Effect of Ticagrelor on High-fat diet and Streptozotocin Induced Diabetes in Male Wistar Rats- An Experimental Study

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LIST OF ABBREVIATIONS

AA	-	Amino acids
ADA	-	American diabetic association
AGEs	-	Advanced glycation endproducts
ANOVA	-	One way analysis of Variance
BMI	-	Body mass index
CPCSEA	-	Committee for the Purpose of Control and Supervision of Experiments on Animals
CVD		Cardiovascular disease
DAG		Diacylglycerol
DC		Diabetic control group
DM		Diabetes Mellitus
DN		Diacerein group
DPP		Diabetes Prevention Program
ER		Endoplasmic Reticulum
FBG		Fasting Blood Glucose
FFA		Free Fatty Acids
FOXO1		Transcription factor forkhead box protein O1
GAD		Glutamic acid decarboxylase
GIP		Glucose-dependent insulinotropic peptide
GK		Goto-Kakizaki
GLP-1		Glucagon-like peptide-1
GWAS		Genome-wide Association Study
HDL		High Density Lipoprotein
HDL		High-density lipoproteins
HFD		High-fat diet
HOMA-IR		Homeostasis model assessment-insulin resistance
IAEC		Institutional Animal Ethics Committee
IAPP		Islet amyloid polypeptide
IAPP		Islet amyloid polypeptide
ICAs		Islet cell autoantibodies
IFN-γ		Interferon gamma
IGF-1		Insulin-like growth factor-1
IGT		Impaired glucose tolerance
IL-1β		Interleukin 1 beta
IL-6		Interleukin 6
IRPs		Insulin resistance-associated proteins

IRS	Insulin receptor substrate
JNK	c-JUN N-terminal kinase
LADA	Latent autoimmune Diabetes in adults
LDL	Low-density lipoproteins
MAPK	Mitogen-activated protein kinase
MF	Metformin group
MHO	Metabolically healthy Obesity
MODY	Maturity-onset Diabetes of the Young
NAFLD	Nonalcoholic fatty liver disease
NASH	Nonalcoholic steatohepatitis
NC	Normal control group
NF-κB	Nuclear factor kappa-B
NLRP3	NLR family pyrin domain-containing 3
PAMPs	Pathogen-associated molecular patterns
PI3K	Phosphatidylinositol-3-kinase
PIP-3	Phosphatidylinositol 3,4,5-trisphosphate
PKB	Protein kinase B
PP	Pancreatic Polypeptide
RAGE	Receptor for Advanced glycation endproducts
RCT	Randomized Controlled Trial
ROS	Reactive oxygen species ROS
SEM	Standard error of mean
SFFA	Saturated free fatty acids
Shc	Src homology and collagen protein
SJS	Stevens-Johnson syndrome
STZ	Streptozotocin
SUR	Sulfonylurea receptor
SYSADOA	Symptomatic slow-acting drug in osteoarthritis
T2DM	Type 2 Diabetes Mellitus
TEN	Toxic epidermal Necrolysis
TLR	Toll-like receptor
TNF-α	Tumor Necrosis Factor alpha
TXNIP	Thioredoxin-interacting protein
WHO	World Health Organization
ZFR	Zucker fatty rats

ABSTRACT

Introduction

The current experimental study aimed to evaluate the effect of Ticagrelor on glycaemic parameters in a High-fat diet and Streptozotocin-induced DM in male Wistar rats. In addition, the effect on the inflammatory markers were also assessed.

Methods

Animals were randomly divided into various groups. One group was Normal control while others consisted of diabetic rats without treatment or one of the treatments. Diabetes was induced by feeding the rats with High-fat diet for 2 weeks followed by a single intraperitoneal injection of Streptozotocin. (30 mg/Kg). Following induction of Diabetes treatment with either Metformin, Ticagrelor (16.2 mg/kg) or Ticagrelor (35mg/kg) was continued for 6 weeks. Body weight, Fasting Blood Glucose and HbA1C were measured at various time intervals. Inflammatory markers were studied at the end of the study.

Results

All three treatments significantly reduced Fasting Blood glucose and HbA1C compared to the untreated rats. In addition, when compared to the Ticagrelor (16.2 mg/kg) group, the Ticagrelor (35 mg/kg) group significantly decreased these values. The inflammatory markers (IL-1β, IL-6, TNF-α) were significantly reduced in all treatment groups compared to untreated rats.

Conclusion

The present study showed that the treatment of diabetic rats with oral Ticagrelor improved the High-fat diet and Streptozotocin-induced biochemical alterations. Furthermore, when compared to Ticagrelor (16.2 mg/kg), Ticagrelor (35 mg/kg) was found to be more efficacious across all trial variables. It can be concluded that Ticagrelor may be a promising option for the management of Type 2 Diabetes with ACS (Acute Coronary Syndrome) like comorbid condition. Future research of Ticagrelor in combination with standard anti-diabetic drugs like Metformin in patients of diabetes with co-morbid condition like ACS, as well as clinical trials with larger sample sizes, are recommended

Keywords

Type 2 Diabetes Mellitus, Ticagrelor, Inflammation, Acute Coronary syndrome, Streptozotocin, High-fat diet, Wistar rats

TABLE OF CONTENTS

SL. NO.	*TOPIC*	*PAGE No*
1.	INTRODUCTION	1-3
2.	OBJECTIVES	4
3.	REVIEW OF LITERATURE	5-48
4.	MATERIALS AND METHODS	49-57
5.	RESULTS	58-68
6.	DISCUSSION	69-73
7.	LIMITATION AND FUTURE RECOMMENDATIONS	74
7.	CONCLUSION	75
8.	SUMMARY	76
9.	BIBLIOGRAPHY	77-88
10.	ANNEXURES	89-90

LIST OF FIGURES

Number	Description	Page No
1	Central role of glucose in carbohydrate, fat and protein metabolism	7
2	Structure of human proinsulin (C-peptide plus A and B chains) and insulin	8
3	Mechanisms of glucose-stimulated insulin secretion	9
4	The three stages of type 1 Diabetes.	13
5	Consequences of insulin resistance	16
6	Mechanisms of insulin resistance	18
7	The 'ominous octet' of hyperglycemia in Type 2 Diabetes Mellitus	19
8	Storage of excessive nutrients in adipose tissues leads to an inflammatory response and insulin resistance	22
9	Islet inflammation in Type 2 diabetes	24
10	The NLRP3 inflammasome	26
11	Adipose tissue inflammatory profile imbalance between metabolically healthy and unhealthy obese.	27
12	Chemical structure of ATP, Ticagrelor and Cangrelor	37
13	Key advantages and disadvantages of different classes of animal models used in obesity and diabetes research	40
14	Chemical structure of Streptozotocin	47
15	Mechanism of action of STZ induced Diabetes Mellitus	48
16	Schematic representation of the study design	54-55
17	Sample images of test kits, instruments and selected procedures	56-57

LIST OF TABLES

NUMBER	DESCRIPTION	PAGE No
1.	Classification of Diabetes Mellitus	5-6
2.	Diagnostic criteria for Type 2 Diabetes Mellitus	29
3.	Summary of drugs available for Type 2 Diabetes Mellitus	30-31
4.	Anti-inflammatory effects of glucose-lowering agents used in the treatment of Type 2 diabetes.	33
5.	Summary of clinical trials demonstrating metabolic effects of anti-inflammatory drugs	35
6	Non-Hemorrhagic adverse events of Ticagrelor	38
7	The experimental animals used to study Diabetes	41
8	Characteristics of T2DM animal models	42-44
9	Advantages and disadvantages of various experimental models for diabetes	45
10	Summary of Mechanism of action of STZ action	48
11.	Composition of High Fat Diet [HFD]	50
12.	Number of rats per group with treatment schedule	52
13.	Details of study parameters	53
14	Effect of various treatments on body weight at various time intervals	59
15	Effect of various treatments on Fasting Blood Glucose at various time intervals	61
16.	Effect of various treatments on HbA1C at various time intervals	64
17	Effect of various treatments on serum inflammatory markers	66

LIST OF GRAPHS

NUMBER	DESCRIPTION	PAGE No
1	Effect of various treatments on body weight at various time intervals	59
2	Effect of various treatments on Fasting Blood Glucose at various time intervals	62
3	Effect of various treatments on HbA1C at various time intervals	64
4	IL-1β - End of study (One-way ANOVA followed by Bonferroni's multiple comparison test)	66
5	TNF-α - End of study (One-way ANOVA followed by Bonferroni's multiple comparison test)	67
6	IL-6 - End of study (One-way ANOVA followed by Bonferroni's multiple comparison test)	68

