glucose dissolved in water.*
OR
A1C ≥6.5% (48 mmol/mol). The test should be performed in a laboratory using a method that is NGSP certified and standardized to the DCCT assay.*
OR
In a patient with classic symptoms of hyperglycemia or hyperglycemic crisis, a random plasma glucose ≥200 mg/dL (11.1 mmol/L).

Complications of Diabetes Mellitus

The importance of protecting the body from hyperglycaemia cannot be overstated; the direct and indirect effects on the human vascular tree are the major source of morbidity and mortality in both type 1 and type 2 diabetes mellitus. Generally, the injurious effects of hyperglycaemia are separated into macrovascular complications (coronary artery disease, peripheral arterial disease, and stroke) and microvascular complications (diabetic nephropathy, neuropathy, and retinopathy)

Microvascular Complications

Diabetic retinopathy

Diabetic retinopathy may be the most common microvascular complication of diabetes mellitus. It is responsible for ~ 10,000 new cases of blindness every year in the United States alone. The risk of developing diabetic retinopathy or other microvascular complications of diabetes mellitus depends on both the duration and the severity of hyperglycaemia. Most patients with type 1 diabetes mellitus develop evidence of retinopathy within 20 years of diagnosis. Retinopathy may begin to

develop as early as 7 years before the diagnosis of diabetes mellitus in patients with type 2 diabetes mellitus.

Diabetic nephropathy

Diabetic nephropathy is the leading cause of renal failure in the United States. It is defined by proteinuria > 500 mg in 24 hours in the setting of diabetes, but this is preceded by lower degrees of proteinuria, or "microalbuminuria". Microalbuminuria is defined as albumin excretion of 30-299 mg/24 hours. Without intervention, diabetic patients with microalbuminuria typically progress to proteinuria and overt diabetic nephropathy. This progression occurs in both type 1 and type 2 diabetes mellitus.

Diabetic neuropathy

Diabetic neuropathy is recognized by the American Diabetes Association (ADA) as "the presence of symptoms and/or signs of peripheral nerve dysfunction in people with diabetes after the exclusion of other causes." As with other microvascular complications, risk of developing diabetic neuropathy is proportional to both the magnitude and duration of hyperglycaemia, and some individuals may possess genetic attributes that affect their predisposition to developing such complications.

Macrovascular Complications of Diabetes Mellitus

The central pathological mechanism in macrovascular disease is the process of atherosclerosis, which leads to narrowing of arterial walls throughout the body.

Diabetes Mellitus is a strong independent predictor of risk of stroke and cerebrovascular disease, as in coronary artery disease. Patients with type 2 diabetes mellitus have a much higher risk of stroke, with an increased risk of 150-400%. Risk of stroke-related dementia and recurrence, as well as stroke-related mortality, is elevated in patients with diabetes mellitus.

Patients with type 1 diabetes mellitus also bear a disproportionate burden of coronary heart disease. Studies of have shown that these patients have a higher mortality from ischemic heart disease at all ages compared to the general population. In individuals > 40 years of age, women experience a higher mortality from ischemic heart disease than men. Observational studies have shown that the cerebrovascular mortality rate is elevated at all ages in patients with type 1 diabetes mellitus.

Practice Recommendations

Patients with type 1 diabetes mellitus of > 5 years' duration should have annual screening for microalbuminuria, and all patients with type 2 diabetes mellitus should undergo such screening at the time of diagnosis and yearly thereafter. All patients with diabetes mellitus should have serum creatinine measurement performed annually. Patients with microalbuminuria or macroalbuminuria should be treated with an ACE inhibitor or ARB unless they are pregnant or cannot tolerate the medication. Patients who cannot tolerate one of these medications may be able to tolerate the other. Potassium should be monitored in patients on such therapy. Patients with a GFR < 60 ml/min or with uncontrolled hypertension or hyperkalemia may benefit from referral to a nephrologist.

Patients with type 1 diabetes mellitus should receive a comprehensive eye examination and dilation within 3-5 years after the onset of diabetes mellitus. Patients with type 2 diabetes mellitus should undergo such screening at the time of diagnosis. Patients should strive for optimal glucose and blood pressure control to decrease the likelihood of developing diabetic retinopathy or experiencing progression of retinopathy.

All patients with diabetes mellitus should undergo screening for distal symmetric polyneuropathy at the time of diagnosis and yearly thereafter. Atypical features may

prompt electrophysiological testing or testing for other causes of peripheral neuropathy. Patients who experience peripheral neuropathy should begin appropriate foot self-care, including wearing special footwear to decrease their risk of ulceration. They may also require referral for podiatric care. Screening for autonomic neuropathy should take place at the time of diagnosis in type 2 diabetes mellitus and beginning 5 years after the diagnosis of type 1 diabetes mellitus. Medication to control the symptoms of painful peripheral neuropathy may be effective in improving quality of life in patients but do not appear to alter the natural course of the disease. For this reason, patients and physicians should continue to strive for the best possible glycaemic control.

In light of the above strong evidence linking diabetes mellitus and CVD and to control and prevent the microvascular complications of diabetes mellitus, the ADA has issued practice recommendations regarding the prevention and management of diabetes complications.

Blood pressure should be measured routinely. Goal blood pressure is < 130/80 mmHg. Patients with a blood pressure ≥ 140/90 mmHg should be treated with drug therapy in addition to diet and lifestyle modification. Patients with a blood pressure of 130-139/80-89 mmHg may attempt a trial of lifestyle and behavioural therapy for 3 months and then receive pharmacological therapy if their goal blood pressure is not achieved. Initial drug therapy should be with a drug shown to decrease CVD risk, but all patients with diabetes and hypertension should receive an ACE inhibitor or ARB in their antihypertensive regimen.

Lipid testing should be performed in patients with diabetes at least annually. Lipid goals for adults with diabetes mellitus should be an LDL < 100 mg/dl (or < 70 mg/dl in patients with overt CVD), HDL > 50 mg/dl, and fasting triglycerides < 150 mg/dl.

All patients with diabetes mellitus should be encouraged to limit consumption of saturated fat, trans fat, and cholesterol. Statin therapy to lower LDL by 30-40% regardless of baseline is recommended to decrease the risk of CVD in patients > 40 years of age. Patients < 40 years of age may also be considered for therapy. In individuals with overt CVD, special attention should be paid to treatment to lower triglycerides or raise HDL. Combination therapy with a statin plus other drugs, such as fibrates or niacin, may be necessary to achieve ideal lipid control, but patients should be monitored closely for possible adverse reactions of therapy.

Aspirin therapy (75-162 mg/day) is indicated in secondary prevention of CVD and should be used in patients with diabetes mellitus who are > 40 years of age and in those who are 30-40 years of age if other risk factors are present. Patients < 21 years of age should not receive aspirin therapy because of the risk of Reye's syndrome. Patients who cannot tolerate aspirin therapy because of allergy or adverse reaction may be considered for other antiplatelet agents.

In addition to the above pharmacological recommendations, patients with diabetes mellitus should be encouraged to not begin smoking or to stop smoking to decrease their risk of CVD and benefit their health in other ways. It should also be noted that statins, ACE inhibitors, and ARBs are strongly contraindicated in pregnancy.(16,17)

Treatment

Diabetes Self-Management Education and Support

Effective self-management, improved clinical outcomes, health status, and quality of life are key outcomes of DSME/S and should be measured and monitored as part of care. There are four critical time points for DSME/S delivery: at diagnosis; annually for assessment of education, nutrition, and emotional needs; when new complicating factors arise that influence self-management; and when transitions in care occur.

Medical Nutrition Therapy

The goals of medical nutrition therapy (MNT) are to promote and support healthful eating patterns, emphasizing a variety of nutrient-dense foods in appropriate portion sizes to achieve/maintain body weight goals; attain glycemic, lipid, and blood pressure goals; and delay/prevent complications of diabetes. MNT addresses individual nutrition needs based on personal and cultural preferences, health literacy, and access to healthful foods. It maintains the pleasure of eating by providing non-judgmental messages about food choices and offers practical tools for developing healthy patterns. All individuals should be encouraged to replace refined carbohydrates and added sugars with whole grains, legumes, vegetables, and fruit. Individuals who take mealtime insulin should be offered intensive education on coupling insulin administration with carbohydrate intake.

Physical Activity

Children with diabetes mellitus or prediabetes should be encouraged to engage in at least 60 minutes of physical activity each day. Adults with diabetes mellitus should be advised

to perform at least 150 min/week of moderate-intensity aerobic physical activity (50–70% maximum heart rate), spread over at least 3 days/week with no more than 2 consecutive days without exercise.

All individuals, including those with diabetes mellitus, should be encouraged to reduce sedentary time, particularly by breaking up extended amounts of time (>90 min) spent sitting. In the absence of contraindications, adults with type 2 diabetes mellitus should be encouraged to perform resistance training at least twice per week. Physical activity is a general term that includes all movement that increases energy use and is an important part of the diabetes management plan. Exercise is a more

specific form of physical activity that is structured and designed to improve physical fitness.

Immunizations

Provide routine vaccinations for children and adults with diabetes mellitus as for the general population according to age-related recommendations. Administer hepatitis B vaccine to unvaccinated adults with diabetes who are aged 19–59 years. Consider administering hepatitis B vaccine to unvaccinated adults with diabetes who are aged ≥60 years.

Prevention or Delay of Type 2 Diabetes Mellitus

Patients with prediabetes should be referred to an intensive diet and physical activity behavioral counseling program adhering to the tenets of the Diabetes mellitus Prevention Program (DPP) targeting loss of 7% of body weight and should increase their moderate physical activity (such as brisk walking) to at least 150 min/week. Metformin therapy for prevention of type 2 diabetes should be considered in those with prediabetes, especially in those with a BMI >35 kg/m2, those aged <60 years and woman with prior GDM. At least annual monitoring for the development of diabetes in those with prediabetes is suggested. Screening for and treatment of modifiable risk factors for CVD is suggested. Intensive lifestyle modification programs have been shown to be very effective (~58% risk reduction after 3 years). In addition, pharmacological agents such as metformin, α-glucosidase inhibitors, orlistat, and thiazolidinediones have been shown to decrease incident diabetes to various degrees. Metformin has demonstrated long-term safety as pharmacological therapy for diabetes prevention.

Glycemic Targets:

Assessment of Glycaemic Control

Patients on multiple-dose insulin or insulin pump therapy should perform self-monitoring of blood glucose (SMBG) before meals and snacks, at bedtime, before exercise, when they suspect low blood glucose, after treating low blood glucose until they are normoglycemic, and before critical tasks such as driving.

SMBG allows patients to evaluate their individual response to therapy and assess whether glycaemic targets are being achieved. Results of SMBG can be useful in preventing hypoglycaemia and adjusting medications (particularly prandial insulin doses), MNT, and physical activity. Evidence also supports a correlation between SMBG frequency and lower A1C.

HbA1c Testing

Perform the HbA1c test at least two times per year in patients who are meeting treatment goals (and who have stable glycaemia control). Perform the HbA1c test quarterly in patients whose therapy has changed or who are not meeting glycaemic goals. Use of point-of-care testing for HbA1c provides the opportunity for more timely treatment changes.

Obesity Management for The Treatment of Type 2 Diabetes

There is strong and consistent evidence that obesity management can delay progression from prediabetes to type 2 diabetes mellitus and benefits type 2 diabetes treatment. In overweight and obese patients with type 2 diabetes mellitus, modest weight loss, defined as sustained reduction of 5% of initial body weight, has been shown to improve glycaemic control and triglycerides and to reduce the need for glucose-lowering medication. Sustained weight loss of ≥7% is optimal.

Pharmacological Therapy for Type 1 Diabetes Mellitus

Most people with type 1 diabetes should be treated with multiple-dose insulin injections (three to four injections per day of basal and prandial insulin) or continuous subcutaneous insulin infusion therapy. Consider educating individuals with type 1 diabetes mellitus on matching prandial insulin doses to carbohydrate intake, premeal blood glucose levels, and anticipated physical activity. Most individuals with type 1 diabetes mellitus should use insulin analogs to reduce hypoglycaemia risk.

Pharmacological Therapy for Type 2 Diabetes

Metformin, if not contraindicated and if tolerated, is the preferred initial pharmacological agent for type 2 diabetes. Consider initiating insulin therapy (with or without additional agents) in patients with newly diagnosed type 2 diabetes mellitus who are symptomatic and/or have markedly elevated blood glucose levels or HbA1C. If noninsulin monotherapy at the maximum tolerated dose does not achieved or maintain the HbA1C target over 3 months, then add a second oral agent, a glucagon-like peptide 1 receptor agonist, or basal insulin.(**16)**

The following drugs were used in the study

1. Glimepiride
2. Metformin
3. Vitamin D

1. GLIMEPIRIDE

Glimepiride is a long-acting, third-generation sulfonylurea with hypoglycaemic activity. Compared to other generations of sulfonylurea compounds, Glimepiride is very potent and has a longer duration of action. This agent is metabolized by CYP2C9 and shows peroxisome proliferator-activated receptor gamma (PPAR gamma) agonistic activity.

4-ethyl-3-methyl-N-[2-[4-[(4methylcyclohexyl)carbamoylsulfamoyl]phenyl] ethyl]-5-oxo-2H-pyrrole-1 carboxamide.17

Medical Uses

It is used for treating type 2 diabetes mellitus in patients who cannot control blood sugar levels by diet and exercise alone. It is used along with diet and exercise. It may be used alone or with other antidiabetic medicines. Glimepiride is a sulfonylurea antidiabetic medicine. It works by causing the pancreas to release insulin, which helps to lower blood sugar. (20)

Mechanism of action

Glimepiride primarily lowers blood glucose by stimulating the release of insulin from pancreatic β cells. Sulfonylureas bind to the receptor in the pancreatic β-cell plasma (**18)** |membrane, leading to closure of the ATP-sensitive potassium channel, thereby stimulating the release of insulin. (21)

Pharmacokinetics

Absorption

Studies with single oral doses of Glimepiride in healthy subjects and with multiple oral doses in patients with type 2 diabetes mellitus showed peak drug concentrations (Cmax) 2 to 3 hours post-dose. When Glimepiride was given with meals, the mean Cmax and AUC (area under the curve) were decreased by 8% and 9%, respectively. Glimepiride does not accumulate in serum following multiple dosing. The pharmacokinetics of Glimepiride does not differ between healthy subjects and patients with type 2 diabetes mellitus. Clearance of Glimepiride after oral administration does not change over the 1 mg to 8 mg dose range, indicating linear pharmacokinetics. In healthy subjects, the intra- and inter-individual variabilities of Glimepiride pharmacokinetic parameters were 15–23% and 24–29%, respectively.

Excretion

When C-glimepiride was given orally to 3 healthy male subjects, approximately 60% of the total radioactivity was recovered in the urine in 7 days. M1 and M2 accounted for 80–90% of the radioactivity recovered in the urine. The ratio of M1 to M2 in the urine was approximately 3:2 in two subjects and 4:1 in one subject. Approximately 40% of the total radioactivity was recovered in faeces. M1 and M2 accounted for about 70% (ratio of M1 to M2 was 1:3) of the radioactivity recovered in feces. No parent drug was recovered from urine or faeces. After intravenous dosing in patients, no significant biliary excretion of Glimepiride or its M1 metabolite was observed. (21)

Side effects and contraindications

The major side effects are Severe allergic reactions (rash; hives; itching; difficulty breathing; tightness in the chest; swelling of the mouth, face, lips, or tongue); chest pain or irregular heartbeat; confusion; dark urine; fainting; fever, chills, or persistent sore throat;

low blood sugar symptoms (eg, anxiety, dizziness, drowsiness, fast heartbeat, headache, light headedness, tremors, unusual sweating, weakness); severe or persistent blurred vision or other vision problems; unusual bruising or bleeding; unusual tiredness or weakness; yellowing of the eyes or skin.

The use of Glimepiride is contraindicated in patients with history of hypersensitivity and during pregnancy. **(22)**

2. METFORMIN

Metformin is a Biguanide. The chemical classification of metformin is Biguanides. Metformin is an agent belonging to the biguanide class of antidiabetics with antihyperglycemic activity. Metformin is associated with a very low incidence of

lactic acidosis. This agent helps to reduce LDL cholesterol and triglyceride levels, and is not associated with weight gain, and prevents the cardiovascular complications of diabetes. Metformin is not metabolized and is excreted unchanged by the kidneys.

Figure 3. 3-(diaminomethylidene)-1,1-dimethylguanidine

Mechanism of action

The major clinical activity of metformin is to reduce hepatic gluconeogenesis and glucose production. It has more inconsistently improved insulin sensitivity in peripheral tissues. Because of its limited duration of action, it is usually taken at least twice daily, although a sustained-release formulation is available. (23) Metformin is a potent antihyperglycemic agent widely used in the management of type 2 diabetes mellitus whose main actions are the suppression of gluconeogenesis and the improvement of glucose uptake and insulin sensitivity. (23)

Medical Uses

Metformin is used with a proper diet and exercise program and possibly with other medications to control high blood sugar. It is used in patients with type 2 diabetes mellitus. Controlling high blood sugar helps prevent kidney damage, blindness, nerve problems, loss of limbs, and sexual function problems. Proper control of diabetes mellitus may also lessen risk of a heart attack or stroke. Metformin works by helping to restore body's proper response to the insulin you naturally produce. It also decreases the amount of sugar that liver makes and that stomach/intestines absorb. (24, 25)

Pharmacokinetics

Metformin is not metabolized and is excreted unchanged in the urine, with a half-life of ~5 h. The population mean for renal clearance (CLr) is 510±120 ml/min. Active tubular secretion in the kidney is the principal route of metformin elimination. The

drug is widely distributed into body tissues including the intestine, liver, and kidney by organic cation transporters. There is a large interindividual variability in metformin pharmacokinetics as measured by differences in trough steady-state metformin plasma concentration ranging from 54 to 4133 ng/ml. (26)

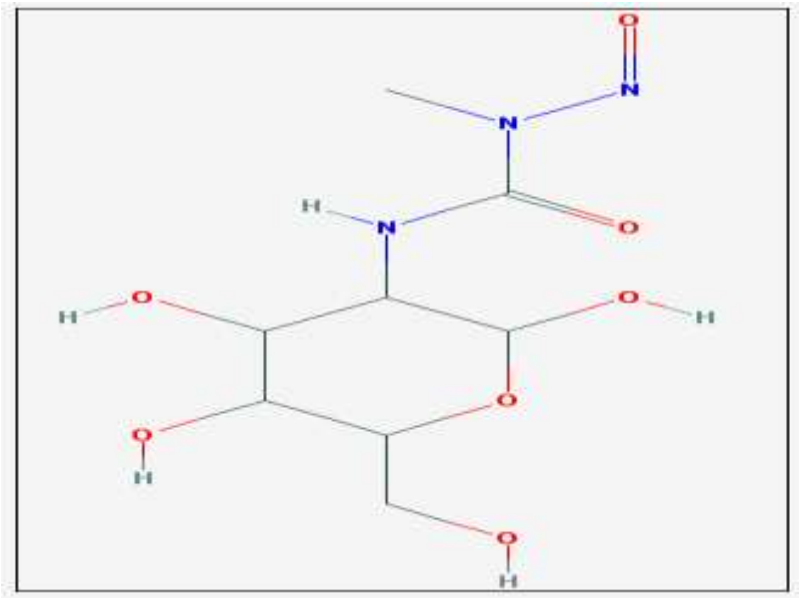
Adverse effects

The most common adverse events are gastrointestinal: nausea, diarrhoea, crampy abdominal pain, and dysgeusia. About one third of patients have some gastrointestinal distress, particularly early in their course of treatment. Metformin also causes lactic acidosis, which is quite rare and occurs almost exclusively in patients who are at high risk for development of the condition independent of metformin therapy. Metformin is also contraindicated in patients with hepatic insufficiency and in the setting of alcohol abuse. Some patients taking metformin develop progressive vitamin B12 deficiency.(24)

OTHER DRUGS USED IN THE STUDY

1. STREPTOZOTOCIN

STREPTOZOTOCIN is an antibiotic that is produced by *Stretomyces achromogenes.* It is used as an antineoplastic agent and to induce diabetes in experimental animals. The chemical structure is a as shown in the figure below



1-methyl-1-nitroso-3-[2,4,5-trihydroxy-6-(hydroxymethyl) oxan-3-yl]urea (17)

Mechanism of action

Streptozotocin (STZ, 2-deoxy-2-(3-(methyl-3-nitrosoureido)-D-glucopyranose) is synthesized by *Streptomycete sachromogenes* and is used to induce both insulin-dependent and non-insulin-dependent diabetes mellitus. Both effects can be attributed to its specific chemical properties, namely its alkylating potency. Its β cell specificity is mainly the result of selective cellular uptake and accumulation.

Beta cell toxicity of Streptozotocin

It is generally assumed that the toxicity of streptozotocin is dependent upon the DNA alkylating activity of its methyl nitrosourea moiety, especially at the O6 position of guanine. The transfer of the methyl group from streptozotocin to the DNA molecule causes damage, which along a defined chain of events, results in the fragmentation of the DNA. Protein glycosylation may be an additional damaging factor. In the attempt to repair DNA, poly(ADP-ribose) polymerase (PARP) is overstimulated. This diminishes cellular NAD+, and subsequently ATP, stores. The depletion of the cellular energy stores ultimately results in beta cell necrosis. Although streptozotocin also methylates proteins, DNA methylation is ultimately responsible for beta cell

death, but it is likely that protein methylation contributes to the functional defects of the beta cells after exposure to streptozotocin.(27)

Dose and route of administration

The frequently used single intraperitoneal dose in adult rats to induce DM is between 40 and 60mg/kg b.w., but higher doses are also used.

Side effects

1. An allergic reaction (difficulty in breathing; closing of the throat; swelling of the lips, tongue, or face; or hives);

2. Kidney damage (little or no urine production, blood in the urine);

3. Liver problems (changes in blood test results, abdominal pain, yellowing of the skin or eyes, decreased appetite, nausea);

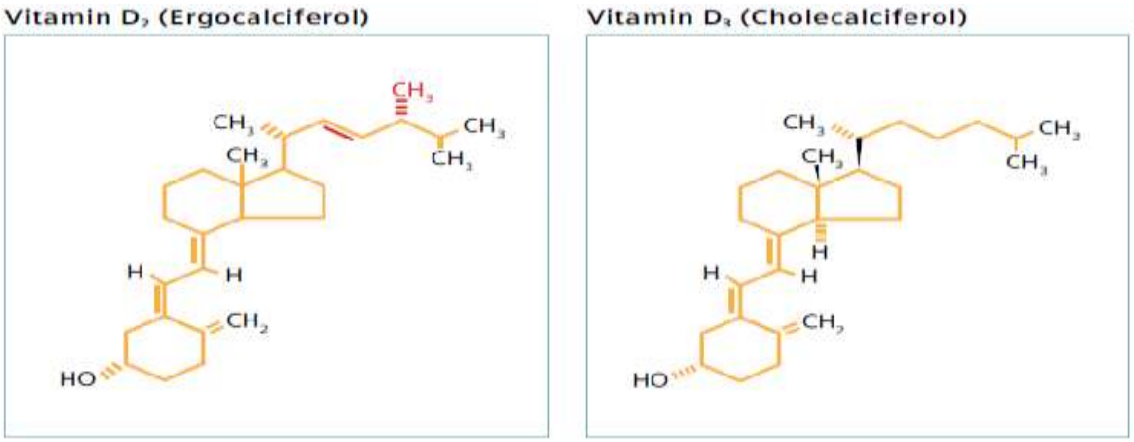
4. Decreased bone marrow function and blood problems (extreme fatigue; easy bruising or bleeding;

VITAMIN D

Vitamin D is a fat-soluble steroid with many roles in the body. In nature, only a few foods products contain Vitamin D, like fatty fish, liver and egg yolk. Many foods, though, are supplemented with Vitamin D, most notably milk and breakfast cereal. Much of the Vitamin D produced in the body is due to exposure of skin to the sun.