INTRODUCTION

Diabetes mellitus (DM) is a group of metabolic disorder characterized by hyperglycemia due to defect in insulin secretion, insulin action or both. It causes secondary pathophysiologic changes in multiple organ systems that impose a tremendous burden on the health care system.[1] Worldwide prevalence of DM has risen dramatically, from estimated 30 million cases in 1985 to 415 million in 2017. Based on current trends of IDF (International Diabetes Federation) 642 million individuals will have DM by 2040.[1] T2DM is known to increase CVS death by 2 fold in men and 4 fold in women.[1] Although the prevalence of both Type 1 and Type 2 DM is increasing, that of Type 2 DM is rising much more rapidly because of increasing obesity and reduced activity levels. The ICMRINDIAB study reported a 7.3 percent prevalence of Diabetes and a 10.3 percent prevalence of preDiabetes, with 77.2 million persons with preDiabetes and 69.2 million with Diabetes.[2] Insulin secretion and responsiveness are impaired by a variety of mechanisms that play a major role in pathophysiology of T2DM that include glucotoxicity, lipotoxicity, oxidative stress, endoplasmic reticulum stress, alterations of gut microbiota, endocannabinoids, formation of amyloid deposits in the islets etc. Interestingly, all of these mechanisms are associated with inflammatory responses. [2]

The pathogenesis of diabetes is linked with hyperglycemia induced inflammation. Various mechanisms have been proposed to explain the role of inflammation in the initiation and progression of DM. Hyperglycemia associated with diabetes is known to increase the metabolic activity of islet cells of pancreas leading to increased formation of ROS (Reactive Oxygen Species) that promote the activation of NLRP3 (Nucleotide binding Oligomerization Domain, Leucine rich repeat and Pyrin domain-3) inflammasome and CASPASE-I. Recent evidence implicates the inflammasome NLRP3 in the pathogenesis of metabolic syndrome and T2DM. NLRP3 and CASPASE-I induced by ROS promotes the secretion of IL-1β that in turn is known to induce secretion of cytokines and chemokines namely IL-6, IL-8, TNF-α, etc. These cytokines promote the accumulation of immune cells in the islet cells that result in insulin resistance.[3] Furthermore in the pancreas, activation of NLRP3 inflammasome by high levels of glucose and fatty acids and subsequent release of IL 1β leads to β cell dysfunction and apoptosis, insulin deficiency and progression of T2 DM.[4] In addition, inappropriate activation of the NLRP3

inflammasome has also been implicated in the other co-morbid conditions like atherosclerosis, acute myocardial infarction etc. DM itself is known to not only increase the risk of CVD (cardio vascular diseases) but also mortality associated with it.[5] NLR appears to be a potential target for investigation in the management of diabetes and its associated co-morbid cardiovascular conditions.

Interestingly, anti-platelet drug Ticagrelor has been found to indirectly inhibit NLRP3 inflammasome. Ticagrelor, a $P2Y_{12}$ receptor antagonist is used in patients with CAD (coronary artery disease) in order to prevent vascular events and deaths in patients with Acute Coronary Syndromes (ACS).[1,5] Ticagrelor has been reported to possess anti-inflammatory action by virtue of inhibition of inflammatory markers like IL-1β and TNF-α which are also involved in pathogenesis of T2DM. [3,5]

Several preclinical and clinical studies have demonstrated that Ticagrelor mitigate inflammation in various models that are known to induce NLRP3. In Nigericin stimulated LPS (lipopolysaccharide) primed mouse bone marrow derived macrophages, Ticagrelor significantly decreased the maturation of CASPASE-I and reduced IL-1β in a dose dependent manner, with decrease in LPS induced expression of pro-IL-1β and TNF-α. These actions were found to be independent of $P2Y_{12}$ signaling.[5] In Alum induced peritonitis mouse model, Ticagrelor treatment was reported to remarkably decrease IL-1β levels and number of peritoneal exudate cells and neutrophils in the peritoneal lavage fluid. Similarly, in LPS induced sepsis model, Ticagrelor markedly reduced serum levels of IL-1β and IL-18. Ticagrelor treatment increased the survival of both wild type of mice and $P2Y_{12}^{-}$ mice, suggesting that its effect on NLRP3 was independent of $P2Y_{12}$ action.[5] In diabetic nephropathy animal model of unilateral nephrectomy followed by STZ injections for 5 days, Ticagrelor treatment prevented diabetes induced mesangial matrix expansion, podocyte effacement and glomerular endothelial cell injury. This study also demonstrated significant reduction in plasma as well as kidney m-RNA expressions and levels of TNF-α and CASPASE 3.[6]

Similarly, in humans, Ticagrelor blocked CASPASE-I maturation and production of IL-1β and TNF-α in LPS primed PBMCS (Peripheral blood mononuclear cells) obtained from ACS patients, thus indicating that it rapidly mitigates NLRP3 associated severity of inflammation in ACS patients. Ticagrelor therefore demonstrates therapeutic potential in

NLRP3 associated diseases like DM and CVD.[5]

However, there is scarcity of information regarding the effect of Ticagrelor on the glycemic parameters in DM per se. Therefore, the present study was planned to investigate the effect of commonly used anti-platelet drug Ticagrelor in an animal model of High-fat diet and Streptozotocin-induced rodent model of Diabetes.

OBJECTIVES

Primary objective:

- To evaluate the effect of Ticagrelor on glycemic parameters in high-fat diet and Streptozotocin-induced DM in male Wistar rats

Secondary objectives:

- To evaluate the effect of Ticagrelor on inflammatory markers viz. (IL-1β, TNF-α, IL-6) in high-fat diet and Streptozotocin-induced DM in male Wistar rats

REVIEW OF LITERATURE

A. Diabetes Mellitus

Introduction:

The American Diabetes Association (ADA) has defined Diabetes as "a group of metabolic disorders characterized by hyperglycemia resulting from defects in insulin secretion, insulin action or both". This definition has been accepted by different international bodies like World Health Organization (WHO).[8] Diabetic Mellitus is a chronic metabolic disorder that affects various organ systems and can cause pathophysiologic changes because of metabolic and genetic dysregulation in the body. A complicated interplay of genetics and environmental variables has been identified as the cause of various distinct types of Diabetes mellitus.[3,8] In the United States, DM is the leading cause of end-stage renal disease (ESRD), non-traumatic lower extremity amputations, and adult blindness. It also predisposes to cardiovascular diseases. With an increasing incidence worldwide, DM is likely to continue to be a leading cause of morbidity and mortality in the future. CVD have substantially increased in individuals with Type 1 and Type 2 DM. The Framingham Heart study revealed that the risk of CAD (coronary artery disease), MI (myocardial infarction), PAD (peripheral artery disease) and CHF (congestive heart failure) is increased from one to five times in DM.[1] India is reported to have the second highest number of diabetic individuals in the world.[2] Results of the Indian Council of Medical Research -India Diabetes (ICMR-INDIAB) study have found the prevalence of diabetes and prediabetes to be as high as 7.3% and 10.3%, respectively with nation-wide projection of 77.2 million people with prediabetes and 69.2 million with diabetes.[2]

Table 1: Classification of Diabetes Mellitus:

Diabetes is classified as follows:[7,8,9]

Type 1 diabetes	• Also known as autoimmune diabetes or Insulin dependent diabetes mellitus (IDDM) • 5 % to 10% of all diabetic cases • Breakdown of beta cells that results in total insulin insufficiency • Type 1 diabetes also has been linked to distinct HLA types, with DR and DQ types being the most common
Type 2 diabetes	• 90 % to 95% of diabetic patients suffer from this condition • Pathophysiology spans from insulin resistance and relatively normal insulin secretion

Specific types of diabetes	I. Exocrine pancreas disorders (eg. Pancreatitis, cystic fibrosis) II. Drug/chemical induced diabetes (eg. Glucocorticoid use, during treatment of HIV, or after organ transplantation III. Monogenic diabetes syndromes (eg. Neonatal diabetes, MODY- maturity onset diabetes of young)
Gestational diabetes mellitus	• Diabetes diagnosed in the second or third trimester of pregnancy that was not clearly overt diabetes prior to gestation

Glucose metabolism and Regulation of glucose homeostasis

Carbohydrates are the chief source of energy in our body. Glucose is a ubiquitous carbohydrate and is utilized for energy by most of our body tissues. The principal sources of carbohydrates in the diet are starch, glycogen, sucrose, and fructose from foods of plant origin, foods of animal origin, cane sugar or beet sugar and from fruits respectively. The majority of these foods contain complex carbohydrates which are to be broken down into simpler forms to facilitate absorption. This process of digestion happens mainly in the small intestine and partially in the stomach.[10]

The body maintains a balance between energy intake from consumed food, gluconeogenesis i.e., glucose produced by the liver, and glucose uptake and utilization by peripheral tissues; a process known as glucose homeostasis. Such a metabolic equilibrium is regulated by multiple factors like neural input, metabolic signals, hormones like glucagon; but the most important regulator being insulin. The metabolic pathways of glucose are decided based upon whether the individual is in a fed state or fasted state. During the fasting stage, the processes are directed to break down the energy depots to guarantee a consistent glucose supply mainly to the brain and some other tissues. This is achieved by lowering the levels of insulin and modestly increasing glucagon and thereby promoting hepatic gluconeogenesis. Low insulin level also leads to a reduction of glucose uptake in tissues like skeletal muscle and adipose tissue which are sensitive to insulin, resulting in the promotion of mobilization of stored proteins and fats. In the fed state the blood glucose levels rise and as a result insulin increases and there is a reduction in glucagon, reversing the process. Insulin stimulates the glucose uptake by skeletal muscle and a majority of postprandial glucose is consumed by skeletal muscles. Insulin is anabolic in nature and aids the storage of carbohydrates, fat and also protein biosynthesis. Insulin either promotes glycogen formation or promotes catabolism by glycolysis, depending

upon the need of the body. Substances secreted by skeletal muscle cells, adipocytes like leptin, adiponectin, resistin, etc. also have a role to play in glucose homeostasis.[3,10]

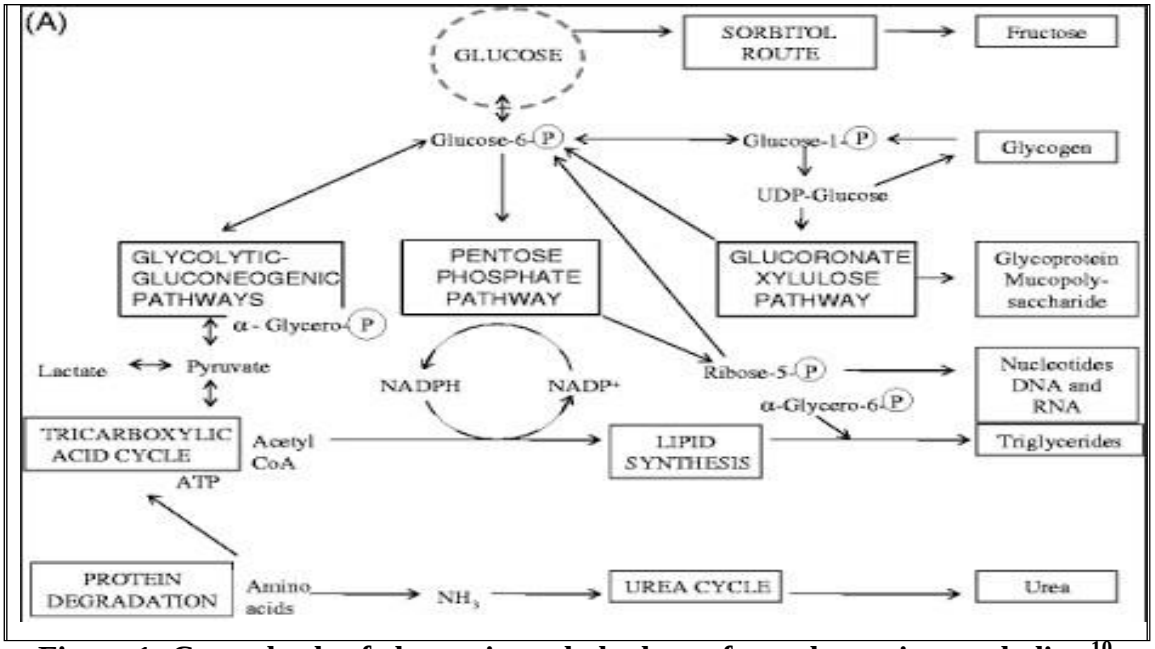


Figure 1: Central role of glucose in carbohydrate, fat and protein metabolism[10]

Insulin

The islets of the pancreas constitute around 1% - 2% of the volume of the pancreas. The pancreatic islet has a rich vascular and nerve supply and is composed of five distinct types of cells. viz. α, β, δ, PP (Pancreatic polypeptide), and ε cells which secrete glucagon, insulin, somatostatin, pancreatic polypeptide, and ghrelin respectively.[11] Initial insulin production begins with the production of preproinsulin, an amino acid precursor polypeptide of 86 amino acids that are produced in a single chain. This is converted to proinsulin as a result of a proteolytic reaction. An internal 31-residue is cleaved off proinsulin and as a result, A and B chains of insulin along with C-peptide are generated. The Golgi complex, the ER (Endoplasmic Reticulum), and β-cells of the pancreas are all involved in this complicated process. β-cell secretory granules secrete insulin and C-peptide simultaneously. C-peptide has no known physiological function or receptor but is useful in the evaluation of β-cell function and the evaluation of insulin-induced hypoglycemia. Islet amyloid polypeptide (IAPP) or amylin, a peptide with 37 AA (Amino Acids), is also secreted by β-cells. Patients with Type 2 Diabetes have amyloid fibrils in their pancreas, which are primarily composed of amylin.[3]

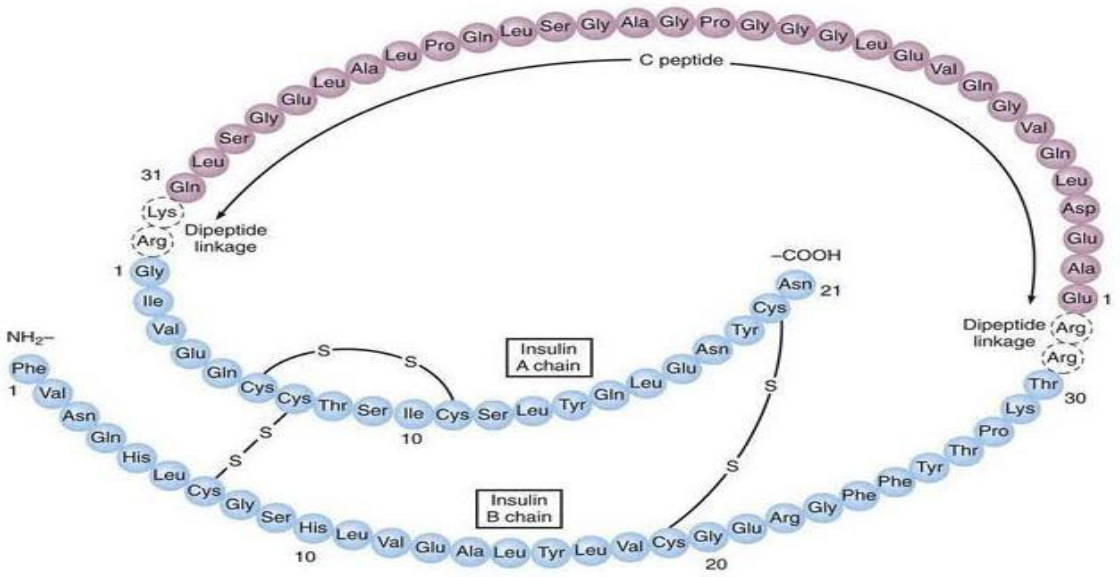


Figure 2: Structure of human proinsulin (C-peptide plus A and B chains) and insulin. Insulin is shown as the shaded (blue color) [12]

Insulin secretion is a tightly regulated mechanism that aims to keep blood glucose levels steady in both fasting and fed states. This is accomplished through the coordinated action of various nutrients and hormones. Fatty acids, amino acids, ketone bodies, etc. facilitate insulin synthesis, but glucose is the primary insulin secretagogue. Insulin secretion is inextricably linked to the extracellular glucose level. The same amount of orally administered glucose induces more insulin as compared to that administered intravenously, which is known as the incretin effect which is attributed to insulinotropic GI (Gastrointestinal) peptides. Pancreatic islets have autonomic innervations; alpha-2 adrenergic receptor stimulation suppresses insulin secretion, while it is enhanced by beta-2 stimulation and vagal nerve stimulation. In general, conditions activating the sympathetic system like hypoglycemia, exercise, hypoxia, hypothermia, surgery, severe burns, etc. inhibit insulin secretion via α2 adrenergic action.[11]

When blood glucose level is >3.9 mmol/L (70 mg/dL) insulin synthesis is stimulated and when the value is in the range of 5–10 mM glucose, insulin release is very prominent. The first step which heralds a series of molecular events stimulating insulin secretion is the entry of glucose into the pancreatic beta-cell via GLUT-1. Immediately this glucose is phosphorylated by the enzyme Glucokinase (hexokinase IV) in the beta cell and is the rate-limiting step of this process. The glucose-6-phosphate thus produced enters the glycolytic pathway and enhances ATP production. This rise in ATP leads to inhibition of an ATP-

sensitive K+ channel (KATP channel), which leads to depolarization of the cell membrane. This KATP channel is heteromeric and consists of two important structures. One is an inward rectifying K+ channel (Kir6.2) and the other is a protein called SUR (sulfonylurea receptor).