An adult in a bathing suit exposed to one small erythematous dose of ultraviolet radiation (a mild pinkness of the skin after exposure) is equal to ingesting 10,000 –

25,000 IU of Vitamin D. A number of factors restrict the development of Vitamin D3 in the skin, such as ageing, sunscreen use and increased pigmentation of the skin. Ultraviolet (UV) wavelength of sun usually between 290-315 nm and the total number of photons absorbed by 7-dehydrocholesterol in the skin



At the cellular level there are two types of responses to 1,25(OH) D : the rapid response which occurs within seconds to minutes and the genomic response which occurs from minutes to days (26). In the rapid response the active Vitamin D binds to either Vitamin D receptor (VDR) 16 or the Vitamin D membrane associated rapid response steroid binding protein (MARRS) in the plasma membrane. This activates the G protein that causes increased intracellular cyclic AMP which increases the activity of protein kinase C and mitogen activated protein kinase leading to biologic responses (27). During the genomic response the 1,25(OH) binds to nuclear VDR and dimerizes with retinoic X receptor (RXR) forming a heterodimer which binds to Vitamin D responsive elements

(VDRE) modulating tissue specific gene expressions and protein synthesis (28) (29). Ultimately this genomic effect will lead to physiological responses.

Vitamin D was originally classified as a nutrient when cod liver oil (a source of Vitamin D) was found to have an antirachitic effect in infants. However, since the discovery of the Vitamin D receptor in 1969 it has been increasingly recognized that

the term “vitamin” D is inappropriate, and that this class of molecules is better defined as a complex endocrine system (28). In terms of its structure and mode of action, Vitamin D is similar to the classic steroid hormones. Indeed, although Vitamin D3 can be found in the diet, the human body can produce it in the skin, by photoconversion of 7-dehydrocholesterol to Vitamin D3. Vitamin D3 can be considered a pro-hormone, which is not known to have any intrinsic biological activity itself. In fact, as will be discussed in detail, Vitamin D3 can be transformed in an active compound, 1,25(OH)2D3 (calcitriol), through two hydroxylation steps: metabolism of Vitamin D3 by the liver to 25(OH)D3 (which is the most abundant form of circulating Vitamin D, transported by the Vitamin D binding protein), and conversion by the kidney to 1,25(OH)2D3. In addition, 25(OH)D3 can be converted to the alternative, much less active dihydroxylated metabolite, 24,25(OH)2D3. Binding of calcitriol to a specific receptor at the target organs is then followed by the generation of specific biological responses (29). Therefore, the reasons for considering calcitriol a hormone are the following: a) endogenous synthesis; b) presence of a specific receptor; c) action in many organ and tissues, different from the organ where it is synthesized; d) induction of specific biologic responses after the interaction with its receptor.

The biological actions of the Vitamin D hormonal system are not limited to the target organs required for mineral ion homeostasis (intestine, bone, kidney, and parathyroid). In addition to endocrine production of circulating 1,25(OH)2D3 by the kidney, a paracrine production of this steroid hormone in several extra-renal organs has been demonstrated. The Vitamin D receptor is present in even more cell types, and calcitriol can elicit specific biological actions in such tissues. In this article, we will summarize the most relevant information on the physiologic aspects of the Vitamin D hormonal system.

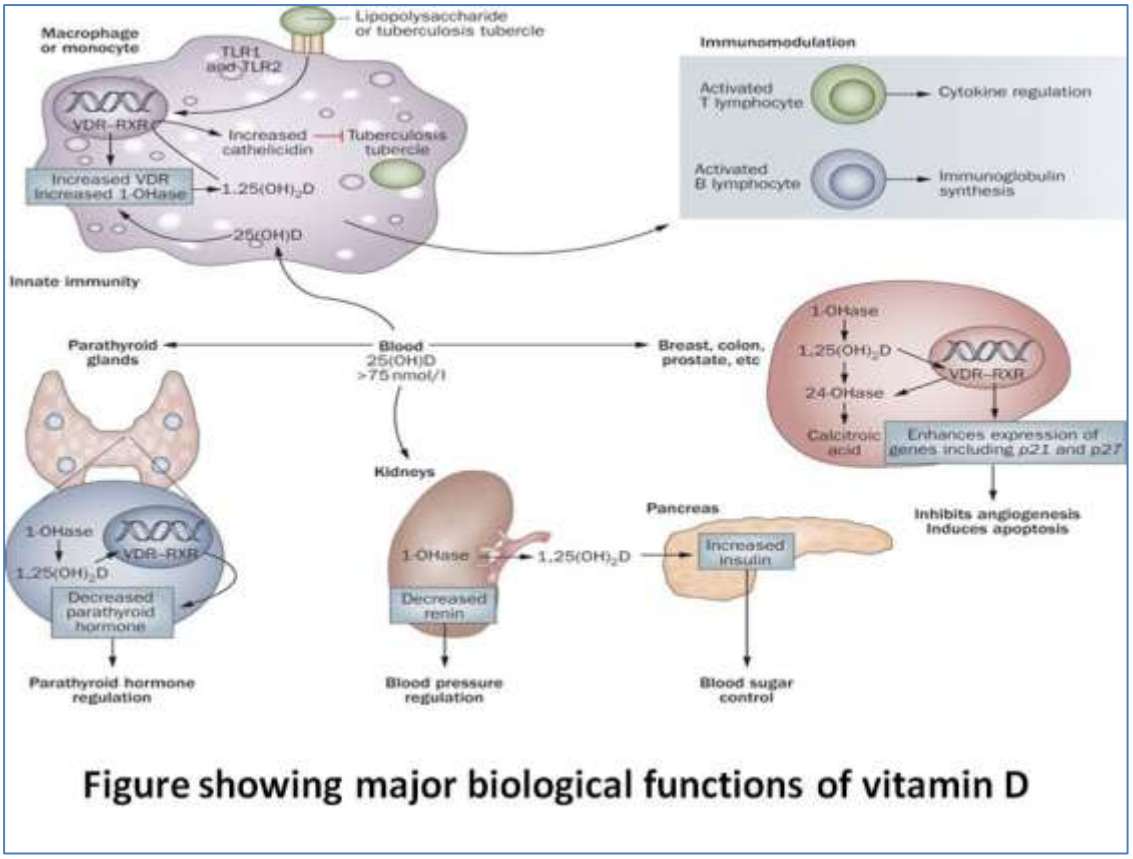
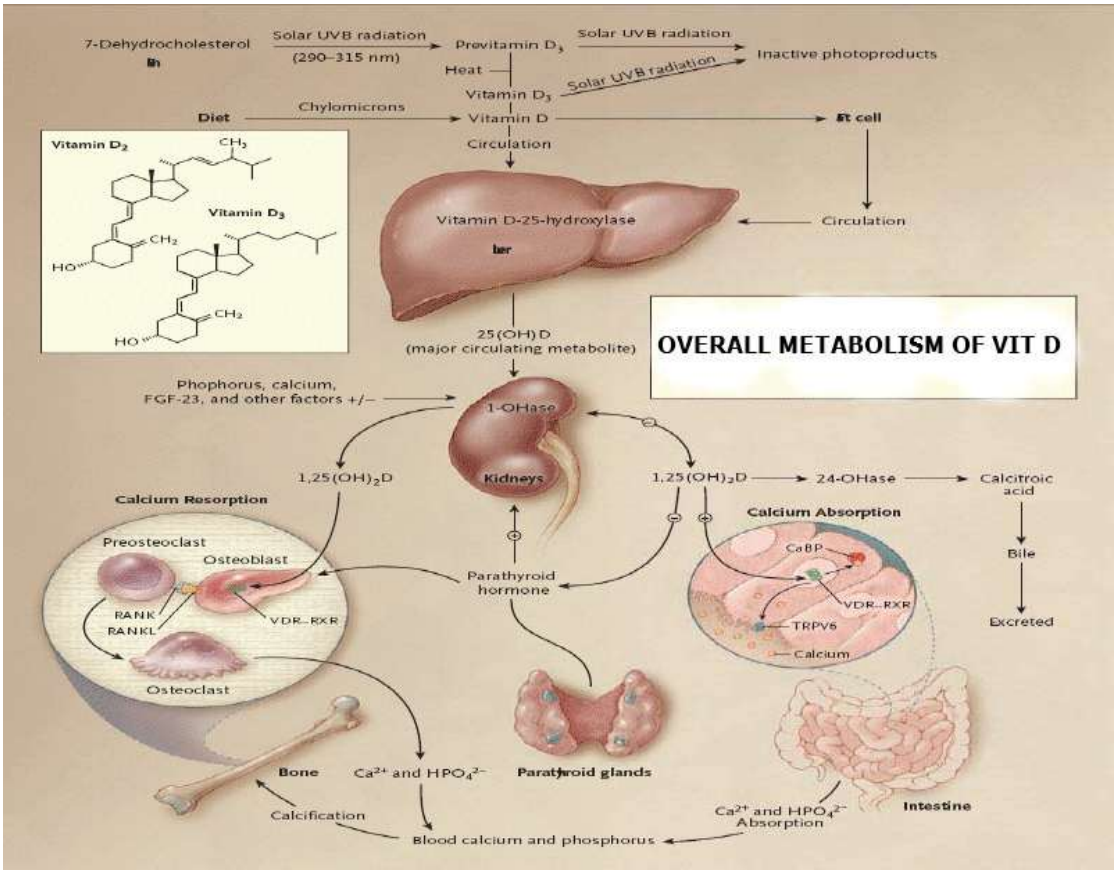


Figure showing major biological functions of vitamin D

VITAMIN D METABOLISM

There are two forms of Vitamin D: cholecalciferol (Vitamin D3) and ergocalciferol (Vitamin D2). Cholecalciferol derives from dietary sources (oily fish, such as salmon and mackerel, animal liver, fish liver oils, eggs), but it is also produced in the skin from 7-dehydrocholesterol (7- DHC, or proVitamin D3). During exposure to light, 7-DHC absorbs solar radiation (ultraviolet B rays or UVB, wavelengths 290-315 nm), which causes its transformation to preVitamin D3. In the following few hours, the latter undergoes a temperature- (heat-) dependent isomerization to cholecalciferol. Cholecalciferol is then transported from the skin to the circulation, where it is bound by the Vitamin D binding protein. No cases of Vitamin D intoxication from an excessive exposure to sunlight have been described, probably because prolonged UVB radiation determines local inactivation of preVitamin D3 and Vitamin D3. Latitude, time of day and season of the year markedly affect skin production of

cholecalciferol. In addition, melanin competes with 7-DHC for UVB photons, therefore decreasing cholecalciferol production. In fact, longer exposures to sun are required in people with darker skin to produce cholecalciferol. The elderly also require longer sun exposure to produce the same amount of cholecalciferol, because their skin content of 7-DHC is reduced up to 70% compared to young adults (30)



Ergocalciferol is created from viosterol, which in turn is created when ultraviolet light activates ergosterol. Ergosterol is a component of fungal cell membranes, serving the same function that cholesterol serves in animal cells. Ergocalciferol is therefore a synthetic molecule, which in some countries can be found in fortified foods (milk, cereals, bread products) or which can be administered as a supplement, orally or parenterally. Recently, Armas et al demonstrated that ergocalciferol is much less effective than cholecalciferol in humans (31), justifying possible different activities of synthetic Vitamin D receptor activators (or Vitamin D analogs), derived from the two

parent molecules, Vitamin D3 and D2. In the absence of adequate exposure to sunlight, administration of Vitamin D is necessary to avoid negative clinical consequences due to Vitamin D deficiency. In western countries, the recommended adequate intake is debated: an intake of 200 – 600 IU/day, depending on age, is generally considered desirable (32), but some authors advocate the use of much higher amounts of between 600 and 1000 UI/day (6). One article suggests the need to increase the tolerable upper intake level for Vitamin D up to 10,000 IU/day (33). Cholecalciferol, and ergocalciferol as well, are enzymatically hydroxylated at carbon 25 in the liver and at carbon 1 in the kidney. The key kidney enzymes, the 25(OH)D3-1α-hydroxylase and the 25(OH)D3-24- hydroxylase, as well as the liver Vitamin D3 25-hydroxylase, are all known to be cytochrome P-450 mixed-function oxidases. Both renal enzymes are located in mitochondria of the proximal tubules. The active metabolite of Vitamin D metabolism is 1,25-dihydroxycholecalciferol (1,25(OH)2D3 or calcitriol), but it should be kept in mind that its serum concentrations do not correlate with Vitamin D stores Calcitriol promotes intestinal absorption of calcium and phosphate, and bone mineralization. Conversely, it suppresses PTH synthesis and parathyroid cell proliferation through a genomic mechanism (34). Genomic effects of calcitriol in target cells are modulated by specific cytosolic receptors for Vitamin D (VDR), which form a heterodimer with the retinoid X receptor (RXR). The complex calcitriol-VDR-RXR interacts with several transcriptional factors (co-activators and/or co-repressors) influencing its binding to a specific Vitamin D response element (VDRE) on the transcription promoters of Vitamin D-sensitive genes. The VDR has been detected in Vitamin D-sensitive tissues (bone, intestine, kidney, and parathyroid glands) and in several other tissues (e.g., myocardium, brain, pancreas, and testis). In addition to the genomic effect, a rapid non-genomic effect of 1,25(OH)2D has been

observed in intestinal cells. Although 25-hydroxy-cholecalciferol, or 25(OH)D3 is 1000 times less active than calcitriol, 25(OH)D3 maintains some biological effects because its serum levels are 1000 times higher than those of calcitriol, thus compensating for the low affinity for VDR (35). Serum concentrations of 25(OH)D3 are the best indicator of Vitamin D body stores. The physiopathological relevance of 25(OH)D has been recently revaluated in population studies showing that low serum concentrations of 25(OH)D3 were associated with higher serum PTH in healthy elderly individuals (36). PTH levels were normal in patients with serum 25(OH)D3 concentrations above 30 mcg/L (75 nmol/L). This threshold for secondary hyperparathyroidsim was also confirmed in elderly individuals with elevated creatinine clearance (37) and in hemodialysis patients (38). Based on these findings, the normal range of 25(OH)D serum concentrations was recently redefined and concentration above 30 mcg/L (75 nmol/L) are now generally recommended to prevent and/or treat secondary hyperparathyroidism (36). Hemodialysis patients with low levels of 25(OH)D3 may more easily show radiological signs of osteomalacia and decreased bone formation at bone histology, regardless of calcitriol levels (38, 39). However, excessively high 25(OH)D levels may be associated with low turnover osteodystrophy (39). The beneficial effects of maintaining normal Vitamin D stores suggest that an unknown Vitamin D metabolite, besides 1,25(OH)2D, may have a beneficial effect on bone and parathyroid metabolism in end-stage renal disease. Regulation of the Vitamin D endocrine system mainly occurs through the stringent control of renal 1α-hydroxylase activity.

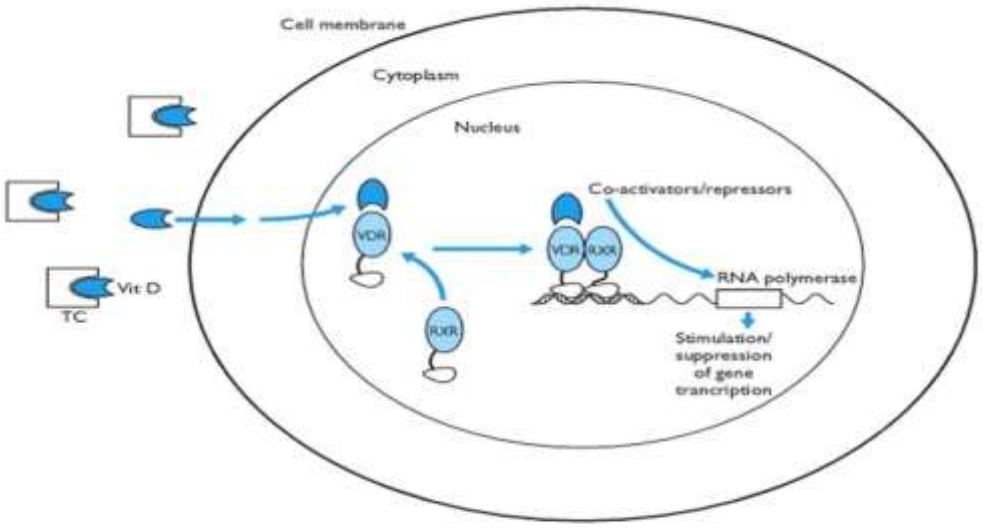


Figure showing interaction of vitamin D receptor(VDR) with retinoic X receptor in regulating gene expression.

Calcitriol production can be modulated in this way according to the calcium and other endocrine needs of the organism. The chief regulatory factors are 1,25(OH)2D3, parathyroid hormone (PTH), the FGF23/Khloto complex, and the serum concentrations of calcium and phosphate. PTH and hypophosphatemia enhance the activity of renal 1α-hydroxylase, but not that of liver 25-hydroxylase. Modulation of 25(OH)D-24-hydroxylase activity, which forms 24,25(OH)2D3 and 1,24,25(OH)3D3, also contributes to the regulation of calcitriol levels and action. Calcitriol is catabolized to the biologically inert, water soluble, calcitroic acid in all of its target tissues, as well as in the kidney and in the liver (30). Vitamin D transport The Vitamin D binding protein (DBP) is the major carrier protein of Vitamin D and its metabolites, because Vitamin D metabolites are lipophilic molecules with low aqueous solubility that must be transported in the circulation bound to plasma proteins. The DBP binds the metabolites with different affinity, highest for 25(OH)D and 24,25(OH)2D, intermediate for calcitriol, and lowest for Vitamin D (40). Unlike other lipophilic hormone-binding proteins, DBP plasma levels are considerably higher, up to 20 times

more, compared to the total amount of Vitamin D metabolites, a feature so far unexplained. Apart from its specific sterol binding capacity, DBP exerts other biological functions, such as actin scavenging, fatty acid transport, macrophage activation and chemotaxis (41). The fact that over 99% of circulating Vitamin D compounds are protein bound has major physiological implications. DBP-bound Vitamin D metabolites have limited access to target cells and, therefore, are less susceptible to hepatic metabolism and subsequent biliary excretion, leading to a longer circulating half-life. Steroid hormones and many other lipophilic compounds were once believed to enter cells solely by free diffusion through the plasma membrane. It is now clear that 25(OH)D does not simply diffuse into the proximal tubule cells containing 1α-hydroxylase: through a megalin-knockout mouse a new endocytic pathway responsible for the delivery of steroids to renal and gonadal tissues has been identified (42). Specifically, entry of 25(OH)D3 into the proximal tubule cells is not by diffusion across the basolateral surface but by megalin-mediated uptake of DBP in the luminal brush border. In addition to megalin, the delivery of 25-(OH)D3 to the 1α-hydroxylase in the renal tubular cells requires the presence of another protein, intracellular Vitamin D binding protein 3 (IDBP-3). Thus, the circulating DBP-bound 25(OH)D3 is filtered by the kidney and taken up by proximal tubular cells via megalin-mediated endocytosis. Once inside the cell, the DBP is degraded, and the released 25-(OH)D3 is delivered to the 1α-hydroxylase by IDBP-3 or reenters the circulation bound to DBP (43).

Extrarenal synthesis of calcitriol

In uremic patients, an increase in serum calcitriol levels following 25(OH)D3 administration has been observed, even in the absence of renal mass, indicating the existence of extra-renal 1α-hydroxilase activity, which has the potential to normalize

serum calcitriol after 25(OH)D3 supplementation (44). The need of supra-physiological levels of substrate to correct serum calcitriol in anephric patients suggests that substrate availability to extra-renal 1α-hydroxilase(s) may play an important role in calcitriol production in uremic states. In the search for the location of the extra-renal source of calcitriol in CKD, Dusso et al found that peripheral macrophages from normal individuals can hydroxylate 25(OH)D3 in the 1α-position, pointing to the existence of a monocyte 1α-hydroxilase, which has also shown a significantly enhanced activity in macrophages obtained from hemodialysis patients (45). Furthermore, in stage 5 CKD patients impaired uptake of 25(OH)D3 by peripheral blood monocytes and a lower apparent affinity for the substrate of the induced 1α-hydroxylase of human monocytes may explain the need for 25(OH)D3 supplementation to better correct serum calcitriol levels. More importantly, calcitriol deficiency in CKD plays a major role in these abnormalities, since both the kinetics of the extrarenal 1α-hydroxylase and 25(OH)D3 uptake by monocytes could be corrected by normalizing serum calcitriol levels (46). Such evidence points to the possible importance of combining 25(OH)D3 and calcitriol supplementation in CKD patients with secondary hyperparathyroidism. Genomic and non-genomic effects of calcitriol Calcitriol exerts its function through the Vitamin D receptor mainly by a genomic mechanism, influencing expression (either upregulation or downregulation) of gene products. Many genes are upregulated by calcitriol, including osteocalcin, osteopontin, calbindin, 24-hydroxylase and many others. On the other hand, calcitriol downregulates inflammatory markers such as IL-2 and IL-12 and has an antiproliferative effect. It also decreases PTH and PTHrP through a negative Vitamin D responsive element. The time-frame required for such genomic action ranges from hours to days. However, in the mid 1980s calcitriol-mediated rapid responses were

discovered (47). These were responses that occurred too rapidly (minutes to an hour) to be explained as the simple consequence of the nuclear VDR regulating gene transcription. Some rapid response examples include the rapid intestinal absorption of calcium (transcaltachia), secretion of insulin by pancreatic β-cells, opening of calcium and chloride channels in osteoblasts, and the rapid migration of endothelial cells. The presence of a second receptor, apart from the nuclear VDR, responsible for the generation of rapid responses was then hypothesized. However, rapid responses are absent in cells isolated from VDR-null mice (48), and VDR was found to be present in caveolae within the plasma membrane (47). Thus, it seems that in some cells the VDR located in the plasma membrane mediates these rapid effects. Furthermore, it has been proposed that the chemical properties of the conformationally flexible 1,25(OH)2D3 allow it to generate different shaped ligands for the VDR that are selective either for genomic or for rapid responses (47).

Vitamin D and type 2 diabetes

After conducting a meta-analysis and review of the impact of Vitamin D and calcium on glycemic control in patients with type 2 diabetes, Pittas et al. (48**)** concluded that insufficient Vitamin D and calcium appears to hinder glycemic control and that supplementing both nutrients may be necessary to optimize glucose metabolism. An observational study from the Nurses Health Study **(**49) that included 83,779 women > 20 years of age found an increased risk of type 2 diabetes in those with low Vitamin D status. A combined daily intake of > 800 IU of Vitamin D and 1,000 mg of calcium reduced the risk of type 2 diabetes by 33%. The National Health and Nutrition Examination Survey (NHANES) III study between 1988 and 1994 **(**50**)** demonstrated that there is a strong inverse association between low levels of 25(OH)D and diabetes prevalence. Low Vitamin D levels have also been shown to be predictive of the future

development of type 2 diabetes (52). Studies (4) showed that increasing Vitamin D serum levels to normal led to a 55% relative reduction in the risk of developing type 2 diabetes. As with most disease states and Vitamin D, prospective studies related to Vitamin D supplementation and diabetes are rare and limited. Prospective trials of Vitamin D and diabetes to date were either too small or used inadequate amounts of Vitamin D (53).