Mutations in the KATP channel may lead to neonatal Diabetes and hyperinsulinemic hypoglycemia, depending on the type of neonatal Diabetes. Depolarization of the membrane results in the opening of a voltage-dependent Ca2+ channel and thus calcium influx. The high intracellular calcium leads to the exocytosis of insulin from storage vesicles. The level of transcription factors, amino acid metabolism, and alterations in cAMP synthesis modulate these processes. Insulin secretion is pulsatile, with small surges of secretion occurring approximately every 10 minutes, superimposed on larger amplitude fluctuations lasting approximately 80-150 minutes. In addition to glucose, multiple metabolic pathways in β-cells and external hormones increase glucose-facilitated insulin secretion. Glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic peptide (GIP) are hormones and are called incretins; these bind to beta-cell receptors and enhance cAMP production in the fed state and ultimately promote insulin secretion. In addition, they also suppress glucagon production and secretion.[13,14]

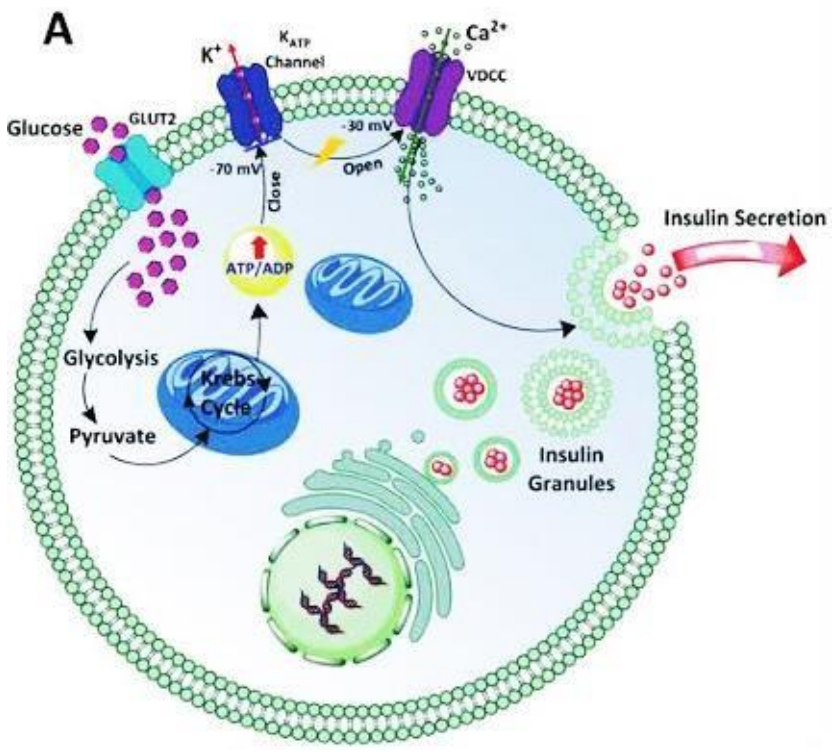


Figure 3: Mechanisms of glucose-stimulated insulin secretion[1]

Insulin acts via a tyrosine kinase receptor that is functionally similar to the IGF-1 receptor.[15] This insulin receptor is a complex dimer having alpha and beta subunits. These subunits are connected via disulfide bonds and give rise to a transmembrane heterotetrameric glycoprotein consisting of two extracellular alpha and two membrane-spanning beta subunits. The β subunits possess an inherent tyrosine kinase activity which is inhibited by the α subunits. The inhibition is overcome as insulin binds to α-subunit, leading to transphosphorylation of β subunits with one another accompanied by autophosphorylation at selected sites. Insulin receptor activation triggers a signaling cascade by phosphorylating several intracellular proteins, like IRSs (Insulin receptor substrate) and Shc (Src homology and collagen protein). Additionally, these proteins interact with effectors, which amplify and prolong the signaling process. Activation of phosphatidylinositol-3-kinase (PI3K) is necessary for insulin action on glucose transport. PI3K gets activated on interaction with IRS proteins, and produces phosphatidylinositol 3,4,5-trisphosphate (PIP3).PIP3 has a role in regulating the localization and activity of various downstream kinases like PKB (Protein kinase B), atypical isoforms of PKC (ς and λ/τ), and mTOR. The isoform Akt2 serves important functions like glucose synthesis in the liver and glucose uptake by skeletal muscle and adipose tissue. Production and translocation of GLUT-4 to the cell membrane of adipose tissue and skeletal muscle is one of the crucial actions of insulin.[11]

Pathogenesis of Type 1 Diabetes Mellitus

Type 1 Diabetes is an autoimmune disorder in which immune effector cells attack pancreatic islets in response to endogenous β-cell antigens. The interplay between genetic, environmental, and immunologic variables that ultimately contribute to the death of β-cells of the pancreas leads to type 1 DM. A diagnosis of this illness can be made at any age; however, the majority of cases are discovered before the age of 20. As the disease is not exclusive to childhood, the old moniker “juvenile-onset Diabetes” is obsolete now. And as many forms of Diabetes eventually require insulin therapy the term “insulin dependent Diabetes mellitus” has been omitted from the present classification. Islet-directed autoimmunity is found in the vast majority of people with type 1 Diabetes. Others without such evidence might develop insulin deficiency by nonimmune mechanisms which are not known yet.[3,16]

The destruction of the islet cells is thought to be caused by three interconnected mechanisms:

- Auto-immunity
- Environmental factors
- Genetic Susceptibility

In susceptible individuals an infectious or environmental stimulus can trigger the autoimmune process, resulting in the production of autoantibodies against pancreatic beta-cell antigens. The rate of deterioration in beta-cell function is variable with some individuals showing rapid progression while others taking several years to evolve into clinical Diabetes. Studies suggest that the features of Diabetes become evident after a certain degree of loss of beta-cell mass which is as high as 70-80% in some patients. Typically, events leading to the emergence of frank Diabetes from covert glucose intolerance are linked to situations that raise insulin demand, such as puberty or infections. Following the initial clinical features of T1DM sometimes patients enter a period where modest insulin doses or even no insulin glycemic control is achieved and this phase is known as the "honeymoon" phase. Eventually, the remaining beta cells stop producing insulin, and the person gets insulin insufficient.[1]

Genetic Susceptibility:

Multiple genes contribute to T1DM susceptibility. Epidemiologic studies have convincingly established a genetic basis for T1D. Because identical twins have a 40 to 60 percent chance of developing type 1 Diabetes, other modifying variables are likely involved in the development of the disease. Recently, GWAS (genome-wide association study) has identified multiple genetic susceptibility loci for T1D.

Some of the candidate genes showing association with type 1 DM are,[17]

1. HLA genes
2. Superoxide dismutase G
3. Insulin gene VNTR
4. CTLA 4
5. Kidd locus

The HLA gene cluster is the most important of these, contributing as much as 50% of the genetic vulnerability to T1D. HLA-A26 B8 DR3, HLA-A24 B8 DR3 (AH8.3), A3 B8

DR3 (AH8.4), and A31 B8 DR 3 (AH 8.5) are the common susceptibility alleles in Indian populations.[18]

Auto-immunity:

Islet cell autoantibodies (ICAs) are a collection of antibodies that target GAD (Glutamic acid decarboxylase), insulin, ZnT-8, and IA-2/ICA-512 among other pancreatic islet components. These are considered autoimmune markers of type 1 DM. ICAs are found in most of the patients (>85%) with new-onset type 1 DM. Around 3–4% of first-degree relatives of such patients also possess these autoantibodies. The presence of ICAs can often predict the development of T1DM, also the risk increases as the number of autoantibodies increases.[1]

Environmental factors:

Only approximately half of monozygotic twins get type 1 Diabetes, implying that environmental variables play a role. However, the precise nature of these variables remains unknown. Prior viral infections have been postulated as triggers, but neither the type of virus nor the mechanism of development of autoimmunity is established. Data suggest that some viruses may share epitopes with pancreatic islet antigens, leading to a cross-reactivity that causes pancreatic islets to be destroyed. This process is known as molecular mimicry. Conversely, some infections may protect against T1DM.

β-Cell Destruction:

Usually beginning of the autoimmune process and the appearance of symptomatic disease has a long lag period. Three distinct stages of type 1 DM have been recognized. In the first stage, individuals develop two or more islet antibodies but the blood glucose level is normal. In stage two, there is a progressive decline of beta-cell density but with no frank symptoms. Finally in stage three classic symptoms of Diabetes appear, and by this time it is estimated that > 90% of cells are destroyed.[19]

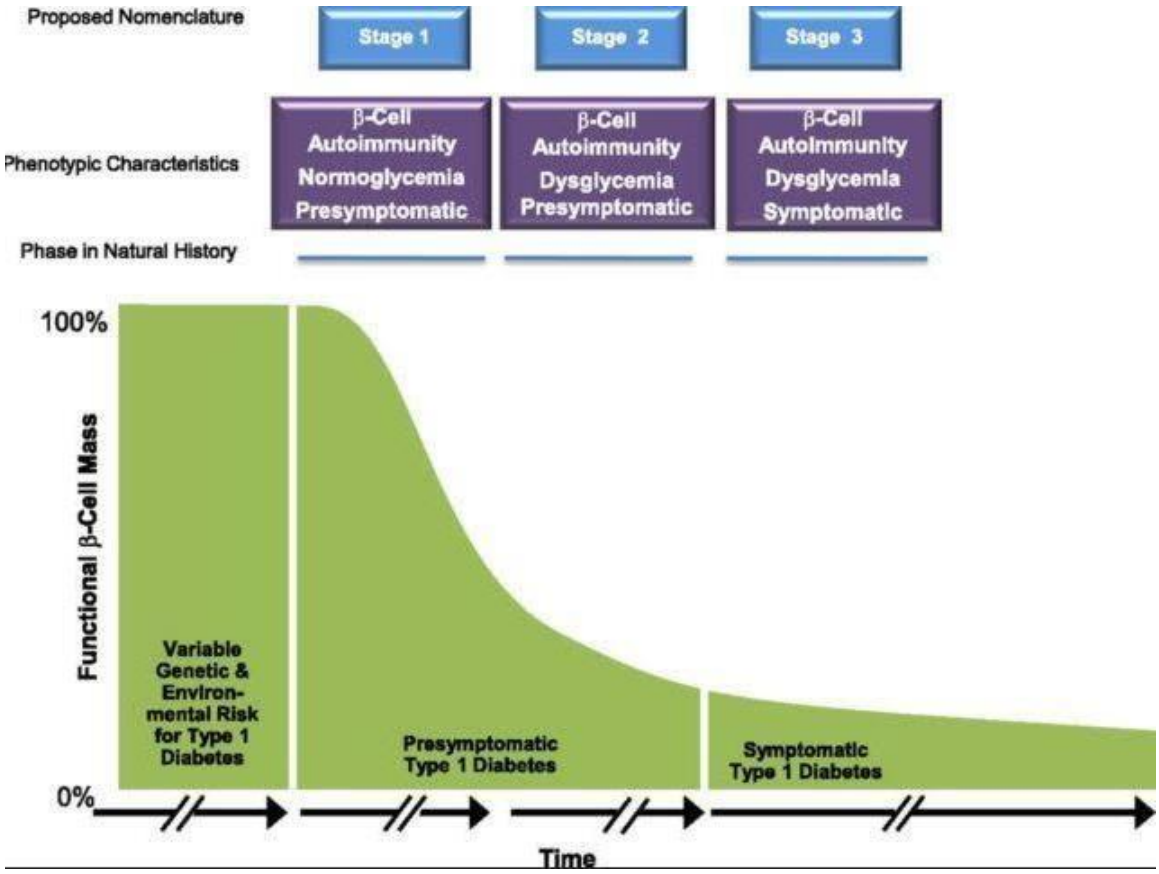


Figure 4: The three stages of type 1 Diabetes[16]

The inability of the islet-specific T cells to self-tolerate is the basic immunological anomaly in type 1 DM. This could be due to a combination of improper clonal deletion of self-reactive T cells and regulatory T cell function abnormalities. As a result, these autoreactive T cells can survive and react to self-antigens. These cells are first activated in the peripancreatic lymph nodes, most likely in response to antigens produced by injured islets. These T lymphocytes migrate to the pancreas after activation and produce beta-cell damage. Th1 cells and CD8+ cells are implicated in this damage and may target insulin, glutamic acid decarboxylase (GAD), and other targets. However, it is unknown whether autoantibodies induce or are a result of islet damage.[16]

Pathogenesis of Type 2 Diabetes Mellitus

T2DM is a complicated disorder characterized by the interaction of hereditary and environmental components as well as a pro-inflammatory condition. The characteristic pathophysiological changes hampering blood glucose control are β-cell dysfunction, insulin resistance, and chronic inflammation. Although the underlying defect in the pathophysiology is debatable, most research supports the notion that insulin resistance occurs before an insulin secretory deficiency, and clinical Diabetes occurs when insulin

secretion is insufficient. Type 2 DM includes a wide spectrum of conditions that share hyperglycemia as a characteristic feature. Our present knowledge of pathogenesis and heredity is based on European data. However, it is becoming clear that Diabetes has distinct pathophysiology in other ethnic groups that is not well understood yet. For instance, patients from East and South Asia have predominant beta-cell dysfunction, Latinos have relatively predominant insulin resistance. East and South Asians develop Diabetes earlier and have lower BMI.[3,16]

Genetic considerations

Type 2 Diabetes runs in families and is inherited. The relative risk of Type 2 Diabetes in a sibling of someone with T2DM is 2-3 if neither of the siblings has the condition, according to research. However, if two siblings have T2DM, the risk rises to 30 percent.[20] When the disease is present in the mother, the risk of developing it is higher than when the condition is present in the father.[21] If a person has a BMI of more than 30 or a fasting glucose of more than >5.5 mmol/ L the chance of developing T2DM rises up markedly.[22] Identification of the genes responsible for the inheritance of Type 2 DM has been challenging. However, genome-wide association studies (GWAS) have recognized over 70 genes, each with a relatively small risk for Type 2 DM. The most important among those is a variant of the transcription factor 7–like 2 genes. Polymorphisms in the genes encoding the peroxisome proliferator-activated receptor (PPAR), inward rectifying potassium channel, zinc transporter, IRS, and calpain 10 have also been linked to Type 2 Diabetes.[1,22]

Environmental Factors

Obesity in the central or visceral region is the most significant environmental risk factor for Type 2 Diabetes. Obesity affects more than 80% of diabetic patients, and the prevalence of T2DM has increased in unison with obesity. Obesity is responsible for the majority of diabetic metabolic abnormalities, and even a modest weight loss can improve insulin resistance and glucose tolerance. Another key risk factor is a sedentary lifestyle; regular exercise combined with weight loss can improve insulin sensitivity. The combination of obesity, hyperglycemia, increased serum cholesterol and triglycerides, and hypertension is known as metabolic syndrome. In several populations across the world like East Asian, South Asian, and Middle Eastern, Type 2 Diabetes is rapidly rising but they do not show a corresponding rise in obesity indicating that the risk is also related to the anatomic distribution of fat as well. Additional environmental risk factors for T2D include

sleep disorders (such as obstructive sleep apnea) and circadian disruption. Circadian disruption is defined as misalignment between the endogenous circadian rhythm and the cycle or rhythm created by individual behaviors. Shift workers, as well as people with sleep disorders or other conditions that limit nighttime sleep and daytime wakefulness, are at risk for circadian disruption. Circadian disruption appears to alter insulin secretion as well as insulin action, according to research. Also, GWAS have shown an association between circadian-controlled genes and Type 2 DM. In addition to insulin secretion and action, these gene disruptions also affect the activity level and feeding behaviors and result in a higher risk for Diabetes.[16,20]

Pathophysiology

Beta cells in the pancreas secrete more insulin to counteract insulin resistance and keep blood sugar levels stable in the early stages of T2DM. Nevertheless, when insulin resistance and reactive hyperinsulinemia proceed, beta cells in some patients are unable to maintain the increased insulin output. As a result, impaired glucose tolerance (IGT) develops which is characterized by high postprandial glucose. An even greater reduction in insulin secretion and a rise in the production of hepatic glucose are responsible for the manifestation of overt diabetes leading to β-cell failure. As a result of poor insulin regulation, glucagon is upregulated and released, boosting hepatic glucose production even more. Both insulin resistance and reduced insulin production contribute to the evolution of Type 2 DM. However, the relative relevance of each differs from one patient to another.[1,21]