Kayaniyil et al (54). Performed a linear regression analysis of 712 subjects after evaluating serum 25(OH) D levels and assessing insulin sensitivity by means of the homeostasis model of insulin resistance. Their results indicated that Vitamin D was significantly correlated to insulin resistance and β-cell function in their multiethnic sample. The researchers concluded that low Vitamin D levels may play a significant role in the pathogenesis of type 2 diabetes.

The NHANES group (2003–2006) evaluated 9,773 U.S. adults > 18 years of age and showed a mechanistic link between serum Vitamin D levels, glucose homeostasis, and the evolution of diabetes (55). Based on their own study, Kositsawat et al (56), concluded that patients with elevated A1C levels should be evaluated for Vitamin D insufficiency.

Vitamin D and type 1 diabetes

Observational studies also suggest that low Vitamin D status may be associated with an increased risk of type 1 diabetes. For example, there is a greater incidence of type 1 diabetes related to geographic variation, with locations at higher latitudes having more type 1 diabetes. This may be the result of less sunshine and, therefore, lower levels of Vitamin D (57).

Hypponen et al. **(**58**)** conducted a cohort study in northern Finland. They collected data during 1 year on 10,821 children regarding Vitamin D supplementation dose and

presence of suspected rickets as it related to the development of type 1 diabetes. Their findings were both significant and astounding; children who took 2,000 IU of Vitamin D daily were 80% less likely to develop type 1 diabetes. This suggests that it may be crucial for all children to take Vitamin D supplementation during their first year of life to help avoid the development of type 1 diabetes.

Another Vitamin D study conducted by Zipitis et al.(59**)** demonstrated that Vitamin D supplementation in early childhood decreased the risk of developing type 1 diabetes by 29% compared to children who were not given Vitamin D supplements. In addition, the researchers found evidence suggestive of a dose-response effect.

Because destruction of β-cells usually begins in infancy or early childhood and continues until type 1 diabetes is diagnosed, studies such as these are intriguing in terms of the utility of Vitamin D in people with type 1 diabetes. It is hoped that starting Vitamin D supplementation soon after birth may be a protective strategy against the development of type 1 diabetes. (60)

Another area of interest is Vitamin D status during pregnancy and lactation and whether a pregnant woman's Vitamin D status plays a role in the development of diabetes in her child. Gregory et al (61), suggest that pregnant women and nursing mothers should take supplements to make sure their Vitamin D serum levels are optimal. This group reasoned that, because Vitamin D is a powerful modulator of the immune system and helps regulate cell proliferation and differentiation, it seems clear that Vitamin D could play a role in preventing type 1 diabetes. Their research showed that adequate Vitamin D status in mothers did have an impact on reducing the development of type 1 diabetes in their children.

However, studies related to the precise dose and duration of Vitamin D supplementation in infants and children are lacking. Currently, 400 IU of Vitamin

D_3 is recommended for supplementation in all infants until enough formula, milk, or other food sources are ingested to sufficiently provide 400 IU/day (62). Although it seems prudent and reasonable to supplement infants, children, and adolescents with Vitamin D to prevent deficiency, there is inconsistency in the recommended Vitamin D dose.

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MATERIAL AND METHODS

MATERIALS & METHODS

Experimental animals

Adult male albino wistar rats (Rattus Norvegicus) weighing 150-200g were used in present study. Animals were divided randomly into 9 groups and total of 56 rats were used in the study, all the rats were kept in polypropylene cages, and were lined with paddy husk which was replaced every day and cages were kept under standard condition of illumination with a 12 - h light-dark cycle at room temperature of 25 ± 1° C and 45-70% relative humidity

Study design: An Experimental animal-based study.

Locus of the study: Central Animal House, BLDEU's Sri BM Patil Medical College Hospital & Research Center, Bijapur.

- Sample size in each group (n) =6

Experimental group

S. No	Groups	Intervention
1.	Group-I	Normal Control (Dimethyl Sulfoxide)
2.	Group-II	Diabetic Control (+STZ 35mg/kg i.p)
3.	Group-III	High Fat Diet (Entire Study Duration)
4.	Group-IV	Normal Control + Vit D (500IU orally)
5.	Group-V	STZ 35mg/kg i.p) +Vit D (500IU orally)
6.	Group-VI	STZ 35mg/kg i.p + Metformin 300mg/kg orally
7.	Group-VII	STZ 35mg/kg i. Metformin 300mg/kg orally + Vit-D (500IU orally)
8.	Group-VIII	STZ 35mg/kg i.p +Glimepiride (200mcg/kg orally)
9.	Group-IX +Vit	STZ 35mg/kg i.p + Glimepiride (200mcg/kg orally) D (500IU orally)

Drugs used:

1. Vitamin D 500IU/kg body weight/day orally,

2. Streptozotocin (STZ) 35mg/kg body weight intraperitoneally,

Purchased from Sigma Aldrich, USA. (CAS [1883-66-4])

3. Glimepiride 500mcg/kg/day orally,

Supplied by IPCA Laboratory, Mumbai.

4. Metformin 300 mg/kg/day orally.

Supplied by IPCA Laboratory, Mumbai.

Except group one and group three all groups rats were fed with High Fat Diet (HFD and *water ad libitum* and group one rats were fed with normal pellet diet.

Total duration: - 7 weeks

Ethical Clearance

The study was reviewed and approved by the **Institutional Animal Ethics Committee (IAEC)** vide letter reference number; (NO-01/BLDE(DU)/2018).

Study was carried out as per Committee for the Purpose of Control and Supervision of Experimentation on Animals (CPCSEA) guidelines. Blood sample was collected from retro-orbital plexus for biochemical investigation and animals were sacrificed and tissues samples of pancreas and liver were preserved for histopathological evaluation.

Experimental protocol followed is depicted in below figure

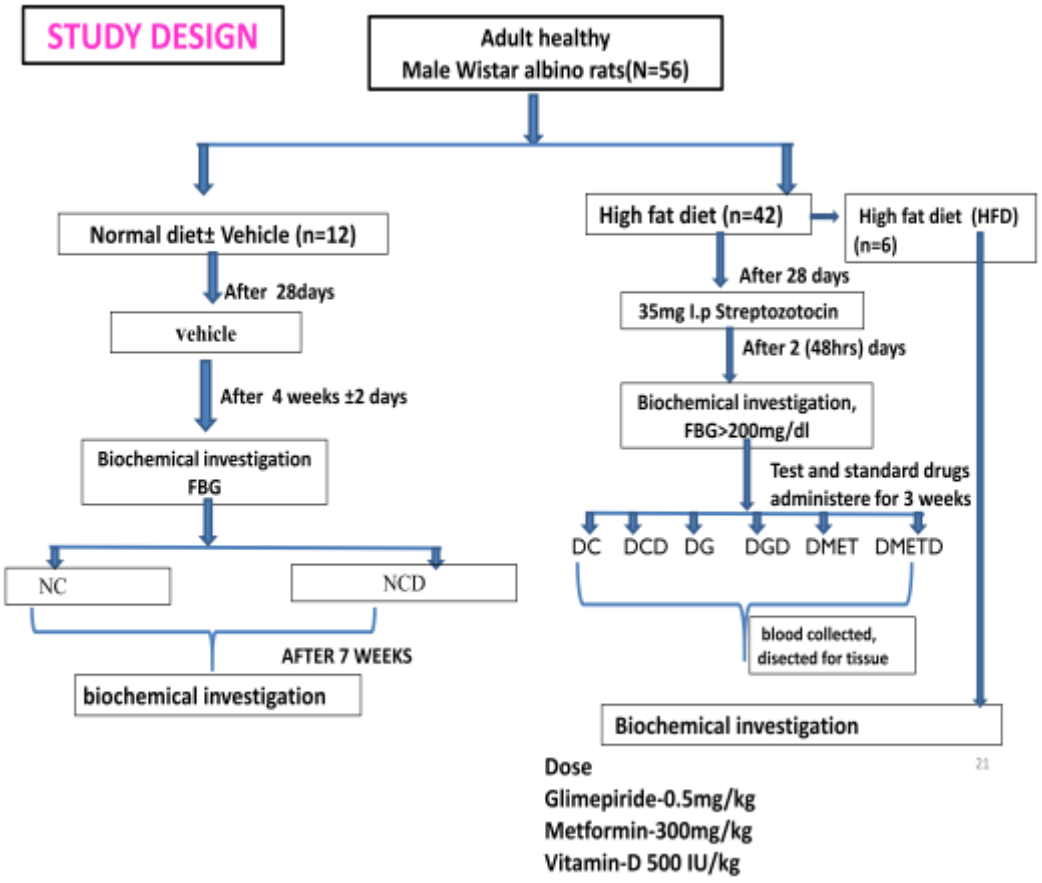


Figure-3 Showing Summary of experimental protocol

Dose calculation:

Rat dose is calculated using average human dose/day and converted into rat dose using following formula66**:**

- Dose of rats(mg/kg) =Human dose(mg/kg)/60 X Km Ratio.

For Glimepiride dose -5/60x6.2

=0.51 (i.e. 500 mcg.)

Similarly, the dose has been calculated for Vitamin D and Metformin.

Streptozotocin induced Diabetes:

Streptozotocin was administered intraperitoneally in the dose of 35mg/kg of the body weight. Streptozotocin induces diabetes by destroying the beta cells of pancreas. Rats with blood glucose levels above 250mg/dl were included in study.

Method of data collection:

Body Weight

Body Weight Weekly: Rats were checked for change in the body weight weekly by using digital balance.

Biochemical analysis

1. Blood glucose levels weekly (glucometer),
2. Serum Insulin (ELISA kit for rat Insulin)
3. Serum HbA1c (ELISA kit for rat HbA1c)
4. Serum Vit D (ELISA kit for rat Vitamin D)
5. Serum ENOS (ELISA kit for rat ENOS)
6. Lipid Profile.

Estimation of fasting plasma glucose.

Estimation of fasting plasma glucose was performed weekly using Accu-chek active glucometer by rat tail vein blood.

Oral Glucose Tolerance Test

OGTT was performed twice - preintervention (day 0). OGTT was performed in the morning hours in overnight fasted rats. The experimental rats were challenged with a glucose load of 2.0g/kg b.wt. administered by oral gavage. Fasting blood sample (0 min) was collected before administration of glucose load and subsequent samples were collected from the tail vein at 30min, 60min, 90min and 120min after administration of glucose load (Bowe et al., 2014). The glucose estimations were made with a commercial glucometer (ACCU-CHEK Active; Roche).

Estimation of Fasting Plasma Insulin

Fasting Plasma Insulin was estimated by ELISA using Rat Insulin ELISA kit (Catalog.No: BEK1243, Chongqing Biospes Co., Ltd, Chongqing, China) following the protocol given in the product manual.

- Principle of the Assay: The kit is based on sandwich enzyme-linked immune-obsorbentassay technique. 96 well plates precoated with anti-Insulin monoclonal antibody was used. The HRP conjugated anti-Insulin polyclonal antibody was used as detection antibodies. The standards, test samples and HRP conjugated detection antibody were added to the wells subsequently, and washed with wash buffer. TMB substrates were used to visualize HRP enzymatic reaction. TMB was catalyzed by HRP to produce a blue colour product that changed into yellow after adding acidic stop solution. The density of yellow is proportional to the insulin amount of sample captured in plate. OD absorbance was read at 450nm in a microplate reader and then the concentration of insulin can be calculated.
- Range: 0µIU/ml-140µIU/ml
- Sensitivity: <5µIU/ml
- Kit components:

1. One 96-well plate precoated with an anti-Rat Insulin antibody
2. Lyophilized insulin standards: 5 tubes (8µIU/ml, 16µIU/ml, 32µIU/ml, 80µIU/ml, 140µIU/ml)
3. HRP conjugated anti-rat Insulin antibody
4. TMB substrate A
5. TMB substrate B
6. Stop solution
7. Wash buffer

Protocol

- Preparation of sample and reagents

1. ***Sample*****:** Blood was collected in EDTA tube. Centrifuged for 30 min at 1000xg within

30 minutes of collection. Plasma was aliquoted and stored at -200C for further analysis.

2. ***Wash buffer***: The concentrated wash buffer was diluted 25-fold (1:25) with distilled

water.

3. ***Standard*****:** Reconstitution of the lyophilized standards: The standards were first equilibrated to room temperature. 0.5ml of double distilled water was added into each vial of the corresponding standard and mixed thoroughly and kept at room temperature

for 10 min for use.

- Assay Procedure:
    1. Standard, test sample and control (zero) wells were set on the pre-coated plate respectively, and then, their positions were recorded. Each standard and sample was run in duplicate.
    2. 50µl of 8µIU/ml, 16µIU/ml, 32µIU/ml, 80 µIU/ml, and 140µIU/ml of standard solutions were aliquoted into the standard wells. 50µl of sample diluent buffer was added into control well.
    3. 50µl of the properly diluted sample (rat serum) was added into test sample wells.
    4. 50µl of HRP conjugated anti-rat insulin antibody was added into above wells (except

control wells) at the bottom of each well without touching the side well.

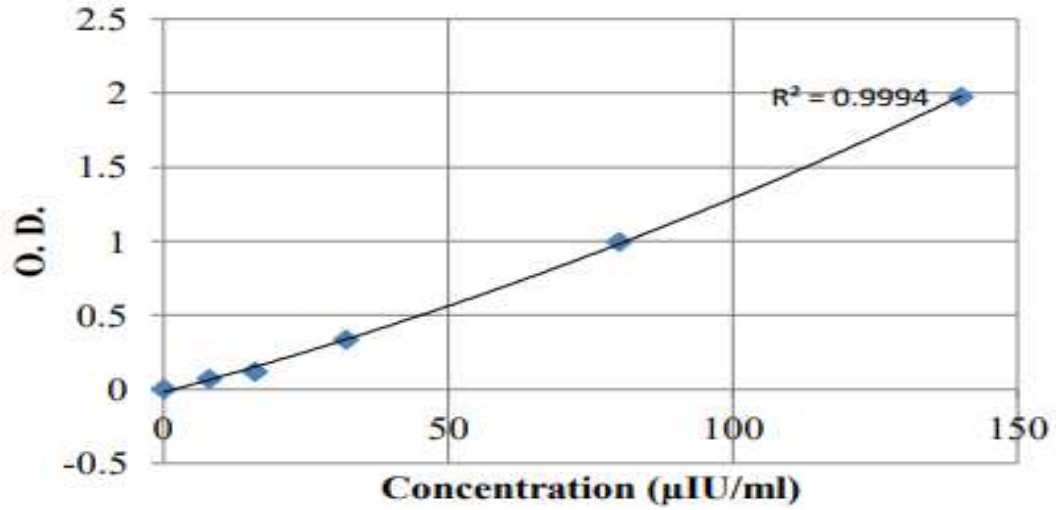
5. The plate was sealed with a cover and incubated at 370C for 60 min.
6. The cover was removed and the liquid of the wells discarded, the plate was clapped on the absorbent filter papers and not washed.
7. The plate was washed three times with wash buffer using manual washing. Each well was completely filled with wash buffer and mildly vortexed for 2 min on ELISA shaker. The contents were then aspirated from the plate and the plate was clapped on absorbent filter paper. The whole procedure was done 3 times.
8. 50µl of TMB substrate A was added into each well and then 50 µl of TMB substrate B was added. The plate was gently shaken by hand for 30 sec. the plate was covered and incubated in dark at 370C for 15 min. The shades of blue were observed in the first 3-4 wells with the highest concentration of rat insulin standard solutions, the other wells showed no obvious colour.
9. 50µl of stop solution was added into each well and mixed thoroughly. The colour changed to yellow immediately.
10. The OD absorbance was read at 450nm in a microplate reader (Model: Merilyzer EIAQUAN, Meril Diagnostics Pvt. Ltd.) within 30 min after adding the stop solution.

Calculations

Relative O.D. 450= (O.D.450 of each well) – (O.D.450 of Zero well).

The standard curve was plotted as the relative O.D.450 of each standard solution (Y) vs. the respective concentration of the standard solutions (X). The rat insulin concentration of the samples was interpolated from the standard chart and curve given below.

X	µIU/ml	0	8	16	32	80	140
Y	OD_{450}	0	0.084	0.1044	0.3405	0.986	2.034



Estimation of serum Vitamin D

This kit was based on standard sandwich enzyme-linked immune-sorbent assay technology. The purified anti- Vitamin D antibody was pre-coated onto 96-well plates. And the HRP conjugated anti- Vitamin D antibody was used as detection antibodies. The standards, test samples and HRP conjugated detection antibody were added to the wells subsequently, mixed and incubated, then, unbound conjugates were washed away with wash buffer. TMB substrates (A & B) were used to visualize HRP enzymatic reaction. TMB was catalyzed by HRP to produce a blue color product that changed into yellow after adding acidic stop solution. The density of yellow is proportional to the Vitamin D amount of sample captured in plate. Read the O.D. absorbance at 450nm in a microplate reader, and then the concentration of Vitamin D can be calculated.

Kit components

1. One 96-well plate pre-coated with anti- rat Vitamin D antibody
2. Standard: 0.5 ml (900 ng/ml)
3. Standard diluent buffer: 1.5 ml
4. Wash buffer (30×): 20 ml. Dilution: 1:30
5. Sample diluent buffer: 6 ml
6. HRP conjugated anti- rat Vitamin D antibody (RTU): 6 ml
7. Stop solution: 6 ml
8. TMB substrate A: 6 ml
9. TMB substrate B: 6 ml
10. Plate sealer: 2
11. Hermetic bag: 1

Preparation of sample and reagents

- **Sample :**Isolate the test samples soon after collecting, then, analyze immediately (within 2 hours). Or aliquot and store at -20℃ for long term. Avoid multiple freeze-thaw cycles.
- **Serum:** Coagulate at room temperature for 10-20 min, then, centrifuge at the speed of 2000-3000 r.p.m. for 20 min to collect supernatant. If precipitation appeared, centrifuge again.
- **Plasma:** Collect plasma using EDTA or citrate plasma as an anticoagulant, and mix for 10-20 min,centrifuge at the speed of 2000-3000 r.p.m. for 20 min of collection. If precipitation appeared, centrifuge again.
- **Cell culture supernatant:** For secretory components: use a sterile container to collect.

Centrifuge at the speed of 2000-3000 r.p.m. for 20 min to collect supernatant. For intracellular components: Dilute cell suspension with PBS（pH7.2-7.4) to make the cell concentration reached 1 million / ml. Damage cells and release of intracellular components through repeated freeze-thaw cycles. Centrifuge at the speed of 2000-3000 r.p.m. For 20 min to collect supernatant. If precipitation appeared, centrifuge again.

- **Tissue samples**: Cut samples and weight, add certain volume of PBS (pH7.4), rapidly frozen with liquid nitrogen. After melting, store samples at 2-8 ℃ . Add certain volume of PBS (pH7.4), homogenize thoroughly, centrifuge at the speed of 2000-3000 r.p.m. for 20 min to collect supernatant. Note:

  1. Coagulate blood samples completely, then, centrifuge, and avoid hemolysis and particle.

  2. NaN3 cannot be used as test sample preservative, since it is the inhibitor for HRP.

  3. After collecting samples, analyze immediately or aliquot and store frozen at -20°C. Avoid repeated freeze-thaw cycles.

- **Wash buffer**

  Dilute concentrated Wash buffer (Kit Component 4) 30-fold (1:30) with distilled water (i.e. add 20 ml of concentrated wash buffer into 580 ml of distilled water).

- **Standard**

  Dilution of Standard: set 10 Standard wells on the pre-coated plates, add 100μl of Standard to the 1st and 2nd well, then add 50μl of Standard diluent buffer to the above two wells and mix thoroughly; transfer 100μl from the 1st and 2nd well to the 3rd and the 4th well respectively, then add 50μl of Standard diluent buffer to the 3rd and the 4th well and mix thoroughly; take out 50μl from the 3rd and the 4th well respectively and discard, and transfer 50μl to the 5th and the 6th well, then add 50μl of Standard

diluent buffer to the 5th and 6th well and mix thoroughly; transfer 50µl from the 5th and 6th well to the 7th and the 8th well, then add 50µl of Standard diluent buffer to the 7th and 8th well and mix thoroughly; transfer 50µl from the 7th and the 8th well to the 9th and the 10th well, add 50µl of Standard diluent buffer to the 9th and the 10th well and mix thoroughly, take out 50µl from the 9th and the 10th well and discard (After diluting, the loading volume for each well is 50µl, and the concentrations are 600 ng/ml, 400 ng/ml, 200 ng/ml, 100 ng/ml, 50 ng/ml).

- **Assay procedure**

1. Equilibrate kit components for 15-30 min at room temperature.
2. Set standard, test sample and control (zero) wells on the pre-coated plate respectively, and then, record their positions. Add 50 µl of diluted standards (600 ng/ml, 400 ng/ml, 200 ng/ml, 100 ng/ml, 50 ng/ml) into the standard wells. Add 50 µl of Standard diluent buffer (Kit Component 3) into the control (zero) well. Do not add sample and HRP conjugated antibody into the control (zero) well.
3. For test sample wells, add 40µl of Sample diluent buffer (Kit component 5) first, then, add 10µl of sample. Add the solution at the bottom of each well without touching the side wall. Shake the plate mildly to mix thoroughly.
4. Cover the plate with Plate sealer (Kit Component 10) and incubate at 37℃ for 30 min.
5. Remove the sealer, and wash plate using one of the following methods.

- **Manual Washing**: Discard the solution in the plate without touching the side walls. Clap the plate on absorbent filter papers. Fill each well completely with Wash Buffer (1×) and vortex mildly on ELISA shaker for 2 min, then aspirate contents from the plate, and clap the plate on absorbent filter papers. Repeat this procedure four more times for a **total of FIVE washes**.

- **Automated Washing**: Aspirate all wells, then wash plates FIVE times using Wash Buffer (1×). After the final wash, invert plate, and clap the plate on absorbent filter papers until no moisture remained. It is recommended that the washer be set for a soaking time of 10 seconds or shaking.

6. Add 50 µl of HRP conjugated anti- Vitamin D antibody (Kit Component 6) into each well (except control well).
7. Cover the plate with Plate sealer (Kit Component 10) and incubate at 37℃ for 30 min.
8. Remove the sealer, and wash the plate. (See Step 5)
9. Add 50 µl of TMB substrate A (Kit Component 8) into each well, and then, add 50µl of TMB substrate B (Kit Component 9), vortex gently the plate on ELISA shaker for 30 seconds (Or shake gently by hand for 30 seconds), and incubate in dark at 37°C for 15 min. The shades of blue can be seen in the wells.
10. Add 50 µl of Stop solution (Kit Component 7) into each well and mix thoroughly. The color changes into yellow immediately.
11. Read the O.D. absorbance at 450nm in a microplate reader within 15 min after adding the stop solution, For calculation, (the relative O.D.450) = (the O.D.450 of each well) – (the O.D.450 of Zero well). The standard curve can be plotted as the relative O.D.450 of each standard solution (Y) vs. the respective concentration of the standard solution (X). The rat Vitamin D concentration of the samples can be interpolated from the standard curve.