Metabolic Abnormalities

Insulin Resistance: Insulin resistance is defined as the inability of target tissues (particularly muscle, liver, and fat) to respond to insulin appropriately. It's a hallmark of Type 2 Diabetes, and is caused by a combination of genetic susceptibility and obesity.[23]

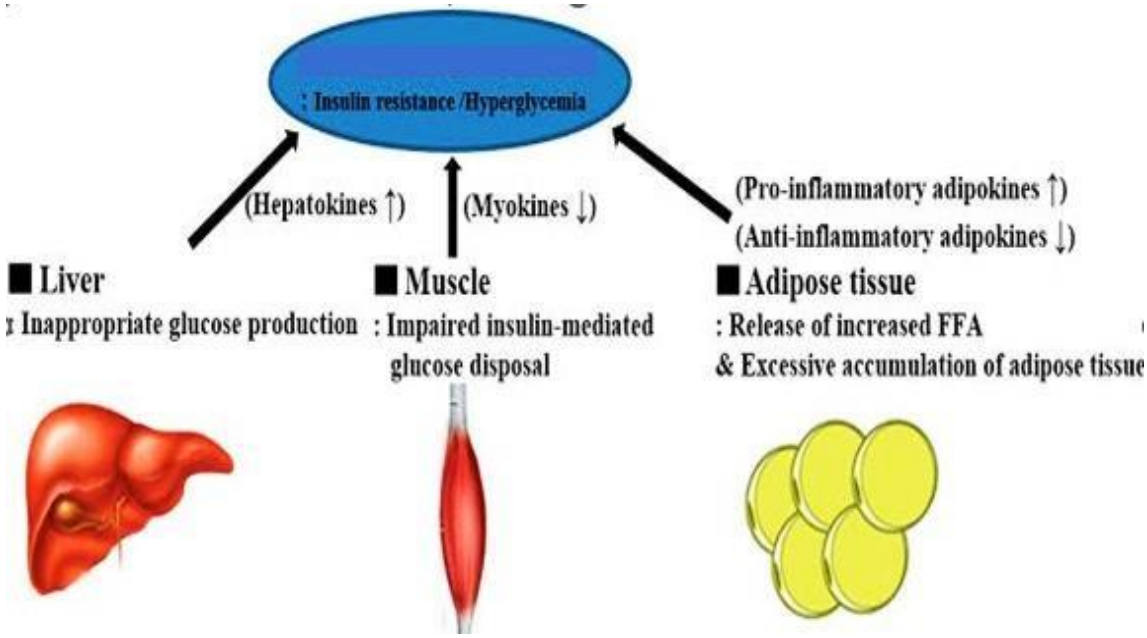


Figure 5 : Consequences of insulin resistance[24,25]

Molecular mechanisms of insulin resistance

Insulin tyrosine kinase receptor is activated by insulin binding, and it phosphorylates multiple insulin receptor substrates (IRSs), notably IRS1 & IRS2. Intracellular signaling molecules, such as phosphatidylinositol 3 kinase (PI3K), can be activated by phosphorylated IRS proteins.[26] By means of translocating GLUT4 to the skeletal muscle plasma membrane PI3K promotes glucose uptake. It further inactivates transcription factor forkhead box protein O1 (FOXO1) by altering downstream gene transcription. Insulin also activates the RAS–mitogen-activated protein kinase (MAPK) pathway. Insulin resistance in obesity and Type 2 DM is mainly attributed to the PI3K pathway; augmented phosphorylation of IRS proteins at serine residues leads to higher resistance.[27] Serine phosphorylation can increase IRS degradation as well, causing higher insulin resistance.[28] This type of phosphorylation can be caused by a variety of factors, including mitochondrial dysfunction, inflammation, ectopic lipid accumulation, and ER stress.[29]

Mitochondrial dysfunction

Mitochondrial dysfunction has been found in the adipose tissue, liver, muscle, and even in the hypothalamus, in rats and humans with obesity, Type 2 Diabetes, and metabolic syndrome.[30] It is due to both reduction in mitochondrial density and reduced functioning of mitochondria, as a result of abnormal expression of several oxidative phosphorylation system components.[31] Mitochondrial failure has been linked to decreased adiponectin release in adipose tissue, powerful insulin-sensitizing adipokine. In other tissues, it has

been postulated that mitochondrial failure leads to an increase in reactive oxygen species (ROS), which in turn activates redox-sensitive serine kinases, which induce a phosphorylation reaction with insulin resistance-associated proteins (IRPs). It is established that ectopic lipid deposition plays a critical role in the evolution of insulin resistance. Hence mitochondrial failure and reduced mitochondrial fatty acid oxidation are likely to be substantial exacerbating factors in this process.[32]

Obesity

Obesity is arguably the most important of the many factors that contribute to insulin resistance. As the BMI rises, so does the risk of Diabetes. Insulin sensitivity is determined not only by the total amount of fat but also by its distribution: Insulin resistance is more likely to be related to central obesity (abdominal fat) than peripheral obesity (gluteal/subcutaneous fat). It has been observed that people from Asia and the Middle East who develop Diabetes without overt obesity have more visceral adiposity. On the contrary people with subcutaneous adiposity may be relatively protected from Type 2 DM. These are known as "metabolically healthy obese" individuals and studies on such a population are an emerging field.

Insulin Resistance Syndromes: Hyperglycemia is the most easily recognized symptom of insulin resistance, which spans a spectrum of illnesses. Various terms are used to refer to a constellation of metabolic abnormalities that include insulin resistance, hypertension, dyslipidemia, central/visceral obesity, Type 2 DM, or IGT/IFG, and accelerated cardiovascular disease viz. metabolic syndrome, insulin resistance syndrome, and syndrome X.[1]

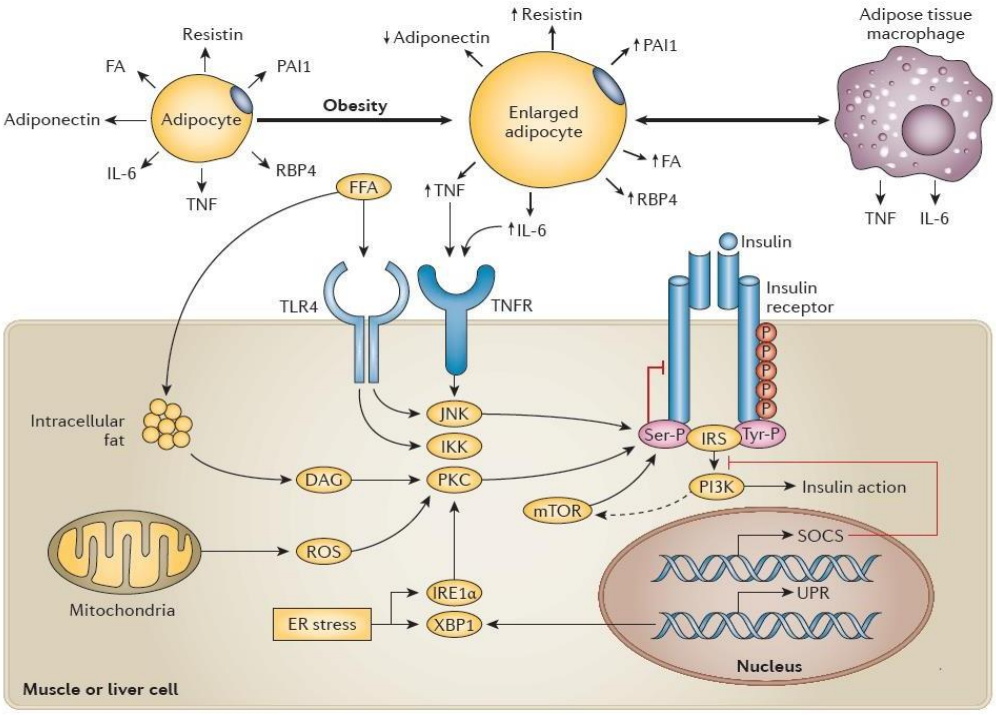


Figure 6: Mechanisms of insulin resistance[29]

Beta-Cell Dysfunction

Insulin resistance can result in reduced glucose tolerance on its own, but the development of overt Diabetes requires β-cell dysfunction. It is not uncommon for patients with "sporadic" T2D to have a significant improvement in their β-cell activity as a compensatory attempt to overcome insulin resistance and maintain euglycemia early on in the disease process, unlike the monogenic forms. However, it appears that β-cells eventually exhaust their ability to adjust to the long-term demands of insulin resistance, and the hyperinsulinemic state is replaced by a situation of relative insulin deficiency.