Precautions

1. Before the experiment, centrifuge each kit component for several minutes to bring down all reagents to the bottom of tubes.
2. It is recommended measuring each standard and sample in duplicate.
3. Do NOT let the plate completely dry at any time, Since the dry condition can inactivate the biological material on the plate.
4. Do not reuse pipette tips and tubes to avoid cross contamination.
5. Do not use the expired components and the components from different batches.
6. Store the TMB substrate B (Kit Component 9) in dark.
7. Prolong the incubation time if the hypochromasia obtained. Heat the water in the water diluting if the crystalloid appeared in Wash buffer (Kit Component 4). 8. Do not remove microplate from the storage bag until needed, and the unused strips should be stored at 2-8℃in their pouch or the provided Hermetic bag (Kit Component 11)

Estimation of serum NOS3

Serum NOS3 was estimated by ELISA using Rat NOS3 ELISA Kit (Catalog No.: BYEK2703 Chongqing Biospes Co., Ltd, Chongqing, China) following the protocol given in the product manual.

- Principle of the Assay

  This kit works on technique of sandwich enzyme-linked immune-sorbent assay. 96 well

  plates precoated with the purified anti-NOS3 antibody are used. The anti-NOS3 antibody

conjugated with HRP was used as detection antibodies. The standards, test samples and HRP conjugated detection antibody were added to the wells, mixed and incubated, and then

unbound conjugates are washed away with wash buffer. TMB substrates (A&B) are used to

visualize HRP enzymatic reaction. HRP catalyzes TMB to produce a blue colour product that changes into yellow after adding acidic stop solution. The density of yellow is proportional to the amount of NOS3 in the sample captured in plate. O.D. absorbance was read at 450nm in a microplate reader, and the concentration of NOS3 is calculated.

- Range: 3pg/ml-120pg/ml

- Kit components:

  c. One 96-well plate pre-coated with anti-rat NOS3 antibody

  d. Standard (180pg/ml)

  e. Standard diluent buffer

  f. Wash buffer (30X): Dilution (1:30)

  g. Sample diluent buffer

  h. HRP conjugated anti-rat NOS3 antibody

  i. Stop solution

  j. TMB substrate A

  k. TMB substrate B

  l. Plate sealer

  m. Hermetic bag

- Protocol

Preparation of sample and reagents

1. ***Sample***: The collected blood is allowed to coagulate at room temperature for 10-20 min, and then centrifuged at the speed of 2000-3000rpm for 20 min to collect the supernatant. The supernatant is aliquoted and stored at -200C. Multiple freeze-thaw cycles were avoided.

2. ***Wash buffer***: Concentrated wash buffer was diluted 30 fold (1:30) with distilled water.

3. ***Standard***: Dilution of the standard: 10 standard wells were set on the pre-coated plates and 100µl of the standard was added to the 1st and 2nd well. 50µl of the standard diluent buffer was then added to the above two wells and thoroughly mixed. 100µl from the 1stand 2nd were transferred to the 3rd and 4th well respectively. 50µl of the standard diluent buffer was added to the 3rd and 4th well and mixed thoroughly. 50µl was taken from the 3rd and 4th well and discarded and 50µl was transferred to 5th and 6th well. 50µl of the standard diluent buffer was added to 5th and 6th well and mixed thoroughly. 50µl wastransferred from 5th and 6th well to 7th and the 8th well. 50µl of standard diluents buffer was added to 7th and 8th well and mixed thoroughly. 50µl from 7th and 8th well was transferred to 9th and 10th well. 50µl of standard diluents buffer was added to 9^{th} and 10^{th} well and mixed thoroughly. 50µl was taken out from 9th and 10th well and discarded. After diluting, the loading volume for each well was 50µl and the concentrations were 120pg/ml, 80pg/ml, 40pg/ml, 20pg/ml, 10pg/ml.

- Assay Procedure

1. Equilibrate kit components for 15-30 min at room temperature

2. Standard, test sample and control (zero) were set in the wells on pre-coated plate respectively. Their positions were recorded. 50µl of diluted standards (120pg/ml,

80pg/ml, 40pg/ml, 20pg/ml, 10pg/ml) were added into standard wells. 50µl of the standard diluent buffer was added into control (zero) well.

3. For test sample wells, 40µl of sample diluents buffer was added and then 10µl of the sample was added. The solution was added at the bottom of each well without touching the side well. The plate was mildly shaken to mix thoroughly.

4. The plate was covered with a plate sealer and incubated at 370C for 30 min.

5. The sealer was removed and the plate was washed manually. For this, the solution in the plate was discarded without touching the sidewalls. The plate was claped on absorbent filter paper. Each well was filled completely with wash buffer (1x) and vortexed mildly on ELISA shaker for 2 min, the contents of the plate were then aspirated. The plate was claped on absorbent filter paper. The same procedure was repeated four more times for a total of five washes.

6. 50µl of HRP conjugated anti-NOS3 antibody was added into each well (except control well).

7. The plate was covered with a plate sealer and incubated at 370C for 30min.

8. The sealer was removed and the plate was washed.

9. 50µl of TMB substrate A was added into each well, followed by 50µl of TMB substrate

B. The plate was gently shaken by hand for 30 sec and incubated in dark at 370C for 15

min. The shades of blue were seen in the wells.

10. 50µl of Stop solution was added into each well and mixed thoroughly. The colour changed into yellow immediately.

11. The O.D. absorbance was read at 450nm in a microplate reader (Model: Merilyzer

EIAQUAN, Meril Diagnostics Pvt. Ltd.) within 15 min after adding the stop solution.

- **Calculations**

Relative O.D.450= (O.D. 450 of each well) – (O.D. 450 of Zero well). The standard curve was plotted as the relative O.D. 450 of each standard solution (Y) vs. the respective concentration of the standard solutions (X). The NOS3 concentration of the samples was interpolated from the standard curve.

Serum total cholesterol (TC), Serum triglycerides (TG), and High-density lipoprotein (HDL) were analyzed using a commercial diagnostic kit (Erba Diagnostic Mannheim GmBH).

b. LDL and VLDL levels were calculated using the Friedwald formula (Friedwald et al.,

1972) as indicated below

 LDL (mg/dl)= Total cholesterol – HDL cholesterol - TG/5

 VLDL= TG/5

1. **Estimation of Serum Total Cholesterol**

Serum cholesterol was estimated by cholesterol oxidase – peroxidase enzymatic method

(CHOD-POD) (Allain et al., 1974)

1. **Estimation of Serum Triglycerides**

Serum triglycerides were estimated by glycerophosphate oxidase peroxidase (GODPOD) method.

2. Estimation of Serum high-density lipoprotein (HDL) by Precipitation method, Phosphotungstate magnesium acetate reagent.

3. LDL and VLDL levels were estimated by calculation using the Friedwald formula (Friedwald et al., 1972)

   - LDL mg/dl = Total cholesterol-HDL cholesterol-Triglyceride/5
   - VLDL = TG/5

Weekly Fasting blood glucose was measured using glucometer in all the groups post STZ and after drug treatment. At the end of study blood was collected from retro-orbital plexus for biochemical investigation and rats were dissected and pancreas and liver were taken for histopathological evaluation and preserved in 10 % formalin.

The liver, pancreas were fixed in 10% neutral buffered formalin were embedded in paraffin blocks, sectioned with a microtome (3-5µm thickness) and finally stained by Haematoxylin & Eosin (H&E) and were subjected to histopathological examination.

STATISTICAL ANALYSIS:

All the values have been expressed as the mean ± SEM and analyzed by paired student-t test and one-way analysis of variance (ANOVA) in order to test differences between groups. The level of statistical significance has been set at $p<0.05$.

RESULTS

ORAL GLUCOSE TOLERANCE TEST (OGTT)

Table-1: Showing values of glucose tolerance test. (mg/dl)

TIME / GROUPS (N=6 RATS)	0 TIME	30 MIN	60 MIN	90 MIN	120 MIN
Group=I (NC)	79 ± 6.32	110±8.32	126±13.44	135±12	148±12.44
Group-II (DC)	425±29.67	400±31.30	377±33	360±36	344±27.12
Group-III(HFD)	82±7.36	123±6.46	133±8.46	141±11.3	155±12.36
Group-III(NCD)	81±5.82	116±6.34	130±8.12	137±11.27	143±10.22
Group-IV (DCD)	436±31.45	385±37.41	365±39	355±27	337±21
Group-V(DG)	350±33	300±27.75*	267±22.44*	220±19.83**	199±14.28**
Group-VI(DGD)	335±41.33	310±29.56*	274±25.48*	180±20.04**	175±11.23**
Group-VII(DMET)	338±27.33*	326±18.87	290±26	300±17.33	295±22.05
Group-VIII(DMETD)	341±33*	310±31.07	289±23.67	285±21.09	280±18.66

**Indicates p <0.001, * 0.01 compared diabetic control

Diabetic rats showed statistically elevated blood glucose levels compared to normal control rats after the glucose load. The increased glucose levels of OGTT in diabetic Group-II (DC) indicated severe glucose intolerance, (p<0.001) compared with Group-I (NC).Diabetic rats treated with Glimepiride and Glimepiride + Vit D (*P<0.001) and Metformin and Metformin + Vit D exhibited reduced glucose levels which was statistically insignificant when compared to diabetic control (DC).

BODY WEIGHT

Diabetic control rats showed a significant increase in body weight compared to normal control (P<0.01), where as rats normal rats treated with Vitamin D also showed elevated body weight when compared with normal rats and it was significant (P<0.01), but when compared to diabetic rats gain in body weight was lesser and the difference is statistically significant (P<0.01). Diabetic rats treated with Vitamin D alone and Glimepiride has also shown increase in body weight which is higher than diabetic rats, where as diabetic rats treated with both Vitamin D and Glimepiride did

not gain weight when compared to diabetic rats and the difference in body is statistically significant when compared to diabetic rats and also rats treated with Vitamin D and Glimepiride individually (P<0.01). whereas diabetic rats treated with Metformin and in combination with Vitamin D did not gain much weight and the difference in weight was statistically significant when compared to diabetic control.(Table-2)

Table 2. Showing values of body weight weekly (gm)

TIME / GROUPS (n=6)	Day 0	Week 1	Week 2	Week 3	Week 4		Week 5	Week 6	Week 7
Group-I (NC)	164.33 ±3.19	179.33 ±2.12	193.66 ±12.04	207.33 ±8.34	212.50 ±8.97		228.66 ±10.49	241.50 ±12.88	253.83 ±15.28
Group-II (DC)	169.66 ±1.42	203.33 ±2.01	245.83 ±2.89	286.66 ±3.33 #	314.33 ±3.05 #		341.00 ±2.62#	345.16 ±3.83#	315.33 ±3.05#
Group-III (DHFD)	169± 7.56	185.47 ±6.45	239± 11.36	288.26±17.75	337.42±16.83		377.31 ± 23.22	421±26.12	452.12 ± 22.36
Group-IV(NCD)	164.33 ±2.71	189.16 ±3.09	214.16 ±2.97	238.83 ±2.78	270.50 ±4.58		296.50 ±3.51# *#	317.00 ±4.88#	270.50 ±4.58# *
Group-V (DCD)	170.33 ±3.39	207.83 ±3.41	251.50 ±3.11	294.66 ±3.33	337.16 ±5.12		370.83 ±5.85	398.00 ±5.93	413.16 ±5.12
Group-VI (DG)	171.66 ±2.33	229.16 ±4.72	236.83 ±9.05	280.26 ±1.85	308.6 ±1.99		341.33 ±1.90	383.16 ±2.18# *#	407.6 ±1.99*
Group-VII (DGD)	164.66 ±3.87	229.16 ±6.03	240.83 ±3.73	279.33 ±2.98	316.00 ±4.93		346.33 ±4.56#	380.00 ±3.75	374.00 ±4.93*
Group-VIII(DMET)	160 ±3.57	234.50 ±10.9	255.33 ±4.04	287.00 ±4.83	322.66 ±4.48		290.50 ±6.28*	280.33 ±5.73*	274,66 ±4.48*
Group-IX (DMETD)	165.66 ±16.65	215.33 ±4.552 45.83±	257.16 ±4.23	247.00 ±2.58	257.00 ±4.38		270.33 ±6.14*	276.16 ±4.76*	287.00 ±4.38*

DRUG TREATMENT

*P<0.01 Compared to Diabetic Control, # P< 0.01 Compared to Normal Control.

BLOOD GLUCOSE LEVELS

Diabetic control rats showed significant rise in blood glucose levels when compared with normal control rats (P<0.001), whereas rats treated with Glimepiride and metformin alone and in combination with Vitamin D able to control rise in sugar levels significantly(P<0.001 and 0.01) respectively when compared with diabetic untreated rats. Diabetic rats treated with Vitamin D alone also shown reduction in blood sugar levels but it was statistically insignificant (Table-3).

Table 3. Blood glucose(FBS) before 1 hour of STZ administration and on subsequently at the end of every week (mg/dL) (Mean±SD)

TIME / GROUPS (n=6)	DAY 0	WEEK 1	WEEK 2	WEEK 3
Group-I (NC)	79±1.58	80±3.14	78±2.98	73±2.52
Group-II(DC)	80.00±3.72	404±20.88	518±10.58	526±13.72
Group-III (HFD)	85±4.22	96±5.56	96±8.56	97±8.44
Group-IV (NCD)	78±1.72	76±2.94	79±6.91	78±2.26
Group-V (DCD)	70.00±1.73	362.5±9.20	423.16±11.09	464.33±17.77
Group-VI (DG)	98±3.78	327±7.46	286±6.65**	236±8.20**
Group-VII (DGD)	108±2.44	288±5.56*	256.33±**	194±2.80**
Group-VIII (DMET)	113±1.92	356±9.29	329±9.78	273±10.46
Group-IX (DMETD)	117±2.32	293±8.47*	280±7.10*	282±4.88*

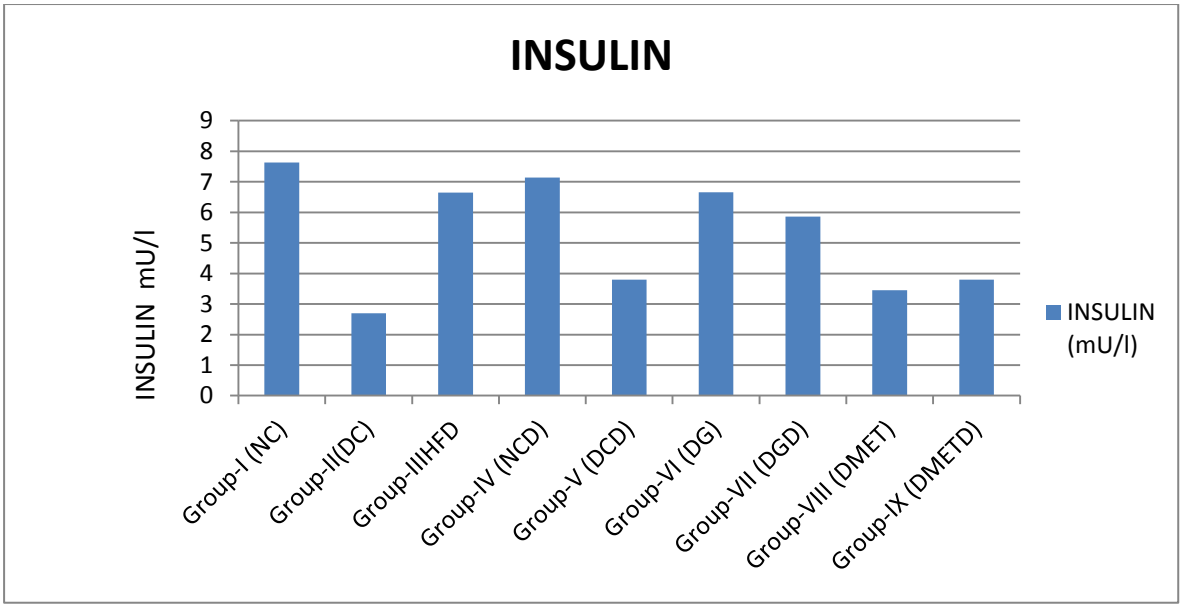
**P<0.001, *P<0.01Compared to Diabetic Control.

INSULIN

Diabetic rats showed drastic reduction in insulin levels compared to normal untreated rats, whereas rats fed with high fat diet also showed decrease in insulin levels but it was found to statistically insignificant. However diabetic rats treated with Vitamin D could able to increase insulin levels (P<0.05) when compared to diabetic untreated rats. However diabetic rats treated with with Glimepiride and in combination with Vitamin D showed drastic increase in insulin levels and the significance was at P<0.001. whereas diabetic rats treated with metformin has slight increase in insulin levels, metformin and Vitamin D combination treated rats showed increase in insulin levels and it significance was at P<0.05 (Table-3 & Graph-1)

Table-3: Showing values of Insulin mU/L in experimental rats

Group -I (NC)	Group-II(DC)	Group-IIIHFD	Group-IV (NCD)	Group-V (DCD)	Group-VI (DG)	Group-VII (DGD)	Group-VIII (DMET)	Group-IX (DMETD)
7.63± 0.21	2.7± 0.23	6.65± 0.43	7.14± 0.4	3.8± 0.39	5.86± 0.53*	6.66± 1.35*	3.45± 0.39	3.80± 0.64



Graph-1 Showing values of Serum Insulin in experimental rats

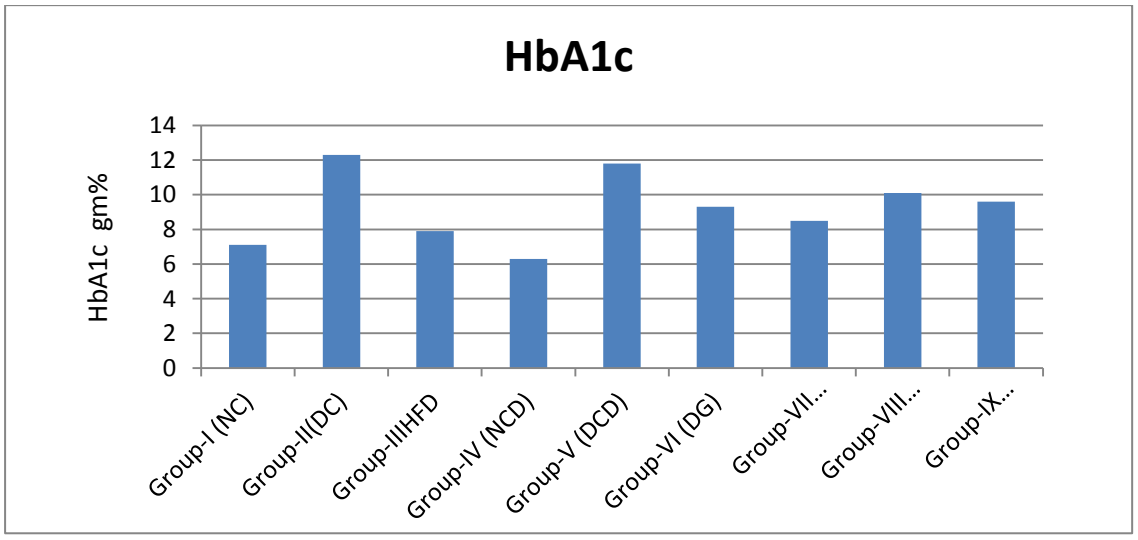
Glcosylated Haemoglobin (HbA1c)

The values of Glycosylated haemoglobin are in consistent with insulin levels in all group of rats. Diabetic rats shown increase in HbA1c levels when compared to normal control rats. However diabetic rats treated with Glimepiride and in combination with Vitamin D showed reduced insulin levels and it was found to be statistically significant (P<0.01 & 0.001). Diabetic rats treated with metformin and along with Vitamin D showed decrease in HbA1c levels at P<0.05 significance level. Whereas rats fed with HFD has shown slight increase in HbA1c levels and which was statistically insignificant.

Table 4. Showing values of glycated haemoglobin HbA1c (gm %) in experimental rats

Group-I (NC)	Group - II(DC)	Group-III(HFD)	Group -IV (NCD)	Group-V (DCD)	Group-VI (DG)	Group-VII (DGD)	Group-VIII (DMET)	Group-IX (DMETD)
7.1± 0.67	12.3± 1.1	7.9± 0.77	6.3± 0.8	11.8± 1.10	9.3±* 1.1	8.5±** 0.85	10.1± 1.07	9.6±* 8.9

*P <0.01, **P <0.001 Compared to Diabetic group.



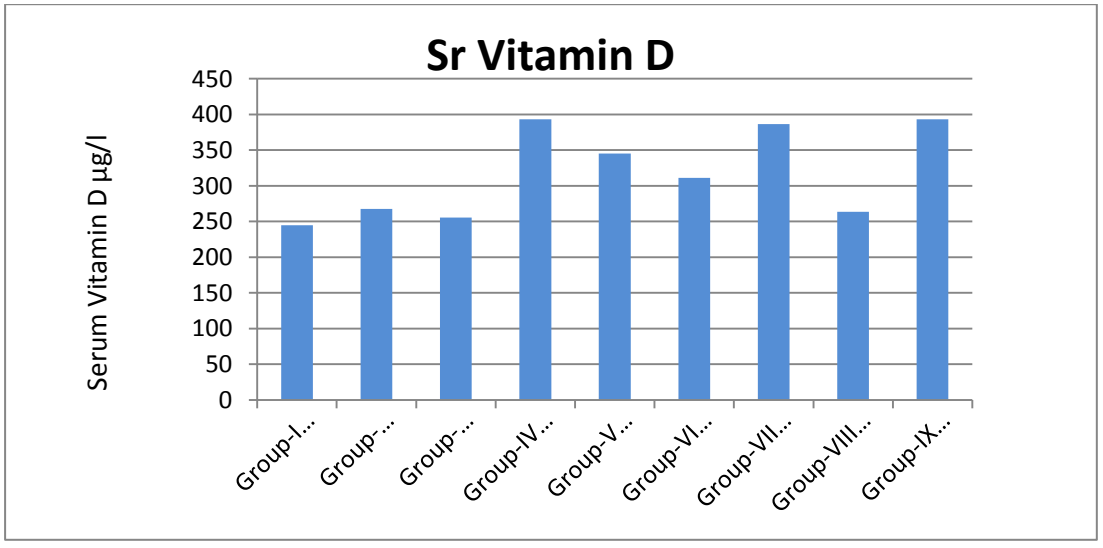
Graph-2 Showing values of glycated haemoglobin HbA1c (gm %) in experimental rats

SERUM VITAMIN D

Serum Vitamin D values were increased in all groups administered with Vitamin D, However the difference in Vitamin D levels is statistically significant when compared to diabetic control($P<0.001$). Diabetic untreated rats did not show any difference in serum Votamin D levels when compared with normal control rats. Where rats treated with Glimepiride alone also shows elevated serum Vitamin D levels ($P<0.01$). Whereas the values of Vitamin D in HFD normal rats and diabetic rats treated with metformin were comparable.

Table 5. Showing values of Serum Vitamin D (µg/l)

Group-I (NC)	Group - II(DC)	Group - IIIHF D	Group -IV (NCD)	Group-V (DCD)	Group-VI (DG)	Group-VII (DGD)	Group-VIII (DMET)	Group-IX (DMET D)
244.83± 22.34	237.6 ± 33.23	255.5± 21.64	393±** * 43.44	345±** 36.33	311±* 28.26	386.33± * 38.24	263.66± 26.12	393±* 41.42



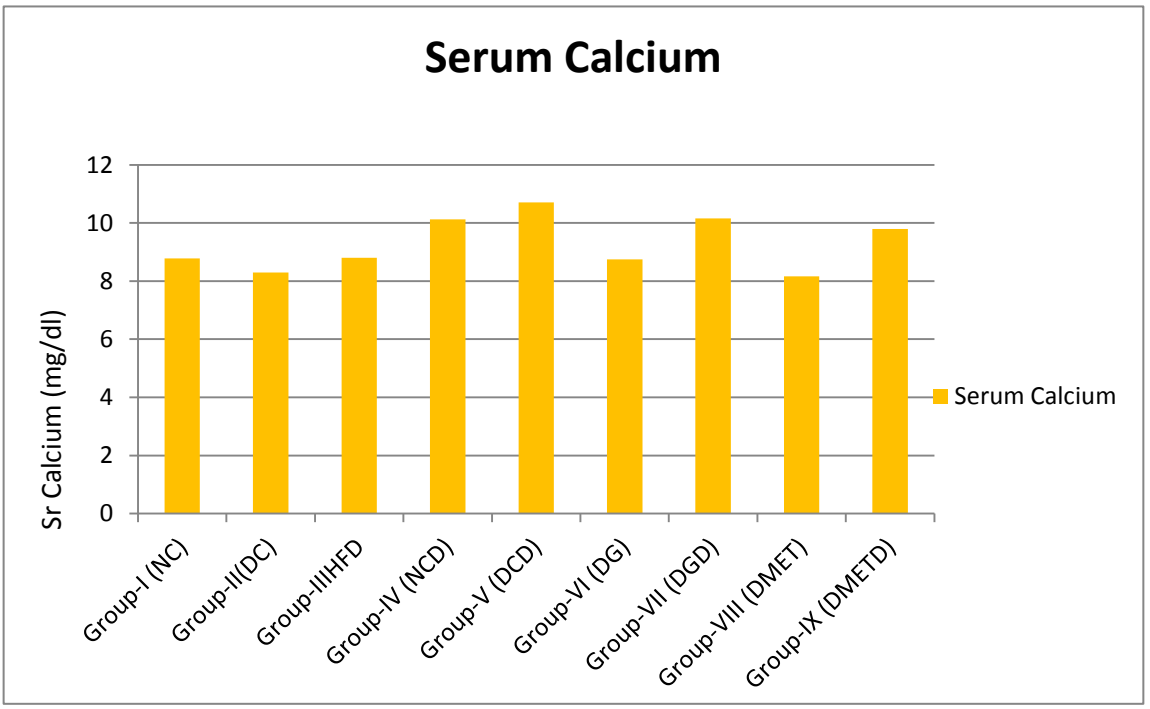
Graph 3. Showing values of serum Vitamin D in experimental rats (µg/l)

SERUM CALCIUM

Serum calcium values were found to be increase corresponding to increase in Vitamin D levels in rats treated with Vitamin D, diabetic rats treated with Vitamin D has shown significant increase in serum calcium levels (P<0.01). Thus supplementation of Vitamin D has positive impact on serum calcium levels.

Table 6. Showing values of Serum Calcium (mg/dl)

Group-I (NC)	Group-II(DC)	Group-IIIHFD	Group-IV (NCD)	Group-V (DCD)	Group-VI (DG)	Group-VII (DGD)	Group-VIII (DMET)	Group-IX (DMETD)
8.78± 0.21	8.30± 0.23	8.8± 0.65	10.13± 0.43*	10.71± 0.39*	8.75± 0.53	10.16± 1.3*	8.16± 0.89	9.80± 1.64*



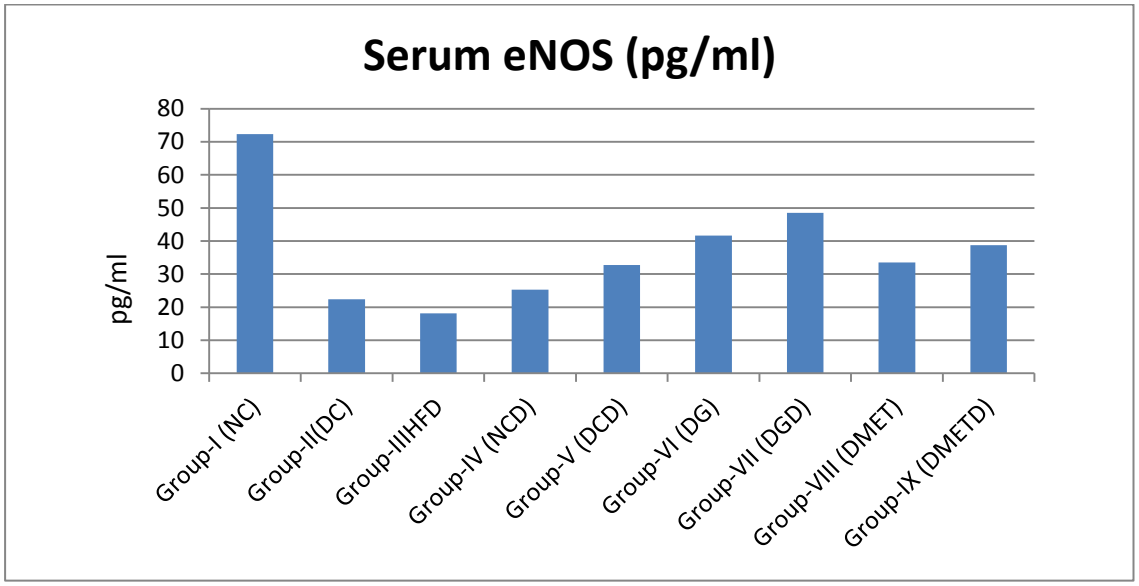
Graph 4. Showing values of Serum Calcium (mg/dl)

SERUM ENDOTHELIAL NITRIC OXIDE SYNTHASE (eNOS)

In all diabetic rats decreased levels of endothelial nitric oxide synthase were seen. Whereas In the Vitamin D and Glimepiride, combination treated group the decreased eNOS levels were increased significantly ($p<0.01$). Whereas Glimepiride and metformin treated alone have increased eNOS levels ($p<0.05$).

Table 7. Showing values of serum eNOS (mg/dl)

Group-I (NC)	Group-II(DC)	Group-IIIHFD	Group-IV (NCD)	Group-V (DCD)	Group-VI (DG)	Group-VII (DGD)	Group-VIII (DMET)	Group-IX (DMETD)
72.31± 14.32	22.37± 6.1	18.67± 2.71	25.29±3.3	32.73± 9.36*	41.64± 9.76*	48.5±6.77**	33.50± 6.42*	38.75± 5.55*



Graph 5. showing values of endothelial nitric oxide synthase enzyme (eNOS)

LIPID PROFILE

Diabetic animals showed significant elevations in triglycerides (TG), total cholesterol (TC), low density lipoprotiens (LDL) and very low density lipoproteins (VLDL) and accompanied with marked decline in HDL relative to the corresponding control.

Diabetic rats treated with Vitamin D (Group-V) showed reduction of total cholesterol, VLDL cholesterol and triacylglycerols. Whereas Vitamin D and Glimepiride combination caused a significant decrease ($p<0.01$) in the serum level of TG, TC, LDL cholesterol, when compared with diabetic rats. However, normal rats fed with HFD showed elevated levels of TG, TC, LDL, and VLDL and decreased levels of HDL ($P<0.01$) levels

The present study also showed increase the HDL levels significantly ($p<0.01$) in Glimepiride and Vitamin D treated rats when compared to diabetic control.

Table 7. Comparison of lipid profile among groups of experimental animals (n=6 in each group)

Parameters	Total Cholesterol	riglycerides	HDL	LDL	VLDL
Group-I (NC)	156.83±5.67	85.33±4.38	27±2.08	65±2.59	20.16±2.12
Group-II(DC)	324.66±9.42	`149.83±6.	13.83±0.79	244.50±8.1	28.66±0.95
Group-III (HFD)	264±11.30#	146.11±7.33#	22.11±3.43#	281±13.45##	29.56±2.06#
Group-IV (NCD)	172±4.55	99.33±4.98	15.83±1.13#	58.50±5.18#	18.41±1.009
Group-V (DCD)	263±2.80	139±3.27#	15.83±1.13#	196.21±13.81##	27.33±1.10#
Group-VI (DG)	205±6.58*	110.50±3.67*	35±0.89#	151±8.83###	17.43±0.87*
Group-VII (DGD)	165±13.90**	96.333±5.30*	31.16±0.87**	131.33±9.34**	16±0.68*
Group-VIII (DMET)	265±17.40	135.66±5.54	18.66±0.6	189.33±11.8*	24.16±1.70
Group-IX (DMETD)	225±8.23**	122.50±7.22*	21.66±0.76**	168±3.05**	23.50±1.11*

*P< 0.01, **P<0.001 compared to Diabetic control , # P<0.01, ##P<0.001 compred to normal control

HISTOPATHOLOGY

Histology of Pancreas

Histopathological examination showed degeneration of islets in diabetic rats, quantity of lobes are more, β cells are seen with unsaturated vacoules (2±), β cells with dark stained degenarated vacoules are seen. Apoptic changes were also seen in daibetic control. Groups treated with Vitamin D, Glimepiride and their combination did now show any pathological changes.

HISTOLOGY OF PANCREAS

Group-I (NC)

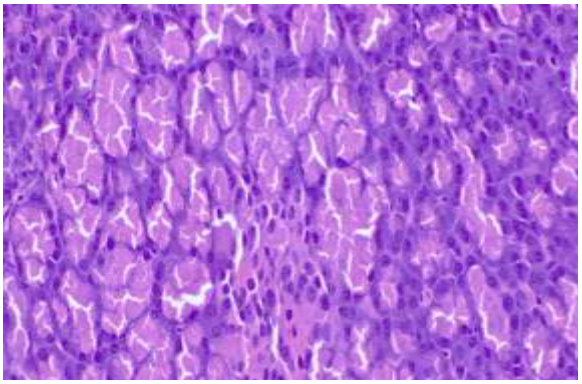


Image of Pancreas H/E stained 40X showing normal islets beta cells.

Group-II (DC)

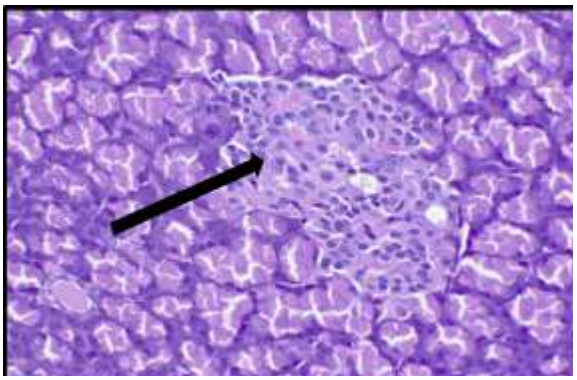


Image of Pancreas H/E stained 40X showing degeneration of islets, increase in lobes and beta cells with saturated vacuoles, some

Group-III (HFD)

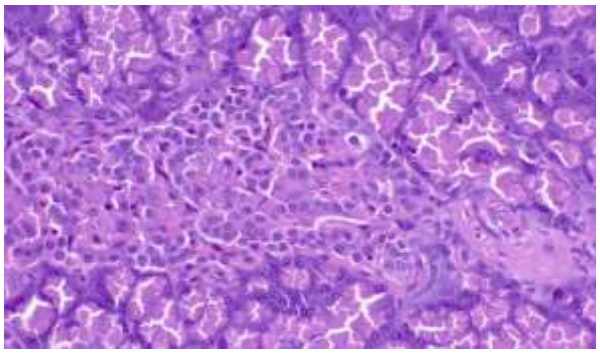


Image of Pancreas H/E stained 40X showing normal islets beta cells. No signs of apoptosis

Group-IV (NCD)

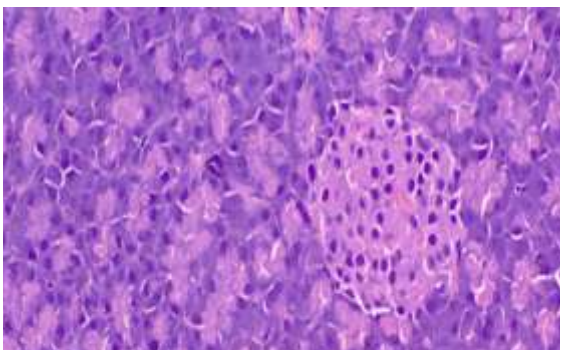


Image of Pancreas H/E stained 40X showing normal islets beta cells. No signs of degeneration in beta cells.

Group-V (DCD)

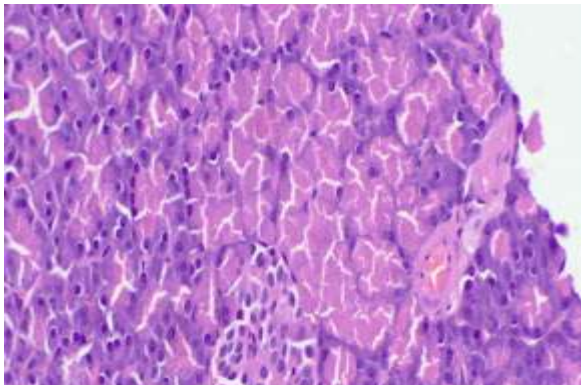


Image of Pancreas H/E stained 40X showing normal islets beta cells. Some degeneration of vacuoles is seen

Group-VI (DG)

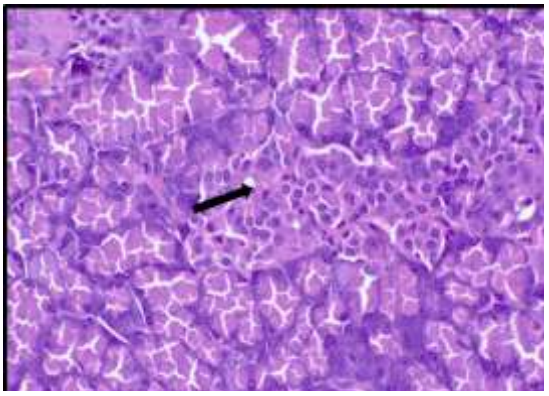


Image of Pancreas H/E stained 40X showing normal islets beta cells. Normal arrangement of islets are seen, beta cell count is more

Group-VII (DGD)

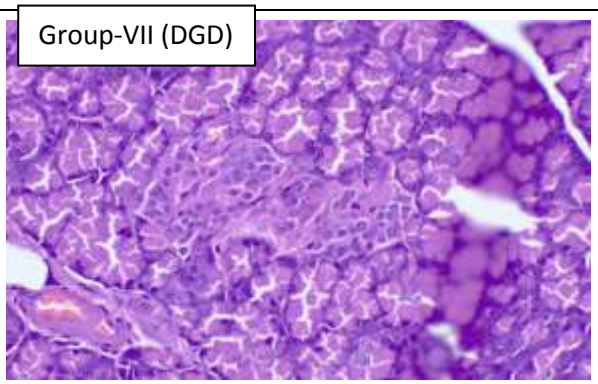


Image of Pancreas H/E stained 40X showing normal islets beta cells. Some degeneration of vacuoles is seen.

Group-VIII (DMET)

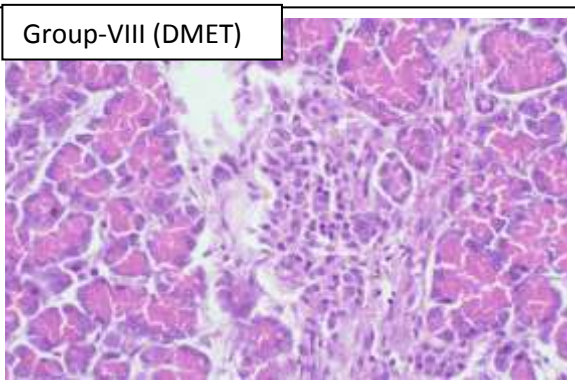


Image of Pancreas H/E stained 40X showing , distorted appearance of islets are seen, beta cells are seen with degenerated vacuoles.

Group-IX (DMETD)

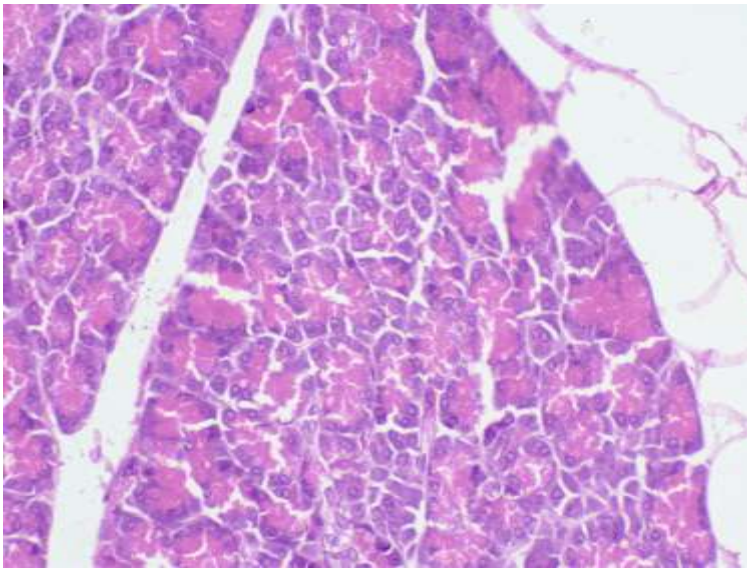


Image of Pancreas H/E stained 40X showing distorted islets with decreased beta cells. Some degeneration of vacuoles is seen.

HISTOLOGY OF LIVER

Group-I (NC)

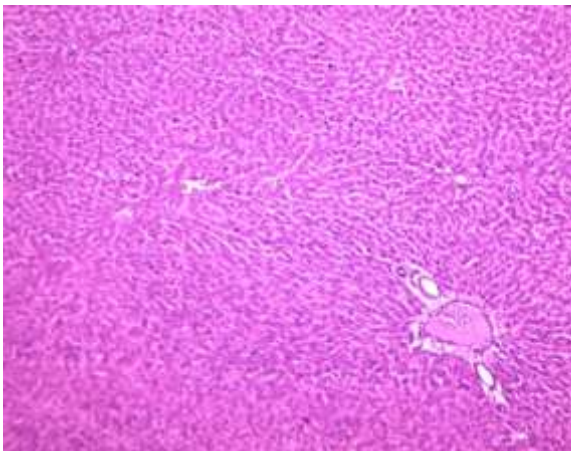


Image of liver H/E 40X Showing classical hepatic lobules; with cords of hepatocytes radiating from the central vein to the periphery of the lobule.

Group-II (DC)

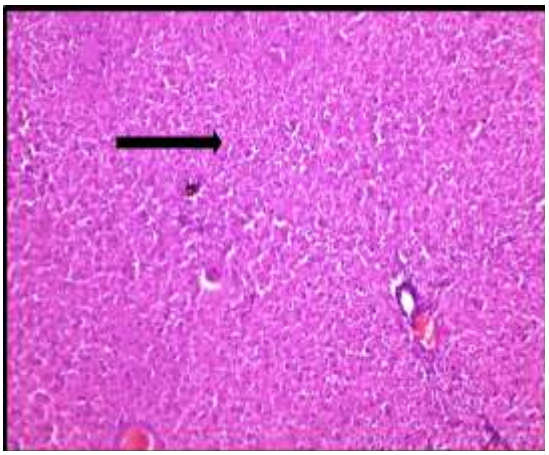


Image of liver H/E 40X Showing Hepatocytes around the central vein. narrow sinusoids, hepatocytes swelling and vacuolations, Some amount of necrosis is seen

Group-III (HFD)

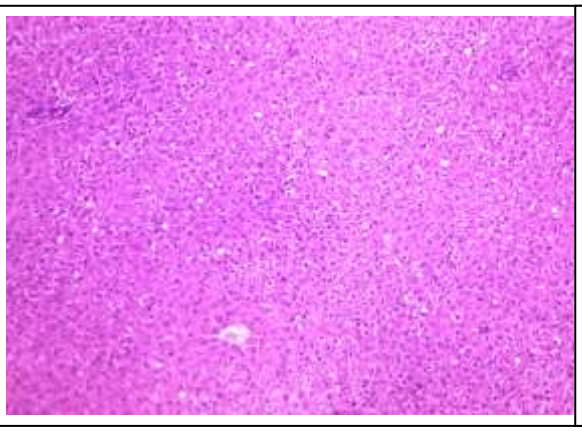


Image of liver H/E 40X Showing fatty changes are noted , all other appearences such as lobes, sinusoids, central vein are normal in appearance

Group-IV (NCD)

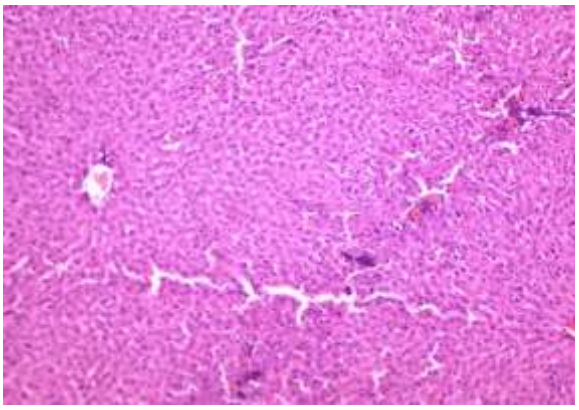


Image of liver H/E 40X Showing Normal in appearance, normal arrangement of hepatocytes around central vein.

Group-V (DCD)

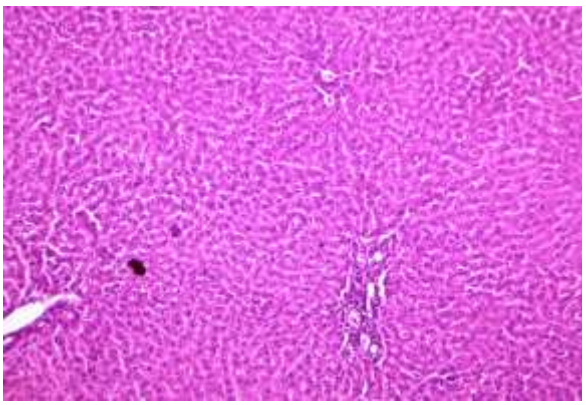


Image of liver H/E 40X Showing Normally arranged cells, with some necrosis. Large cells with a centrally placed prominent nucleolus. portal area and lobular architecture, sinusoids and central vein appear relatively narrowed.