Several mechanisms contribute to β-cell dysfunction in T2D:[29,33]

i	Excess FFA (Free fatty acids) that impair β cell function and diminish insulin release (lipotoxicity)
ii	The impact of chronic hyperglycemia (glucotoxicity)
iii	An abnormal incretin effect and reduced secretions of GIP and GLP-1, hormones that enhances insulin secretions
iv	Amyloid deposition within islets: a characteristic finding in patients with long standing history of diabetes
v	Genetic polymorphisms

In summary, the comprehensive model incorporating all the varying pathophysiology is known as the 'ominous octet' of hyperglycemia. Recent evidence suggests that two additional pathophysiological anomalies viz. activation of inflammatory pathways and impaired insulin-mediated vasodilation that can be added to the 'ominous octet'.[29]

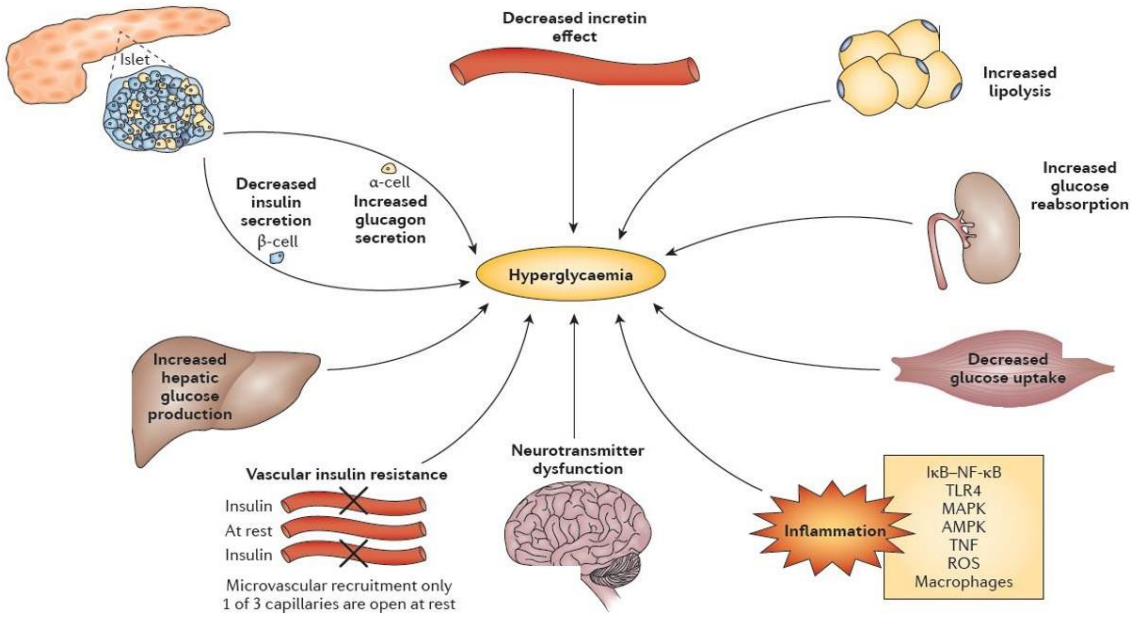


Figure 7: The 'ominous octet' of hyperglycemia in Type 2 Diabetes Mellitus[29]

Inflammation and Diabetes

Traditionally, metabolism and immunity were thought to be separate entities with discrete activities. The immune system's main job is to help the body recover from stress. Recent research suggests that this function is not only restricted to infections and injuries but also overnutrition.[34] Obesity and T2DM are linked to inflammation. Inflammation is a symptom of an illness, not a cause of it. Early effects of inflammation include limiting infection spread and promoting tissue regeneration. However, prolonged or excessive exposure might aggravate disease by destroying tissue. This is probably true for Type 2 DM pathophysiology. Inflammation aggravates insulin resistance and pancreatic islet inflammation, causing aberrant beta-cell secretion and Diabetes. Inflammation may be the cause of the greater risk of cardiovascular disease in diabetic and obese patients.[35]

Numerous investigations have shown that pancreas, muscle, liver, and adipose inflammatory sites in obesity and T2DM. Obese people with metabolic syndrome or Diabetes have macrophages infiltrating these tissues. These cells help produce pro-

inflammatory cytokines like TNF-, IL-6, and IL-1.[36] They promote insulin resistance by altering insulin signaling in peripheral organs by activating the JNK (c-JUN N-terminal kinase) and NF-kB (nuclear factor-kappa B) pathways.[37] Activated in obesity and T2DM, these pathways lead to tissue inflammation.

Triggers of innate immunity in obesity and Type 2 DM patients

Inflammation is found in all energy-balancing tissues as well as in the arteries that transport nutrients.[38] Experimental evidence shows linkage between metabolic stress and inflammation. The saturated long-chain Free fatty acids palmitate and stearate and monounsaturated oleate which account for nearly 80 percent of FFAs in human circulation are proinflammatory in vitro.[39] Toll-like receptor (TLR)-dependent and TLR- independent mechanisms have been suggested to explain FFA-induced inflammation. TLRs are innate immune pattern recognition receptors that are activated by bacterial wallproducts like LPS-containing long-chain fatty acid moieties. As a result, FFAs may directly interact with TLRs, causing an inflammatory reaction.[40] Recent research, however, has ruled this out, and many indirect mechanisms by which FFAs engage TLRshave been revealed. FFAs have been demonstrated to promote the formation of lipid raftsin cell membranes, enabling TLR dimerization.[41] Others proposed an endogenous ligand between FFA and TLR. For example, the TLR2 interacts with fatty acid transporter CD36.Most recently, it has been shown that the liver-derived glycoprotein fetuin-A works as an endogenous ligand, connecting FFA and TLR4, causing inflammation and insulin resistance in the body.[42] Moreover, lipid-mediated toxicity may amplify damage signals like HMG1. TLRs recognize these chemicals and trigger pro-inflammatory pathways. FFAs trigger inflammatory pathways without TLRs by releasing reactive oxygen species (ROS), which then activate stress kinases. ROS may also activate the IL-1 system by producing NLRP3 inflammasomes.[43] In human and mouse islets, FFAs generate a broad proinflammatory response that is primarily dependent on IL-1 receptor activation. The generation of cytokines and chemokines by FFAs was completely prevented by suppressing IL-1 receptor activation in human islets with either the natural antagonist IL-1Ra or a neutralizing anti-IL-1 antibody. The lack of Myd88, a universal intracellular docking protein required for both TLR and IL-1 receptor activation, completely protectedTLR2 or TLR4-deficient mouse islets from FFA-induced proinflammatory cytokine production. This shows TLR-dependent and TLR-independent pathways may coexist.[44]

Glucose, like FFAs, can cause systemic inflammation that can be explained by two processes. Firstly, chronic hyperglycemia induces nonenzymatic glycation of proteins and lipids, forming AGEs (Advanced glycation endproducts) that activate the pattern recognition receptor RAGE (Receptor for Advanced glycation endproducts) that in turn activates NF-kB and the stress kinases ERK1 and ERK2.[45] RAGE is expressed on smooth muscle cells, macrophages, podocytes, T cells, cardiomyocytes, and neuronal cells. The second process includes reactive oxygen species (ROS) produced by excessive glucose oxidative phosphorylation. Inflammasome activation by ROS and FFAs results in the release of active IL-1 and the production of IL-1-dependent cytokines and chemokines.[44,45]

Inflammation in adipose tissue

Hotamisligil et al first demonstrated that adipose tissue from obese people has higher levels of TNF-α than normal. Their findings implicated TNF in obesity-induced insulin resistance.[46] An abundance of pro-inflammatory genes, cytokines, and chemokines were found in enlarged adipose tissue, according to growing data. The improvement in insulin sensitivity linked with weight loss also reduced the expression of pro-inflammatory genes. Thus, adipose tissue inflammation was regarded as a precursor to metabolic syndrome, T2DM, and atherosclerotic cardiovascular illnesses. Obesity has lately been linked to an increase in immune cells in the stromovascular component of adipose tissue infiltrated by macrophages.[36] Although larger adipocytes produce pro-inflammatory cytokines and chemokines, macrophages significantly contribute to the generation of pro-inflammatory cytokines. Systemic inflammation, metabolic syndrome, and insulin resistance are all associated with increased macrophage recruitment, which is related to obesity. Weight loss through surgery or lifestyle modifications reduces the number of macrophages and pro-inflammatory markers in adipose tissue and plasma of obese people.[47]