Group-VI (DG)

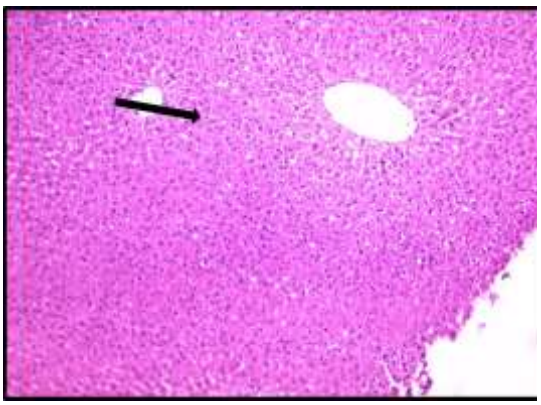


Image of liver H/E 40X Normal in appearance of cell and arrangements, fatty changes are noted

Group-VII (DGD)

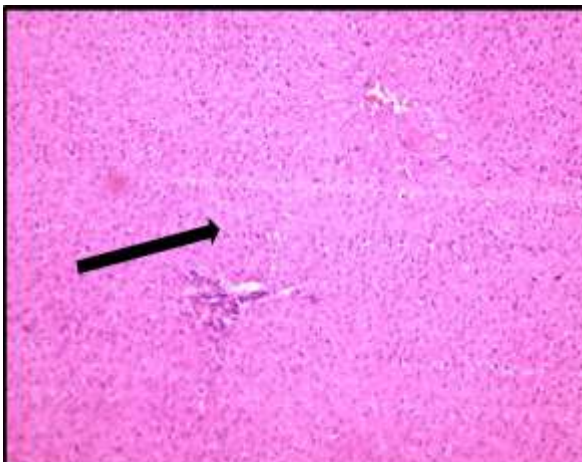


Image of liver H/E 40X Normal in appearance of cell and arrangements, fatty changes are noted

Group-VIII (DMET)

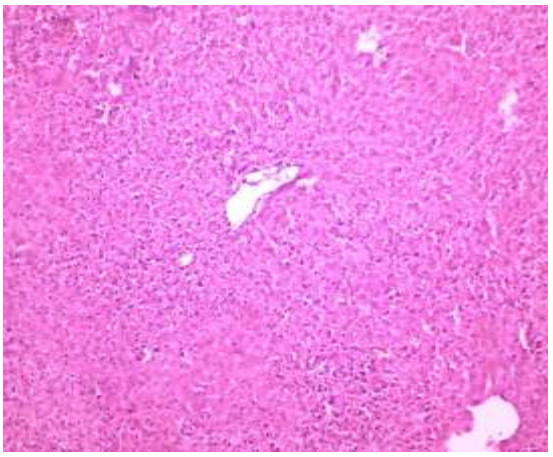


Image of liver H/E 40X Cells are large in size with more or less centrally placed prominent nucleolus, fatty changes are seen

Group-IX (DMETD)

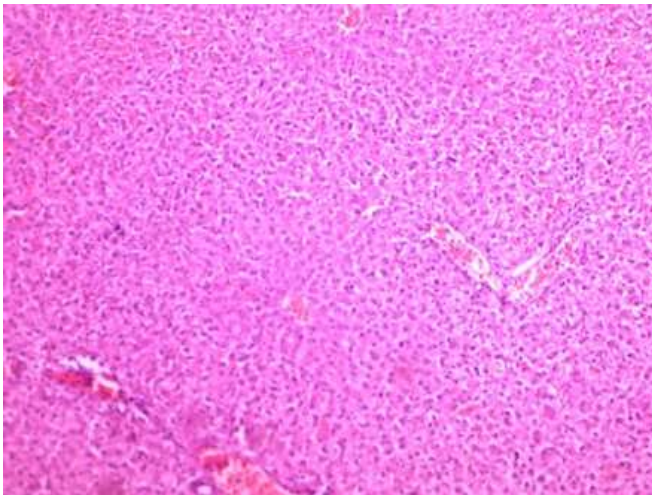


Image of liver H/E 40X Normally arranged cells with fatty changes are noted.

DISCUSSION

DISCUSSION

Diabetes mellitus is a multiorgan disorder characterized by early direct or indirect defects in muscles, hepatocytes, adipocytes, and β-cells of pancreas. (1) It has shown that dysregulation in insulin signalling mechanisms results in glucose intolerance, insulin resistance and T2DM. (2) The increasing prevalence of T2DM has stimulated the development of many new approaches to treat hyperglycemia effectively and safely. The main goal of these therapies is to reduce glucose levels and prevent diabetes complications. Several antidiabetic drugs are well accepted worldwide because of relatively low incidence of adverse effects and good therapeutic efficacy. The mechanism of action for most of these drugs has been well established. However, individual responses to these medications can differ significantly, possibly due to the heterogeneity of T2DM pathophysiology. (3) Vitamin D is a fat-soluble vitamin that mainly functions in calcium homeostasis and bone mineralization. Vitamin D deficiency, which has been considered a pandemic recently, is associated with skeletal manifestations, including rickets in the early years of life and osteomalacia in older ages. The effects of Vitamin D deficiency extend to include a wide range of extra-skeletal including cardiovascular, neuropsychiatric, endocrinal, gastrointestinal, and renal effects. (4) Evidence of the association between Vitamin D deficiency and T2DM has been accumulating over the past decade.

Researchers have shown that Vitamin D3 is a potent modifier of the risk of developing type 2 diabetes (T2DM). (5) Studies in humans have confirmed these findings showing that individuals with reduced concentrations of Vitamin D3 were at a higher risk of developing DM. (6) Therefore, this study is designed to evaluate the effects of Vitamin D on glucose homeostasis parameters, calcium levels, Vitamin D

and endothelial nitric oxide synthase and lipid profile in HFD/STZ diabetic rats in combination with oral anti-diabetic drugs.

HFD/STZ-induced diabetic experimental rat model synergistically simulates natural T2DM in human beings via a combination treatment of a diet high in fat to cause hyper-insulinemia, insulin resistance and glucose intolerance followed by STZ (β-cell toxin), injection to reduce functional β-cell mass. HFD/STZ diabetic experimental obese rat model is designed to simulate the early stages of human T2DM pathology characterized by various complex metabolic dysfunctions.

Effect on Body weight:

In the present study, we found that the supplementation of Vitamin D has an appreciable effect on body weight. We found that diabetic group had a continuous increase in body weight till STZ administration, but in subsequent weeks weight was not increased. Whereas rats in group III have a persistent increase in body weight till the end of the study. Our study did not show significant differences in diabetic and Vitamin D group. Whereas, in animals supplemented with the standard antidiabetic drug Glimepiride alone and along with Vitamin D, weight gain was significant ($P<0.01$) as compared to diabetic control rats. However, with Metformin and Vitamin D combination treated rats increase in body weight is not significant. Our study is in accord with the study done by Ahmad and Schucn NJ et al.(7)

Effect on Blood Glucose, Insulin and HbA1c

Beaulieu et al. showed that the glucose-stimulated insulin release is reduced in Vitamin D-deficient rats. In the present study, Glimepiride and its combination with Vitamin D had a glucose-lowering effect. This finding was in accord with results from previous studies compared with the sulfonylurea glibenclamide in T2DM patients. (8)

Supplementation of Vitamin D to a diabetic group has reduced blood sugar levels compared to the diabetic group ($p< 0.05$). Diabetic rats supplemented with Vitamin D, Glimepiride and Metformin combination have also shown a reduction in blood glucose, and it is significant ($p<0.001$ and 0.01), respectively. Liu et al. found a decrease in fasting glucose levels after supplementation with Vitamin D in adults and concluded that there is an inverse relationship between blood concentration of Vitamin D3, fasting glucose and insulin resistance. Supplementation of Vitamin D alone and along with Glimepiride has a significant reduction in blood sugar levels ($P<0.01$), ($P<0.001$). Metformin alone and in combination with Vitamin D has a positive effect but is not statistically significant. The present study correlates with the study done by Bourlon PM et al. similar results were shown by Mohamed I Saad et al. Several lines of evidence support a role for Vitamin D in pancreatic b-cell function and regulation of insulin secretion, which correlates with that of sugar levels in the present study. (9) In in-vitro and in-vivo studies, Vitamin D deficiency impairs glucose-mediated insulin secretion in rat pancreatic b-cells, whereas Vitamin D supplementation restores insulin secretion. (9) Insulin levels were increased significantly in rats treated with Vitamin D alone $P<0.05$, and in combination with Glimepiride, insulin levels were increased significantly ($P<0.001$). This increase in insulin levels correlates with HbA1c levels in Glimepiride, and Vitamin D supplemented group ($p<0.01$).

Mathieu and Lee JH et al. reported that the Vitamin D receptors are present in pancreatic β cells. Lack of Vitamin D is associated with impaired insulin synthesis and secretion. (10,11) It has also been reported that Vitamin D may improve glycemic control by increasing glucose transporter type 4 (GLUT4) expression and improving glucagon-like peptide-1 (GLP-1) function. (12) Vitamin D deficiency also increases

the level of parathyroid hormone, which contributes to increased insulin resistance and increased risk of hyperglycemia. (13)

The results of present study may be endorsed to that supplementation with vitamin D which has been shown to restore insulin secretion in animals by several mechanisms (Bourlon et al. 1999). Firstly, the β cell in the pancreas that secretes insulin has been shown to contain vitamin D receptors as well as the 1 alpha-hydroxylase enzyme. Secondly, researchers have also found an indirect effect of vitamin D on insulin secretion, potentially by a calcium effect on insulin secretion. Vitamin D contributes to the normalization of extracellular calcium, ensuring normal calcium flux through cell membranes (Pittas et al. 2007). Other potential mechanisms associated with vitamin D and diabetes include improving insulin action by stimulating expression of the insulin receptor, enhancing insulin responsiveness for glucose transport, and having an indirect effect on insulin action potentially via calcium effect on insulin secretion (Pittas et al. 2007). Our results are in concordance with Scragg et al. (2004) and Parker et al. (2010) who stated that there is a strong inverse association between low levels of Vitamin D and diabetes and increasing Vitamin D serum levels to normal led to a 55% relative reduction in the risk of developing type 2 diabetes.

Glimepiride is an insulin secretagogue which enhances insulin secretion from β-cells by different mechanisms. Glimepiride is a second-generation sulfonylurea that acts directly by binding to the ATP-dependent potassium channels on the β-cells, the closure of these channels by sulfonylurea's results in depolarization of the β-cells and a successive calcium influx which leads to glucose independent insulin release. Moreover, sulfonylurea's are reported to inhibit glucagon secretion from pancreatic α-cells (14)

A systematic review conducted by George et al. suggested significant improvement in fasting glucose, glycated haemoglobin or insulin resistance in individuals treated with Vitamin D when compared to placebo. Derakhshanian H et al. showed that high Vitamin D resulted in a significant reduction in fasting blood glucose and a significant increase in insulin levels, and a decrease in Hba1c values.

Several lines of evidence support the role of Vitamin D in pancreatic b-cell function and regulation of insulin secretion. (15, 16) In in-vitro and in vivo studies, Vitamin D deficiency impairs glucose-mediated insulin secretion in rat pancreatic b-cells, whereas Vitamin D supplementation restores insulin secretion. (17,18) Vitamin D may have a direct effect on b-cell function mediated by binding of the active circulating form, 1,25- dihydroxyVitamin D [1,25(OH)2D], to the Vitamin D receptor, which is expressed in pancreatic b-cells. (19, 20)

Thus all the above studies are in accord with the present study

Effect on eNOS

In the present study, it has been found that there are decreased levels of endothelial nitric oxide synthase enzyme in all diabetic rats. In the Vitamin D and Glimepiride, combination-treated group, the decrease in eNOS levels increased significantly ($p<0.01$), whereas standard drugs alone have increased eNOS levels ($p<0.05$). The above results are in line with the study by Paolo Tessari et al., An impaired NO metabolism is found in type 2 diabetes. (21)

In diabetes, an endothelial injury may be a consequence of glucometabolic and peroxidative stress. Experimental evidence suggests that the endothelium-dependent relaxation induced by acetylcholine is significantly reduced in the aorta of diabetic rats. Failure of vascular endothelium to evoke NO-mediated vasorelaxation may be

due to decreased formation of NO, increased degradation of NO, or a combination of both processes.

High glucose inhibits endothelial NOS activity through a protein kinase C–associated mechanism. Stimulation of NOS activity by insulin is impaired in the muscles of type 2 diabetic patients. (22) According to Muniyappa R et al., Nitric oxide is likely to be involved in the defective insulin-mediated stimulation of blood flow in type 2 diabetes. It has been demonstrated in previous in vitro studies that Vitamin D administration increased intracellular calcium content through the formation of intracellular second messengers, such as (cAMP),(DAG), and (IP3). (23) These signalling molecules are potent activators of protein kinase A (PKA) and protein kinase C (PKC), which trigger calcium release from intracellular stores and calcium uptake through the voltage-sensitive calcium channels in the plasma membrane. (24)

An increase in the intracellular calcium concentration promotes the formation of the calcium/calmodulin (CaM) complex, which plays an essential role in activating eNOS. Furthermore, Vitamin D may activate eNOS activity in a phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) pathway. (25) Study done by Mario Felaco et al. showed that levels of eNOS protein and its co-enzyme are significantly decreased in T2DM. Francesco Cosentino et al. stated that increased sugar increases eNOS expression. Thus the present study underlines the previous studies. The increase in eNOS values in Glimepiride and Vitamin D combination treated rats is significant ($p<0.01$) compared to diabetic control. Whereas other test drugs Glimepiride and Metformin-treated rats, also exhibited increased eNOS levels, and significance was found to be $p<0.05$.

Effect on calcium and Vitamin D

The present study showed elevated calcium and Vitamin D levels in Vitamin D-supplemented groups and resulted in a significant reduction in blood glucose and HbA1c. ($P<0.01$). So the present study found a significant correlation between serum calcium and blood glucose, HbA1c and insulin levels. The above results of the present study are comparable with a study done by Hoda D et al.

Effect on Lipid Profile

According to Cornell et al. 2006 lipids play a vital role in the pathogenesis of diabetes mellitus represented in dyslipidemia. Diabetic dyslipidemia is characterized by high plasma triglyceride, low-density lipoprotein (LDL) cholesterol, and reduction in high-density lipoprotein (HDL) cholesterol levels that are in consistant with our results. Wang et al. 2009 reported that there were direct and indirect effects of vitamin D on adapting the lipid profile, and serum levels of TG can be decreased through regulatory action that increases the activity of lipoprotein lipase in tissue.

According to Christensen et al. (2009) vitamin D improves absorption of calcium from intestine, This calcium could then reduce fatty acid absorption via the formation of insoluble calcium–fatty complexes in the gut the decreased absorption of fat, particularly saturated fatty acids, this leads to reduction in serum levels of total and LDL cholesterol. In addition, it has also been suggested that vitamin D receptors improve lipid metabolism by reducing acetylated low-density lipoprotein cholesterol uptake (Al-Daghri et al. 2012). More direct effects include the promotion of highdensity lipoprotein cholesterol particle formation and the regulation of serum apolipoprotein A-1 levels by vitamin D, both of which contribute to increased

cholesterol transport and overall improved lipid profiles (Carbone et al. 2008; Wehmeier et al. 2008; Al-Daghri et al. 2012).

Diabetic animals showed significant elevations in BG, TG, TC, LDL and VLDL and were accompanied by a marked decline in HDL relative to the corresponding control. Diabetic rats treated with Vitamin D showed a reduction of total cholesterol, VLDL cholesterol and triacylglycerols. in the present study, Combination of Vitamin D and Glimepiride caused a significant decrease ($p<0.01$) in the serum level of TG, TC, and LDL cholesterol when compared with diabetic rats. The present study also showed increase in HDL levels significantly ($p<0.01$) in Glimepiride and Vitamin D-treated rats when compared to diabetic control.

Effect on Histopathology

Histopathological examination showed degeneration of islets in diabetic rats. The quantity of lobes are more, β cells are seen with unsaturated vacuoles (2±), and β cells with dark stained degenerated vacuoles are seen. Apoptotic changes were seen in diabetic control rats. Groups treated with Vitamin D, Glimepiride and their combination did now show any pathological changes. Present study results are inconsistent with the study done by Furman BL et al. Lee HA et al. has demonstrated alterations of the endocrine pancreas in STZ-induced diabetic rats and mice similar to our findings. The present study results, supported by the histological findings in the liver of diabetic rats treated with Vitamin D and Glimepiride, suggest the hepatoprotective effects of Vitamin D in diabetes and comply with previous studies. (26) The liver histoarchitecture is nearly preserved in rats treated with Vitamin D, Glimepiride and Metformin.

CONCLUSION

CONCLUSION

The present study shows the beneficial effects of Vitamin D alone when combined with oral antidiabetic agents in improving insulin, BG, and lipid profile, as observed in STZ-induced diabetic rats. Reduction in TG, TC, LDL, and VLDL, increased HDL levels and decreased enzyme eNOS have been seen in Vitamin D-supplemented groups of rats. Present study results suggest that the inclusion of Vitamin D in a regular regimen may help in reducing the dose of OHA. Thus, preventing the metabolic load and its complications and improving quality of life. A study can be further carried out to evaluate the efficacy of Vitamin D and oral antidiabetic agents in diabetic subjects. Once established as a potent modifier of diabetes mellitus in larger patients.

LIMITATIONS AND FUTURE PROSPECTS

LIMITATIONS AND FUTURE PROSPECTS

As the study has been carried out in a limited number of animals, it requires further studies and the inclusion of other parameters, such as detecting Vitamin D receptors and estimating Vitamin D-dependent enzyme activities in glucose homeostasis in various body tissues to validate this study's outcome.

Large clinical trials are needed to include Vitamin D in regular treatment of type-II diabetes mellitus.

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ANNEXURES

B.L.D.E(DEEMED TO BE UNIVERSITY)

(Declared vide notification No. F.9-330/97-U.3 (A) Dated. 29-2-2004 of the MHRD, Government of India under Section 3 of the UGC Act,1956)

The Constituent College

SHRI. B. M. PATIL MEDICAL COLLEGE, HOSPITAL AND RESEARCH CENTRE, VIJAYAPUR.

DEPARTMENT OF PHARMACOLOGY

CERTIFICATE

This is certify that the project title: "An Evaluation Of role of vit D in the pathophysiology of streptozotocin induced type-II diabetes mellitus in rats and its impact on oral hypoglycemic/antidiabetic agents" . (Proposal No. 01/ BLDE (DU)/ 2018)

Has been approved by the I A E C.

Mr. Gurudatta Moharir has been permitted to utilize 54 Albino Wistar Rats to carry out his research work at the earliest.

Dr. A A Naikwadi
Name of the Chairman/member Secretary I A E C

03-10-2018
Signature with date.

Chairman/ Member Secretary of I A E C

Dr. Viswantha Swamy A.H.M.
Name of C P C S E A Nominee:

03/10/18

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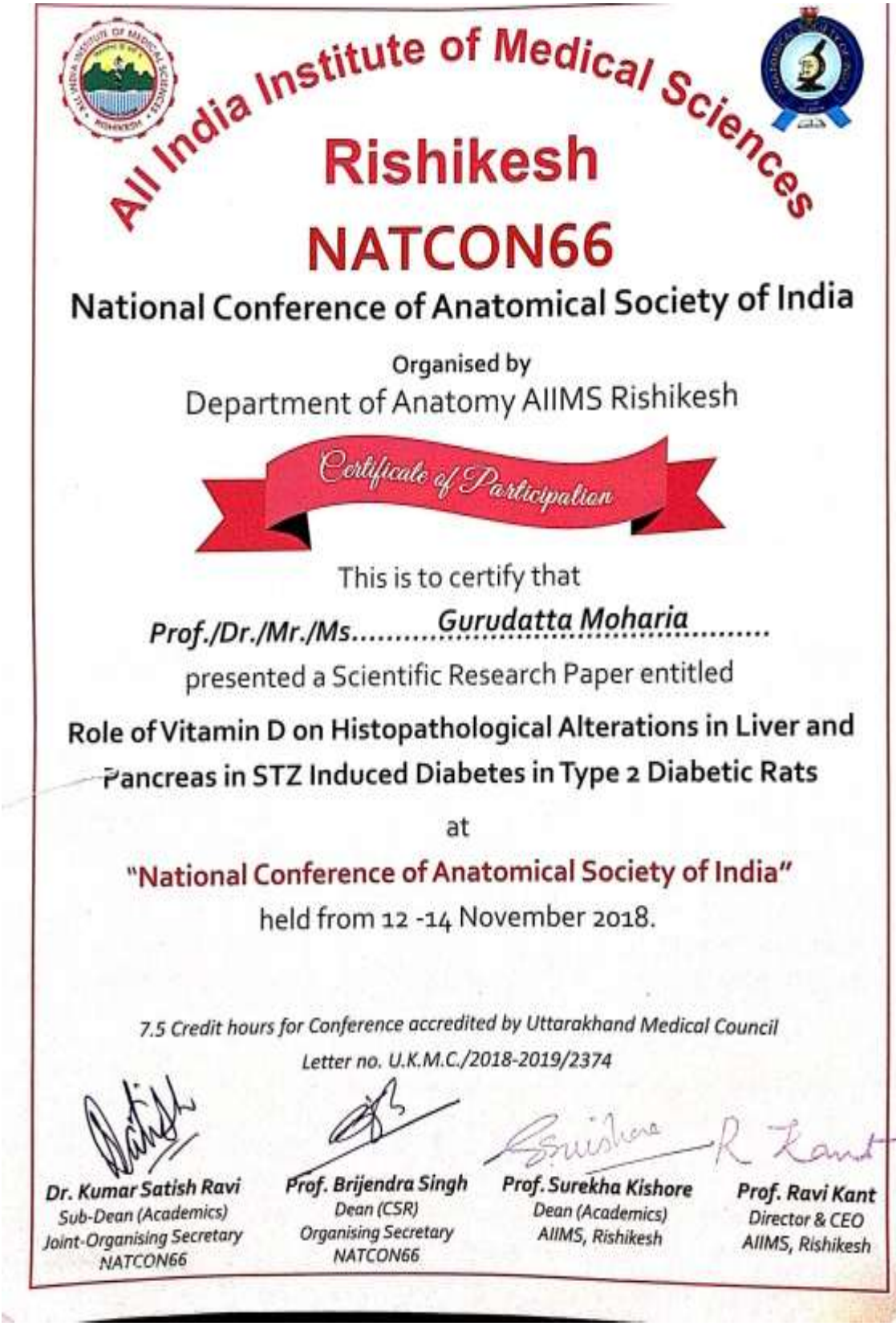

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This is to certify that

Prof./Dr./Mr./Ms.....Gurudatta Moharia.....

presented a Scientific Research Paper entitled

Role of Vitamin D on Histopathological Alterations in Liver and Pancreas in STZ Induced Diabetes in Type 2 Diabetic Rats

at

"National Conference of Anatomical Society of India"

held from 12 -14 November 2018.

7.5 Credit hours for Conference accredited by Uttarakhand Medical Council

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He / She is granted 4 CPD credit points by Maharashtra Medical Council.

Prof. (MS) Syed Mudassar	Prof. Abbas Ali Mehadi	Prof. Bipin M. Tiwale	Dr.R.K.Sharma	Dr.Sandeep S. Kadam	Dr.Shailesh Kore
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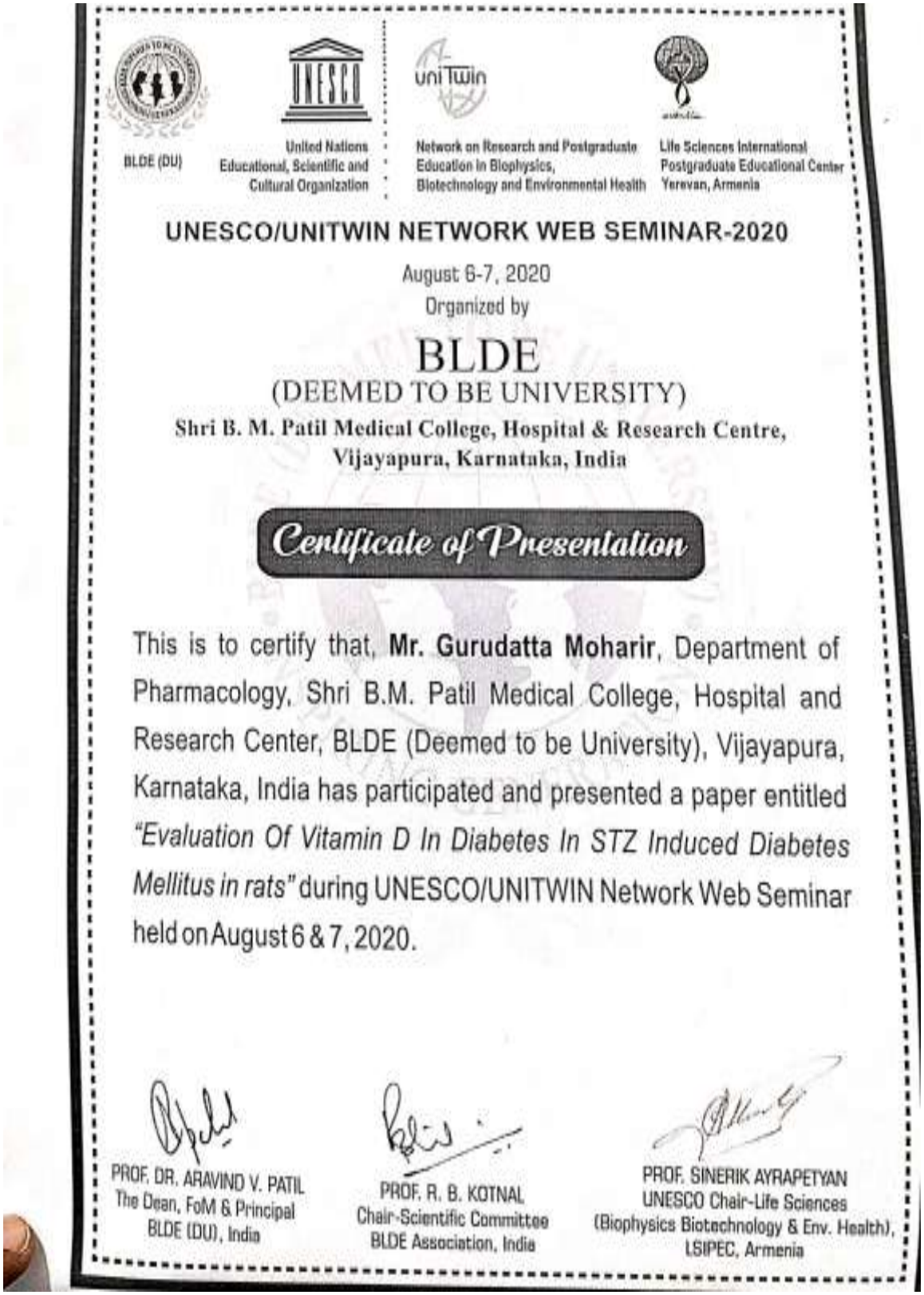

BLDE (DU)

United Nations Educational, Scientific and Cultural Organization

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Life Sciences International Postgraduate Educational Center Yerevan, Armenia

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Certificate of Presentation

This is to certify that, **Mr. Gurudatta Moharir**, Department of Pharmacology, Shri B.M. Patil Medical College, Hospital and Research Center, BLDE (Deemed to be University), Vijayapura, Karnataka, India has participated and presented a paper entitled *"Evaluation Of Vitamin D In Diabetes In STZ Induced Diabetes Mellitus in rats"* during UNESCO/UNITWIN Network Web Seminar held on August 6 & 7, 2020.

PROF. DR. ARAVIND V. PATIL
The Dean, FoM & Principal
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PROF. R. B. KOTNAL
Chair-Scientific Committee
BLDE Association, India

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MÆDICA - a Journal of Clinical Medicine

ORIGINAL PAPER

Effect of Vitamin Don Blood Sugar, HbA_{1c} and Serum Insulin Levels in Streptozotocin-Induced Diabetic Rats

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BACKGROUND

Background: *Type 2 diabetes mellitus (T2DM) is an increasingly common disorder characterized by chronic hyperglycemia and marked dyslipidemia. This study evaluates the effect of vitamin D supplementation alone and in combination with glimepiride in streptozotocin-induced T2DM in rats.*

Materials and methods: *A total of 30 Wistar albino rats of either sex weighing 150-200 g were included in the study. The effect of oral administration of vitamin D was evaluated in streptozotocin-induced T2DM in rats. Blood glucose, serum insulin, serum HbA_{1c}, and serum vitamin D were evaluated.*

Results: *Vitamin D treatment has significantly improved hyperglycemia, hyperinsulinemia, and insulin sensitivity compared with the non-treated diabetic rats. Oral administration of vitamin D in streptozotocin-induced T2DM reduced blood sugar levels, increased insulin levels (more prominently when administered along with glimepiride) and decreased HbA_{1c} levels ($p<0.005$).*

Conclusion: *Administration of vitamin D can improve hyperglycemia and hyperinsulinemia in streptozotocin-induced T2DM in rats. Thus, it could be considered as an add on therapy along with other antidiabetic drugs.*

Keywords: type 2 diabetes mellitus, streptozotocin, HbA_{1c}, antidiabetic drugs.

INTRODUCTION

Type 2 diabetes mellitus (T2DM) is a multifactorial disease such as chronic hyperglycemia, altered insulin secretion, and insulin resistance – a state of decreased responsiveness to normal concentrations of circulating insulin (1, 2). It is also defined by impaired glucose tolerance (IGT) that results from islet β-cell dysfunction, followed by insulin deficiency in skeletal muscle, liver, and adipose tissues (3, 4). In individuals with IGT, the development of T2DM is governed by genetic predisposition and environmental variables (a hypercaloric diet and the consequent visceral obesity, or increased adiposity in liver and muscle tissues) and host-related factors (age, imba-

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lances in oxidative stress, and inflammatory responses) (5, 6). Clinical complications of T2DM include both microvascular diseases (*e.g.*, retinopathy, nephropathy, and neuropathy) and macrovascular complications (*e.g.*, myocardial infarction, peripheral vascular disease, and stroke) (1). Macrovascular complications are the leading cause of mortality among diabetic persons (7). Although important knowledge has been acquired on the etiology of diabetes, its precise etiopathogenesis is still under debate. Inflammatory factors, reactive oxygen species, and autoimmune reactions have all strongly emerged as the major pathogenic effectors for diabetes.

The involvement of vitamin D in pathogenesis and prevention of diabetes has recently generated widespread interest. Many studies highlighted a correlation between vitamin D and incidence of T2DM. Thus, a seasonal variation in glucose control was reported to be worse in winter in T2DM patients (4), which may be due to prevalent hypovitaminosis D as a result of reduced sunlight during the cold season. Insulin resistance is a recognized precursor for the development of T2DM. Vitamin D has a favorable effect on insulin action either directly – by stimulating the expression of insulin receptors and thereby, enhancing insulin responsiveness for glucose transport (7) – or indirectly *via* its role in regulating extracellular calcium, ensuring normal calcium influx through cell membranes and adequate intracellular cytosolic Ca^{2+} pool. Calcium is important for insulin-mediated intracellular processes in insulin-responsive tissues (skeletal muscle and adipose tissue), with a very narrow range of Ca^{2+} needed for optimal insulin-mediated functions. Changes in Ca^{2+} in primary insulin target tissues may contribute to peripheral insulin resistance *via* impaired insulin signal transduction, leading to decreased GLUT-4 activity (7). It also was shown to improve glucose tolerance (2). Vitamin D, by its function as an important antioxidant, may also prevent T2DM.

It was found that few animal studies were showing the effect of vitamin D on blood sugar levels, especially in combination with oral hypoglycemic agents. Many exertions are shifted towards disease prevention and a search for safer drugs. Several studies showed that T2DM was associated with low vitamin D levels. For this reason, our study aimed to test the effect of vitamin D supplementation alone and along with an oral hypoglycemic agent (glimepiride) in T2DM. More precisely, the study objectives were to evaluate the effect of vitamin D supplementation alone and in combination with glimepiride on serum insulin, HbA_{1c}, and blood glucose levels in streptozotocin (STZ) induced T2DM in rats receiving a high-fat diet (HFD).

MATERIALS AND METHODS

A total of 30 Wistar albino rats of both sexes, weighing150-200 g, were included in the study. The work was carried at the central animal house of BLDE (Deemed to be University), Sri. B.M. Patil Medical College Hospital and Research Center, Vijayapur, Karnataka, India. Animals were kept at room temperature (22-28°C), with 55±10% relative humidity, for 12 hours, being exposed to dark-light cycle, and were given standard pellet chow, HFD, and water ad libitum. Institutional Animal Ethics Committee (IAEC) approval was obtained (NO-01/BLDE(DU)/2018).

Streptozotocin was purchased from Sigma Aldrich USA. Glimepiride was procured from IPCA laboratories, Mumbai, and vitamin D was purchased from Fermenta Biotech. HFD was prepared as per Guo *et al*. (8). Insulin, HbA_{1c}, and Vitamin D kits were purchased from Chongqing Biospes Co., Ltd, China.

Experimental design

Rats were divided into five experimental groups (n=6), each kept in a separate cage, as follows: group 1=control; group 2=diabetic control; group 3=diabetic control with vitamin D; group 4=diabetic with standard treatment (glimepiride); and group 5=diabetic with standard treatment + vitamin D.

Rats in groups 2-5 were fed with HFD till completion of the study (during three weeks), and STZ was administered intraperitoneally (IP) in a dose of 35 mg/kg (8) body weight (dissolved in distilled water). After five days, blood samples were tested for glucose levels, and values above 250 mg/dL were included in the study. Group 1 received liquid paraffin daily and standard pellet chow.

Post diabetes, rats in group 3 were given vitamin D 4 000 IU/kg body weight orally, while groups 4 and 5 received glimepiride and glimepiride + vitamin D, respectively, for three weeks. Glimepiride was administered in a dose of 100 mcg/kg body weight orally. Liquid paraffin

was used as a vehicle for the administration of vitamin D and glimepiride. At the end of the study, blood samples were collected through retro-orbital plexus using heparinized capillary tubes, centrifuged, and serum was used for estimation of insulin, HbA_{1c} and vitamin D.

RESULTS

When compared to group 1, all rats had a significant gain in body weight ($p<0.05$), but in group 2, the gain in body weight has been not substantially increased, being rather constant after one week of study. At the same time, body weight had a significant decrease ($p< 0.05$) in rats treated with glimepiride (group 4), glimepiride, and vitamin D (group 5) (Figure 1).

At baseline, blood glucose levels were comparable in all groups. In group 2, there was an increase in blood glucose levels (hyperglycaemic), which remained consistently high from week 1 to the end of the study; in groups 4 and 5, blood glucose levels were significantly ($p<0.05$) reduced; moreover, addition of vitamin D to the treatment given to group 5 rats lead to a marked reduction compared to glimepiride alone (Table 1).

Among diabetic control rats (group 2) there were decreased serum insulin levels and significantly increased HbA_{1c} levels ($p<0.05$) compared to the control group 1, whereas vitamin D levels were comparable. Diabetic rats treated with vitamin D (group 3) showed a slight increase in serum insulin levels and a decrease in those of HbA_{1c} compared to group 2, while vitamin D levels were significantly elevated ($p<0.05$). Glimepiride treatment (group 4) lead to an increase of serum insulin levels and a significant reduction in those of HbA_{1c} ($p<0.05$), as compared to untreated diabetic rats (group 2). A significant increase in levels of serum insulin and vitamin D as well as a significant decrease in HbA_{1c} levels was seen when glimepiride and vitamin D were administered concomitantly (group 5) compared to group 2, and serum insulin values were comparable to group 1 (Table 2).

DISCUSSION

In this study, experimental induction of T2DM in rats resulted in increased fasting blood glucose and decreased fasting serum insulin. Also, vitamin D supplementation has significantly lowered glucose levels and raised insulin levels; this may be endorsed to supplementation with vitamin D which has been shown to reinstate insulin secretion in animals (9).

Our study showed that vitamin D treatment has significantly improved hyperglycemia, hyperinsulinemia, and insulin sensitivity compared with non-treated diabetic rats. The reduction in glucose levels was found to be significantly higher in combined therapy with vitamin D and glimepiride than glimepiride monotherapy. The improvement obtained with vitamin D supplementation was in agreement with the study of Zeitz *et al* (10). Others provided evidence that vitamin D supplementation exerted beneficial effects in obese spontaneously hypertensive rats and Wistar

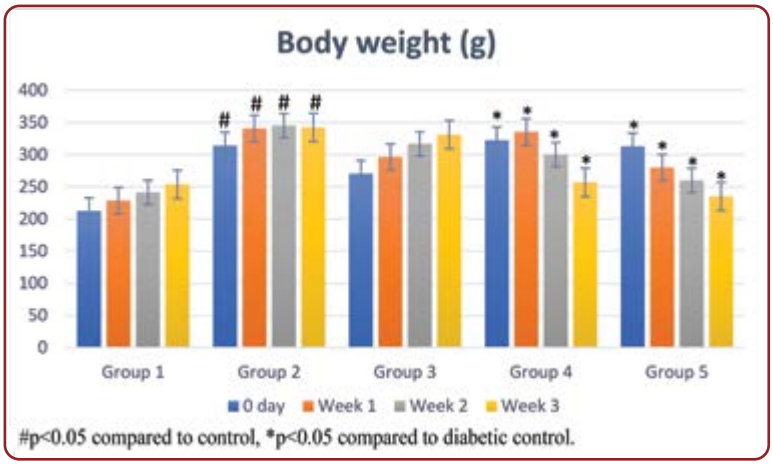

FIGURE 1. Body weight (g) in all groups (mean±SD)

GROUPS	0 day	Week 1	Week 2	Week 3
Group 1	77.00±8.50	80.66±3.14	78.16±7.30	73.66±6.18
Group 2	259.67±12.22	404.66±20.88	418.83±23.47	409.33±17.16*
Group 3	265.33±11.18	320.83±22.55*	298.83±16.94*	313.33±19.05*
Group 4	267.16±5.49	241.83±7.35*	212.00±3.22*	179.66±6.40*
Group 5	255.50±4.03	222.00±8.19*	176.00±4.14*	154.33±5.04*

#p<0.05 compared to control, *p<0.05 compared to diabetic control.

TABLE 1. Blood glucose levels (mg/dL) in all five study groups (mean±SD)

TABLE 2. Serum insulin, HbA_{1c} and Vitamin D levels (mean±SD)

GROUPS (n=6)	Serum Insulin (mU/L)	HbA_{1c} (nmol/L)	Vitamin D (µg/L)
Group 1	7.65±0.25	718±91.04	244.81±24.02
Group 2	2.7 ±0.53#	1426.5±110.03#	267.66± 19.52
Group 3	3.14±0.40	1299.33±83.32	393.5±23.17*
Group 4	5.8±0.46*	981±90.26*	300.16±20.76
Group 5	6.66±0.58*	856±40.21*	386.33±30.42*

#p<0.05 compared to control, *p<0.05 compared to diabetic control.

rats, where there was a reduction in glucose levels in vitamin D-supplemented animals (5, 11, 12).

Insulin secreted from pancreas has been shown to contain vitamin D receptors as well as the 1 alpha-hydroxylase enzyme. Also, researchers have found an indirect effect of vitamin D on insulin secretion, potentially by a calcium effect on insulin secretion. Vitamin D contributes to the normalization of extracellular calcium, ensuring normal calcium flux through cell membranes (13). Other possible mechanisms related to vitamin D and diabetes include convalescing insulin action by stimulating expression of the insulin receptor, increasing insulin responsiveness for glucose transport, and having an indirect effect on insulin action potentially *via* calcium effect on insulin secretion (13). Our results were in concordance with those reported by Scragg *et al* (14), who stated that there was a strong inverse association between low levels of 25(OH)D and diabetes, and increasing vitamin D serum levels to normal led to a 55% relative reduction in the risk of developing T2DM.

In the present work, improvements in insulin secretion with vitamin D supplementation were observed in a rat model of T2DM. There was also a decrease in HbA_{1c} levels with vitamin D alone and in combination with glimepiride. Our study showed that vitamin D supplementation alone has raised insulin levels and exerted an additive effect on glimepiride action. The reduction in glucose levels was found to be significantly higher when glimepiride and vitamin D were administered as combination therapy. The improvement seen after vitamin D treatment is consistent with the findings of Kayaniyil *et al* (15).

In a study by Calle *et al* (16), treatment with vitamin D to streptozotocin-induced diabetic rats improved the decreased basal glucose transport by 107%, which was correlating to a reduction in glucose levels in our study. Treatment with vitamin D resulted in a noteworthy increase in insulin concentration, which could improve hyperglycemia in diabetic rats. Serum HbA_{1c} has significantly decreased in vitamin D treated rats, which is consistent with a study by Hoda *et al* (17).

Control rats had constantly increased their body weight, whereas diabetic rats dropped body weight during the first week after STZ injection, probably because of decreased glucose metabolism and increased fat metabolism (18). Subsequently, the body weight of rats in the STZ group was further increased until the completion of the study, while the groups with vitamin D and glimepiride and glimepiride + vitamin D group did not have an increase in body weight similarly to diabetic control – therea was a sugnificant difference, and the p-value was <0.001. The authors considered that the reduced insulin and increased body weight induced by STZ had a higher resemblance to T2DM. The difference in body weight between group 2 and groups 4 and 5 was significant ($p < 0.001$).

The possible mechanisms by which vitamin D can influence the metabolism of glucose may be the result of a rapid non-genomic effect or a slower genomic effect of vitamin D by stimulating insulin release through increased VDR expression (19). Another possible mechanism is the suppression of the release of proinflammatory cytokines that are believed to mediate insulin resistance. The latter hypothesis is supported by studies showing an association between low serum 25(OH)D and increased C-reactive protein levels. Besides, vitamin D may indirectly influence the extracellular and intracellular calcium regulation, which is essential in mediating glucose transport in target tissues. In an attempt to understand the role of vitamin D in β-cell function, Nyomba *et al* (20) found that plasma calcium levels, vitamin D binding protein (DBP), circulating vitamin D, and bone mass were reduced in STZ-induced diabetic rats. These defects were related to altered metabolism of vitamin D due to an inhibitory effect of insulin deficiency on renal function 25(OH)D3 1α-hydroxylase. The concentration of HbA_{1c} decreased marginally, but insignificantly, in group 3, and had a significant decrease in groups 4 and 5. In addition, a conspicuous enhancement was observed in the insulin level. Previous studies suggest several different mechanisms to explain the role of vitamin D in normalizing the glucose level. Lack

of vitamin D has been shown to result in impairment (disturbance) of glucose metabolism by increasing insulin resistance, which in turn occurs due to decreased adipose expression and degradation of the β-cell function and mass (21).

Supplementation with cholecalciferol was found to restore the alteration in IP3 and AMPA receptor, a non-NMDA-type ionotropic transmembrane receptor for glutamate expression in the pancreatic islets, which helped to restore calcium-mediated insulin secretion, indicating the therapeutic role of vitamin through the regulation of glutamatergic function in diabetic rats (22).

CONCLUSION

Administration of vitamin D can improve hyperglycemia, hyperinsulinemia in streptozotocin-induced type 2 diabetes mellitus in rats, thus can be potentially considered as add on therapy along with other antidiabetic drugs. However, further randomized clinical control trials are required to confirm the dose and duration before it is included in the therapy of T2DM. ❑

Conflicts of interest: none declared.

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Effect of vitamin D on blood glucose and lipid profile in streptozotocin-induced diabetic rats

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***Abstract*---**Introduction and Objectives: One of the vital nutrients, vitamin D, has a role in fat metabolism and other metabolic processes in addition to its direct impact on calcium and bone metabolism. This study intends to look at how vitamin D intake affects the levels of

lipids, glucose, and insulin in rats with experimentally induced diabetes mellitus. Materials and Methods: 24 male albino Wistar rats, weighing between 250 to 300 g, were placed into four groups at random (n=6). Group 1 served as the control group, while the other three groups were given an i.p. injection of 35 mg/kg streptozotocin (STZ) to induce diabetes and a high-fat diet for three weeks. Then, for the following three weeks, groups III and IV received treatment with vitamin D (400 IU/kg) and Vitamin D + Glimepiride (50 mcg/kg). Results: Vitamin D supplementation was observed to dramatically lower lipid profile, raise insulin, and decrease HbA1c concentration. Additionally, when Vitamin D and Glimepiride were administered together, a substantial decrease in the serum concentrations of blood glucose, HbA1c, and lipid profile was seen (p 0.05). Conclusion: According to the findings of this study, treatment with glimepiride and vitamin D could dramatically lower the fasting plasma glucose, insulin, and lipid profiles in an experimental type 2 diabetic animal.

***Keywords*---**vitamin D, diabetes mellitus, streptozotocin, HbA1c, rats.

Introduction

Diabetes mellitus type 2 is a non-communicable disease that is spreading over the world at an alarming rate [1]. According to the WHO, the prevalence of diabetes will increase from 17.1 crores people in 2000 to 36.6 people in 2030 [2]. Type 2 DM is linked to hypertension[3] cardiovascular [4,5] disease, blindness, nephropathy, and neuropathic consequences [6,7]. The main role of metabolism and bone health is played by vitamin D, a fat-soluble vitamin. Vitamin D insufficiency has been linked to a variety of non-skeletal ailments [8], including osteoporosis, cancer [9], and immunological disorders; type 1 and type 2 diabetes; hypertension; cardiovascular disease; and others. The vitamin D receptor (VDR) and the 1-hydroxy enzyme (25-hydroxyvitamin D is converted to 25-hydroxyvitamin D) are both found in numerous organs [10].

Vitamin D insufficiency has been linked to reduced glucose tolerance and diabetes mellitus in numerous cross-sectional and interventional investigations [11,12,13] . As a result, blood hydroxyl cholecacitriol concentrations in T2DM patients are lower than in healthy individuals. The adverse association between vitamin D and type 2 diabetes has been explained by many processes. Through genetic and non-genomic processes, On insulin synthesis, beta-cell function, and insulin resistance, vitamin D has both direct and indirect effects [14,15]. Contrarily, the major cause of death in those with type 2 diabetes is cardiovascular disease. Vitamin D is necessary for endothelial function, blood pressure regulation, calcification of the coronary vasculature, raising vascular resistance, and avoiding CVD, claims a study [16]. One of the hypothesised mechanisms for the association between vitamin D levels and lipid profile is vitamin D's action on lipid profile modulation. The goal of our research is to see if there's a link between vitamin D levels in the blood and lipid profiles such as cholesterol, TG, HDL, and LDL in type 2 diabetic rats.

Materials and Methods

24 male Wistar rats weighing 200–250 g were used in the experiment. The investigation was conducted at BLDE University in Vijayapur's main animal house. Prior to and after the experiment, rats were housed with free access to food and water, a 12:12 h light/dark cycle, and stable environmental conditions. The handling of the animals during the experiment, sampling, and sacrifice all adhered to CPCSEA regulations. The IAEC of BLDE University, Vijayapur, authorised the study. Four groups of rats were randomly assigned; group 1 served as the control group, while groups 2, 3, and 4 were fed a high-fat diet [17]. for three weeks and received a single intraperitoneal (i.p.) 35 mg/kg injection of freshly prepared STZ, dissolved in 0.1 M sodium citrate buffer with pH 4.5, within a few minutes of preparation. Three days following STZ injection, diabetes was assessed by checking the blood glucose level using glucometer (Aqua Chek Active) strips. Diabetic rats were defined as those with blood glucose levels greater than 250 mg/dL and were included in the study. Following 72 hours of diabetes induction, the treatments started and continued each day for 21 (three weeks) straight days.

Animals were grouped as follows

Group 1: oral distilled water was administered.
Group 2: diabetic untreated wistar rats were given vehicle daily orally.
Group 3: diabetic rats received 400 iu/kg body weight of vitamin d daily orally.
Group 4: diabetic treated 400 iu/kg body weight of vitamin d and glimepiride (glim) 50 mcg/kg body weight daily orally.

Experimental procedures and laboratory measurements

At the end of the study period, anaesthetic was administered to the animals, and blood samples were taken through the retro-orbital plexus for evaluation of the following parameters: HbA1c, Triglycerides (TG), Total cholesterol (TC), High-Density Lipoprotein-Cholesterol (HDL-C), and Low-Density Lipoprotein-Cholesterol (LDL-C). A 50mcg/kg body weight oral dosage of glimepiride was given. Carboxymethylcellulose was used as a vehicle for the administration of glimepiride and vitamin D. Throughout the study, all efforts were taken to reduce rats' suffering. Statistical analysis was done by using SPSS version-17 and methods used were oneway ANOVA and Student’s t-test.

Results

Table 1. Body weight at the beginning and after the end of every week in gm (Mean±SD)

Groups	Week 0	Week 1	Week 2	Week 3
Control	212±8.2	228±7.6	241±8.1	253±5.7
Diabetic Control (DC)	345±10.1	380±11.5	398±8.4	402±9.2
DC + Vit D	314±6.1	341±4.4	345±6.6	342±9.5
Glim + Vit D	315±4.4	280±4.7	260±6.4	235.5±5

Animals in group 1-3 gained body weight consistently. However, rats in group 2

(Diabetic control) gained significant ($p<0.05$) body weight compared to normal control rats. Whereas, body weights in group 4 (Diabetic + Glimiperide and Vitmain D) rats were decreased signicantly compared to the group 1 (normal control) and group 2 (diabetic control).

Table 2. Blood glucose(FBS) before 1 hour of stz administration and on subsequently at the end of every week (mg/dL) (Mean±SD)

Groups	Week 0	Week 1	Week 2	Week 3
Control	80±4.3#	80±4.1#	78±3.4#	73±4.7#
Diabetic Control (DC)	125±6.1*	404±25.7*	518±34.1*	526±37.7*
DC + Vit D	118±6.2*	368±16.5*	459±12.1*	471±9.4*#
Glim + Vit D	120±8.1*	130±8.3*#	123±10.3*#	90±5.2*#

*p<0.05 compared with the control group. #p<0.05 compared with the diabetic group

The blood glucose levels of diabetic rats and diabetes treatment groups (groups 3 and 4) were significantly ($p<0.05$) elevated compared to the normal control group. Group 3 rats treated with Vitmain D alone demonstrated a modest reduction in blood glucose levels. Vitmain D in conjunction with Glimepiride significantly decreased blood glucose levels compared to Group 2 (Diabetic control).

Table 3. Lipid profile of diabetic male rats treated with glimepiride, vitamin D, and their mixtures (MeanSD) and control rats

Groups	TG (mg/dL)	TC (mg/dL)	LDL(mg/dL)	VLDL (mg/dL)	HDL (mg/dL)	CAL (mg/dL)
Control	85±10.7	156±13.9	65±6.3	20±5.2	27±5.1	10.78±0.5
Diabetic Control (DC)	149±15.1#	324±23.1#	244±22.2#	28±2.3#	13±1.9#	7.3±0.6
DC + Vit D	135±12.2*	298±11.1*	222±5.4*	24±2*	15±1.7*	9.53±1*
Glim + Vit D	111±8*	220±6.9*	156±11.7*	20±2.7*	20±2.4*	10±0.9*

#p < 0.05 compared with the control group. *p < 0.05 compared with the diabetic group.

After Vitmain D administration, lipid profile (TG, TC, LDL-c, and VLDL-c) levels reduced in comparison to the diabetic group., by 9.39%, 8.0%, 9%, and 14.2%, and Vitmain D + Glimepiride by 25.50%, 32%, 36%, 28.57%, respectively. Whereas, there is increase in HDLc and Calcium levels in group 3 and 4 by 15.38%, 30.54%, and 53.86%, 36.98%, respectively. Similarly the values of calcium in group 3 and 4 are comparable with group 1 ($p < 0.05$).

Table 4. Insulin, HbA1c, and Vitamin D levels in control and diabetic male rats treated with vitamin D, glimepiride and their combinations (Mean±SD)

Groups	Insulin (mU/L)	HbA1c (mmol/mol	Vit D (µg/L)

		%)	
Control	7.7+0.3	7+1	272.5±21
Diabetic Control (DC)	2.7 +0.5	14.26.5+2	236.7±22.7
DC + Vit D	3.1+0.4*	12.19.33+1.32*	415.2±35.6*#
Glim + Vit D	6.6+0.6#	8.6+1.2#	387.2±35.6*#

#p value <0.05 Compared to diabetic group. *p value <0.05 Compare to control group.

Normal insulin levels were observed in the normal control group (group 1). These levels were decreased significantly ($p<0.05$) in the diabetic control group compared to normal rats. Vitamin D treatment increased insulin levels marginally in diabetic rats but failed to achieve normal levels. HbA1c levels were significantly ($p<0.05$) increased in the diabetic control rats compared to the normal control. Treatment with Vitamin D did not reduce HbA1c levels significantly. However, Vitamin D in combination with Glimepiride reduced the HbA1c levels significantly ($p<0.05$) compared to diabetic untreated rats. Vitamin D levels were reduced slightly in the diabetic rats compared to normal rats. Whereas Vitamin D levels were significantly high in Vitamin D alone and in combination groups (groups 3 and 4).

Discussion

There is accumulating proof that from both animal and human studies through both direct and indirect methods, vitamin D may potentially lower the risk of acquiring both type 1 and type 2 diabetes mellitus [18]. According to several report [19,20] , low vitamin D levels have been associated with an elevated risk of type-I diabetes mellitus incidence and type-II diabetes mellitus development in general population. The evidence from experimental studies and clinical trials, however, is scant and ambiguous, making it insufficient to establish a link between vitamin D intake and glycemic control. In our study, vitamin D intake led to a slight drop in FPG. Additionally, there was a slight improvement in the insulin level. The significance of vitamin D in bringing the glucose level back to normal is explained by a number of different mechanisms, according to earlier research. Vitamin D insufficiency has been demonstrated to impair glucose metabolism by increasing insulin resistance, which is brought on by a drop in adipose PPAR- expression and a decline in beta-cell mass and function [21]. The restoration of calcium-mediated insulin secretion was facilitated by the reversal of the altered IP3 and AMPA expression level in the pancreatic islets, this demonstrated the vitamin's therapeutic impact on glutamatergic function in diabetes patients [22]. In our investigation, it was shown that vitamin D administration not only brought blood sugar levels close to normal but also raised insulin levels (p 0.05). Additionally, it has been noted that 1, 25$(OH)_2$ D_3 therapy restored the pathogenic alterations in the mTOR signaling pathway brought on by high hyperglycemia, successfully preventing apoptosis in beta-cells [23]. Additionally, vitamin D appears to improve the IRS-1-mediated intracellular mechanisms of insulin action and increase the expression of the total protein GLUT4 [24,25] . Numerous studies show that oral administration of dietary vitamin D to older mice enhances glucose metabolism by enhancing GLP-1 [26]. According to some reports, cholecalciferol supplementation

in skeletal muscle can reverse drug-induced reductions in Pik3r1 expression, a crucial gene acting downstream of the insulin receptor, and concurrent calcitriol can improve impaired insulin-stimulated glucose uptake into the myotube in a PI3K-dependent manner [27]. The significant dyslipidemia in untreated diabetic rats was indicated by an increase in plasma triacylglycerol, total cholesterol, very-low-density lipoprotein (VLDL) cholesterol, and low-density lipoprotein (LDL), and low-density lipoprotein cholesterol, and a decrease in high-density lipoprotein (HDL). Similar findings were found in a number of investigations using animal or experimental models of diabetes[28,29,30,31]. The hypertriglyceridemia associated with diabetes is thought to be caused by a number of metabolic processes, one of these is a rise in hormone-sensitive lipase activity, which catalyses the mobilisation of fatty acids from triacylglycerols stored in adipocytes [29, 32]. The volume of fatty acids that reach the liver increases as a result, and it subsequently reassembles those fatty acids into triacylglycerols that are then secreted in VLDL. The activity of the enzyme lipoprotein lipase, which catalyses the hydrolysis of triacylglycerols in VLDL and chylomicrons, has also been shown to be decreased by diabetes [32,33]. This encourages hypertriglyceridemia in diabetics. In the current investigation, vitamin D treatment in STZ-induced diabetic rats, there was a reduction in VLDL, triacylglycerols, and LDL and an increase in HDL. This may be related to better glycemic management through a process involving more effective insulin due to vitamin D [34,35].

Overall, this study demonstrated that supplementing with vitamin D might considerably lower blood glucose levels, increase insulin sensitivity, and alters lipid profiles in an experimental diabetic model. The combination of vitamin D and glimepiride had the greatest positive impact. To include vitamin D in the treatment of diabetes mellitus, further research including the general population, interventional studies, and clinical trials are required.

Conclusion

In the above study we found that Vitamin D is one of the important vitamins which not only has its direct action on calcium and bone metabolism, it is also involved in other metabolic functions including glucose homeostasis and fat metabolism, in the above study it was found to be effective in combination with glimepiride in controlling blood glucose levels and also in reducing all the components of lipid profile and increasing HDLc levels.

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EFFECT OF VITAMIN D SUPPLEMENTATION ON HEPATIC FUNCTION, LIPID PROFILE, AND DIABETIC PROFILE IN STREPTOZOTOCIN-INDUCED DIABETIC RATS

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Abstract

Non-alcoholic fatty liver disease (NAFLD) is considered one of the leading liver disorders in type 2 diabetes mellitus (T2DM). The progression of NAFLD is faster in T2DM patients than in nondiabetic patients, consequently leading to serious complications such as cirrhosis and hepatocarcinoma. vitamin D3 has been reported to protect the liver from non-alcoholic fatty liver disease (NAFLD) by attenuating liver damage in type 2 diabetes mellitus (T2DM). vitamin D3 also regulates inflammation by reducing the release of pro-inflammatory cytokines and affects insulin action and lipid metabolism. The present study evaluates the role of vitamin D3 in protecting the liver in NAFLD. In the present study, rats were injected intraperitoneally with 30 mg/kg of streptozotocin and fed a high-fat diet to induce diabetes. All rats were administered vehicle or vitamin D3 (300 ng/kg and 600 ng/kg) by oral gavage for 4 weeks. To assess the status of liver Alanine transaminase, Aspartate transaminase was estimated other parameters such as blood glucose, and vitamin D3 lipid profiles were done. Results showed vitamin D3 treatment improved insulin resistance, liver damage and plasma lipid profiles and in diabetic rats. Finally, the present study provides evidence that vitamin D3 could improve dyslipidemia and prevent NAFLD in T2DM.

Keywords: NAFLD, Type 2 Diabetes Mellitus, Vitamin D3, Insulin Resistance.

INTRODUCTION

Type 2 diabetes mellitus (T2DM) is one of the chronic metabolic diseases with increasing prevalence worldwide and serious health problems. An estimated 425 million people had DM worldwide in 2017, and the number is expected to rise to 629 million by 2045.1 In T2DM, high blood glucose triggers blood vessel damage and causes micro- and macro-vascular complications, contributing to increasing disability and mortality. Non-alcoholic fatty liver disease (NAFLD) is considered one of the leading liver disorders in T2DM.2 Hepatic lipid dysregulation due to insulin resistance increases fat accumulation in the liver.

The progression of NAFLD is faster in T2DM patients than in nondiabetic patients, 3consequently leading to serious complications such as cirrhosis and hepatocarcinoma. In insulin resistance, insulin signaling to suppress gluconeogenesis is impaired, while signaling to stimulate de novo lipogenesis (DNL) continues to be activated through sterol response element-binding protein 1c (SREBP1c).4

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Meanwhile, impaired insulin signaling suppresses peroxisome proliferator-activated receptor (PPAR)-a and carnitine palmitoyltransferase 1 (CPT1) to decrease b-oxidation. The imbalance between lipid synthesis and breakdown causes excess triglyceride (TG) accumulation in hepatocytes. In addition, hyperglycemia-induced reactive oxygen species (ROS) increase hepatic apoptosis by disturbing the balance between pro- and anti-apoptotic molecules.5

Vitamin D is a well-known hormone precursor that regulates calcium-phosphate homeostasis and bone mineralisation. Vitamin D also regulates inflammation by reducing the release of pro-inflammatory cytokines and affects insulin action and lipid metabolism.6 Recent studies have shown that vitamin D deficiency can cause insulin resistance through inflammation, as vitamin D deficiency is associated with increased inflammation.7 Furthermore, the data collected suggest that 1,25(OH)2D3, a biologically active form of vitamin D, may prevent liver injury by regulating lipogenesis or beta oxidation.8,9

It has been reported that the blood vitamin D levels were generally decreased in NAFLD patients.10, 11 An association between low blood levels of vitamin D3 and the risk of T2DM has also been reported. A previously published study showed that 1,25 (OH)2D3 reduced hepatic triglyceride accumulation and glucose output under insulin-resistant conditions in patients with NAFLD. Furthermore, the benefits of vitamin D3 supplementation for improving insulin resistance depend on the baseline 25 (OH) D3 status.12

Materials and methods

Rats weighing 200-250 gm were used in the study. All rats were housed in three cages in a room maintained at 220C and 50% humidity on a 12-hour light/dark cycle, with ad libitum access to food and distilled water. All rats were assigned to two groups, a control group and a diabetic (DM) group. While the control group was fed a normal chow diet, the DM group was fed a high-fat diet (HFD 40% kcal fat). After four weeks of diet treatment, 12-hour fasting rats were injected with streptozotocin (30 mg/kg body weight, Sigma Aldrich) in citrate buffer (pH 4.5) twice a week intraperitoneally (IP) to induce T2DM.13 Normal saline was administered IP to the normal control rats.13 12-hour fasting blood glucose (FBG) levels were measured once a week from the tail vein using an Accu-Chek Active glucometer (LifeScan Inc., Milpitas, USA) once a week. Rats with FBG above 200mg/dl were considered diabetic and included in further study. All protocols for the animal experiment were approved by the Institutional Animal Ethics Committee of Shri B. M. Patil Medical College, Vijayapur, Karnataka, India.

The diabetic rats were divided into four groups (each n=8) [see table 1] and treated with different levels of vitamin D3 supplement. The normal control rats (NC) fed with a normal chow diet were supplemented with a vehicle (olive oil). The diabetic control rats (DC) fed HFD were supplemented with the vehicle. Diabetic rats treated with vitamin D3 were fed HFD and supplemented with 300 ng/kg (low dose vitamin D, DLD300) and 600ng/kg (high dose vitamin D3 DHD) (Sigma Aldrich, USA) dissolved in olive oil, respectively. The vehicle and vitamin D3 were administered daily for 4 weeks by oral gavage.

Table 1. Grouping of Rats

Groups	**Drugs Intervention**
Normal Control (NC)	Olive oil
Diabetic Control (DC)	STZ + Olive oil
Vit D Low Dose (DLD)	HFD + 300 ng/kg DLD orally in olive oil
Vit D High Dose (DHD)	HFD + 600 ng/kg DHD orally in olive oil

Food intake, body weight and 12-hour FBG of the rats were measured every week. After 4 weeks of treatment, rats fasted for 12 hours and blood was collected from the retro-orbital plexus of the rats using heparinised capillary tubes. Rats were sacrificed with a high dose of diethyl ether. (The collected blood was centrifuged to obtain plasma and used for biochemical estimation).

Oral glucose tolerance test

One week before sacrifice, rats fasted for 12 hours and were treated with 2 g/kg glucose by oral gavage to perform OGTT. After glucose administration, the blood glucose level was determined after 0, 15, 30, 60, 90 and 120 minutes using the Accu-Chek Active blood glucose meter. Liver function test Plasma aspartate transaminase (AST) and alanine transaminase (ALT) were measured using commercially available kits.

Plasma and hepatic lipid profiles and plasma vitamin D concentration

For plasma lipid profiles, triglyceride (TG), total cholesterol (TC), and high-density lipoprotein-cholesterol (HDL-C) were measured by using commercial kits. Analysis of plasma vitamin D3 (EAGLE BIOSCIENCES, INC., NH, USA) was performed according to the manufacturer's protocols.

Statistical analysis

Comparison of significant differences between the groups was analysed by one-way analysis of variance (ANOVA) with post hoc Duncan's multiple range test using SPSS (version 22 for Windows, SPSS Inc., IL, USA). Statistical significance was determined at a P-value <0.05. The results were expressed as the mean ±standard error of the mean (SEM).

Results

Effect of vitamin D3 supplementation on food intake, body weight, and liver weight in T2DM rats Food intake was not

significantly different between all groups. Body weight gain was similar in the diabetic groups. However, a low-dose vitamin D3 supplementation significantly reduced liver weight (% BW) compared to the DC group. On the other hand, the HC group showed no significant difference in liver weight (Table 2).

Table 2. Effect of vitamin D3 supplementation on food intake, body weight, and liver weight in type 2 diabetic rats.

	Groups			
	NC	**DC**	**DLD**	**DHD**
Body weight (g)				
Before treatment	28.32 ± 0.69[a]	32.42 ±0.91[b]	33.31 ± 1.70[b]	34.76 ± 1.05[b]
After treatment	31.41 ± 0.86[a]	41.29 ± 1.23[b]	44.30 ± 2.90[b]	41.29 ± 1.60[b]
Weight gain	3.11 ± 0.33[a]	8.88 ± 0.94[b]	10.99 ± 2.01[b]	8.53 ± 1.14[b]
Liver weight (%BW)	3.23 ± 0.28[a]	5.77 ± 0.30[c]	4.52 ± 0.35[b]	4.93 ± 0.28[bc]

Note: Data were presented as mean±SEM (n8). Values with the different superscript letters were significantly different (P<0.05; ANOVA with post hoc Duncan's multiple range test).

There was no statistical difference in plasma vitamin D3 concentration between the control and the DC groups. However, high-dose vitamin D3 supplementation in diabetic rats significantly increased the plasma vitamin D3 levels compared to the DC group (Table 2).

There was no statistical difference in plasma vitamin D3 concentration between the control and the DC groups. However, high-dose vitamin D3 supplementation in diabetic rats significantly increased the plasma vitamin D3 level compared to the DC group (Table 3).

Table 3. Effect of vitamin D3 supplementation on plasma vitamin D3 concentration, blood glucose, hepatic function, and lipid profiles in type 2 diabetic rat.

	Groups			
	NC	**DC**	**DLD**	**DHD**
Plasma vitamin D_3 (ng/mL)	78.00 ± 5.61	83.32 ± 3.74	89.54 ± 10.28[ab]	106.96 ± 4.16[b]
FBG (mg/dL)				
Week 0	111.00 ± 8.35[a]	155.20 ±6.65[b]	169.40 ± 6.69[b]	175.80 ± 7.26[b]
Week 2	119.00 ± 4.95[a]	160.80 ± 5.38[b]	153.40 ± 13.67[b]	176.20 ± 9.23[b]
Week 4	138.40 ± 8.59[a]	177.70 ± 11.96[b]	183.70 ± 14.24[b]	163.00 ± 7.29[ab]
OGTT AUC (mg/dL x 120 min)	21233.40±1679.21[a]	35329.00 ± 1369.67[c]	31208.20 ± 3229.82[bc]	27625.40 ± 1828.19[ab]
Hepatic function (IU/L)				
Plasma AST	88.63 ±15.26[a]	151.04 ± 15.83[b]	90.43 ± 11.72[a]	86.57 ± 25.08[a]
Plasma ALT	43.53 ± 6.95	71.24 ± 3.705[b]	38.85 ± 3.91[a]	51.00 ± 9.51[a]
Plasma lipid (mg/dL)				
TG	51.10 ± 2.79[a]	119.77 ± 39.23[b]	56.13 ± 5.93[a]	53.71 ± 5.46[a]
TC	113.72 ± 4.41	156 ± 6.73 [b]	150.12 ± 7.67[b]	152.16 ± 8.73[b]
HDL-C	42.10 ± 2.83[a]	53.4 ± 7.72[ab]	59.53 ± 5.37[ab]	73.33 ± 8.90[b]

Note: Data were presented as mean±SEM. (n¼8). Values with the different superscript letters were significantly different (P<0.05; ANOVA with post hoc Duncan's multiple range test).

FBG: Fasting blood glucose; OGTT: Oral glucose tolerance test, AUC: area-under-the curve; AST: aspartate transaminase; ALT: alanine transaminase; TG: triglyceride; TC: total cholesterol; HDL-C: high density lipoprotein cholesterol

The FBG level in the DC group was significantly higher than in the NC group. However, the administration of vitamin D3 showed no significant difference compared to that of the DC group in FBG level during the experiment. OGTT was performed to determine glucose homeostasis. The OGTT AUC was higher in the DC group than in the NC group. At the same time, the high-dose vitamin D3 treatment showed a significant decrease in the AUC level compared to the DC group. AST and ALT plasma levels were significantly increased in the DC group compared to the NC group. However, vitamin D3 treatment in the diabetic rats significantly reduced AST and ALT levels, which were comparable to those in the NC group, regardless of the dose. Plasma TG and TC levels were higher in the DC group than in the NC group, and vitamin D3 administration significantly reduced plasma TG levels in diabetic rats. While plasma TC levels were not significantly altered after vitamin D3 administration, plasma HDL-C levels were significantly increased after treatment with high vitamin D3 among type 2 diabetic rats.

Discussion

The current study demonstrated the ameliorating effect of vitamin D3 supplementation on NAFLD. Although vitamin D3 is known to improve insulin signaling,15,16 previous studies reported that vitamin D3 administration showed no significant difference in FBG in diabetic animals.7,15,16 The current study also supported that treatment with Vitamin D3 had no direct effect at the FBG level. However, in a recent study, Benetti E. et al.17 confirmed that vitamin D3 administration improved glucose tolerance in non-diabetic obese rats. Our result showed that an increased vitamin D3 plasma concentration correlated with a reduced OGTT AUC. The result suggests that high-dose vitamin D3 supplementation would be effective in improving insulin sensitivity in T2DM.

Elevated levels of AST and ALT in the blood indicate liver damage, such as B. hepatocellular necrosis.18 Previous studies demonstrated the beneficial effect of vitamin D3 administration on reducing AST and ALT levels in diabetic animals.19,20 In addition, treatment with 2100 IU vitamin D3 for 48 weeks was well tolerated and reduced serum ALT levels in patients with nonalcoholic steatohepatitis.21 Similar results in this study suggest that vitamin D3 treatment may improve hyperglycemia-induced liver dysfunction by attenuating liver damage.

Insulin resistance stimulates the over secretion of VLDL from the liver into the blood and causes hypertriglyceridemia.21 In hypertriglyceridemia, reduced plasma HDL-C levels but increased small dense LDL particles lead to dyslipidemia, which accelerates the deposition of hepatic lipids. Previous studies reported that vitamin D3 normalised serum TG, TC and HDL-C levels in HFD-induced NAFLD rats22 and decreased hepatic TG levels in diabetic rats7. Control studies had significantly lower levels of 25(OH)D3 in patients with NAFLD than in controls.8,25 Researchers believe that lower levels of vitamin D3 in patients with NAFLD may contribute to NAFLD progression. The exact mechanism of vitamin D3 deficiency and NAFLD is not fully established. A reduction in triglycerides and an increase in HDL-cholesterol were observed in the 25 mg calcitriol group compared to the placebo during the 4 weeks of the intervention.23,24 In our research, vitamin D3 treatment significantly reduced plasma TG levels regardless of dose. In addition, vitamin D3 supplementation decreased hepatic TG levels and increased plasma HDL-C levels in a plasma vitamin D3 concentration-dependent manner. Our results indicate that vitamin D3 could improve dyslipidemia and prevent NAFLD in T2DM.

Conclusion

AST and ALT levels in the vitamin D3-treated group of rats suggest that it might improve hyperglycemia-induced liver dysfunction by attenuating liver damage. The present study indicates that treatment with vitamin D3 significantly reduces plasma TG levels, regardless of dose. In addition, vitamin D3 supplementation decreased hepatic TG levels and increased plasma HDL-C levels in a plasma vitamin D3 concentration-dependent manner. Our results demonstrate that vitamin D3 could improve dyslipidemia and prevent NAFLD in T2DM.

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