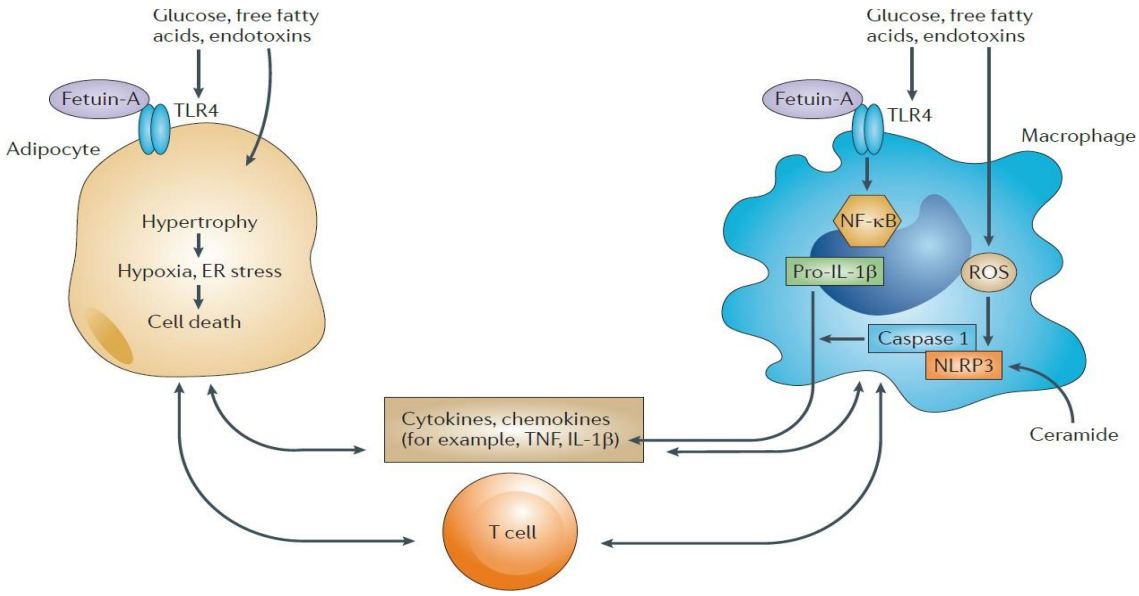


Figure 8: Storage of excessive nutrients in adipose tissues leads to an inflammatory response and insulin resistance[48]

Macrophages are of 2 types. "Classically Activated macrophages" secrete cytokines like IL-1, IL-6, and TNF -α which are pro-inflammatory, while "alternatively activated macrophages" produce IL-10, an anti-inflammatory substance. A phenotypic flip from M2 to M1 is seen with obesity, which coincides with insulin resistance. Insulin signaling and adipose synthesis in adipocytes can be impaired by M1 macrophages via direct and paracrine signals, but M2 macrophages are mostly protective against insulin resistance due to obesity.[36]

In addition to macrophages, the adaptive immune system may be involved in obesity-induced inflammation. During early insulin resistance, infiltrating lymphocytes precede macrophage infiltration in obese adipose tissue, possibly contributing to adipose tissue inflammation through controlling macrophage number and activation state. Obesity also affects the balance of pro- and anti-inflammatory CD4+ cell subsets, causing newly recruited adipose tissue macrophages to secrete cytokines. In patients with obesity and metabolic syndrome, there is a fall in anti-inflammatory regulatory T cells. The regulatory T cells produce a lot of IL-10, which slows macrophage migration and promotes M2 differentiation. In obese mice it was found that increased numbers of these cells enhance insulin sensitivity and oppose macrophage infiltration. These data imply that regulatory T cells can reduce inflammation in adipose and protect against insulin resistance and obesity-related inflammation. [49] Obesity alters many other immune cells in adipose tissue, perhaps

affecting inflammation and insulin resistance. The balance between innate and adaptive immune cells seems to be crucial for adipose tissue homeostasis and regulation in obesity and Type 2 DM. However, immune cell recruitment and activation molecular pathways remain unclear.[47]

Inflammation in Islets cells

After discovering that hyperglycemia promotes β cell necrosis in the pancreas islets, researchers began to suspect an inflammatory process.[50] Experimenting further, researchers discovered that increased glucose levels activated the Fas receptor, which in turn activated IL-1 production. Recent research shows that fatty acids also increase inflammation. Immune cells and cytokines and chemokines were found in the islets of diabetics and animal models. The presence of acute inflammation in islets is supported by the well-described fibrosis seen in tissue sections of individuals with Type 2 Diabetes, characterized by amyloid deposits. Fibrosis is a symptom of persistent inflammation. It's worth noting that IL-1, which is increased in the islets of Type 2 diabetics plays a major impact. This master cytokine is involved in the regulation of a large number of other cytokines and chemokines. This promotes the migration of immune cells responsible for triggering widespread inflammation. In addition, IL-1 induces itself in β-cells, resulting in a vicious cycle. Thus, insulitis is a key player in the etiology of Type 2 Diabetes. Islets β-cells exhibit up to three times the mitochondrial activity of other cells due to glucose oxidation in the mitochondria. Consequently, they are more vulnerable to ROS generation. This may explain why glucose-induced IL-1β affects β-cells. By dissociatingthioredoxin-interacting protein (TXNIP) from thioredoxin, ROS triggers the development of the inflammasome and thus IL-1 release. Hyperglycemia with FFAs induces a proinflammatory phenotype in pancreatic islets and monocytes.[44,51,55]

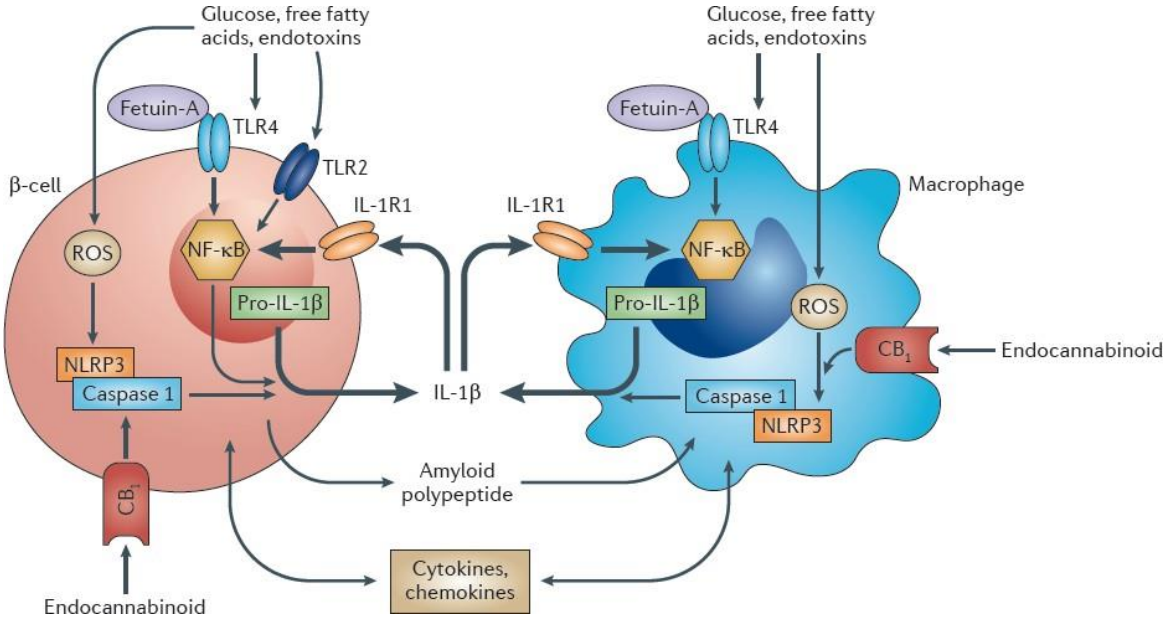


Figure 9: Islet inflammation in Type 2 Diabetes[48]

Protein flux through the ER of beta cells is high under normal settings, but it rises with insulin resistance. Endoplasmic reticulum stress may lead to islet cell malfunction in Type 2 DM. It is believed that TXNIP is caused by endoplasmic reticulum stress, which results in beta cells producing interleukin-1 (IL-1) when NLRP3 is present.[52] IAPP (Islet amyloid polypeptide) deposition is another hallmark of human pancreatic islets, which is observed in over 90% of islets from Type 2 diabetics. A 37 amino acid peptide, IAPP is converted to its mature form and subsequently secreted in combination with insulin. Amyloid deposition is caused by the fast aggregation and formation of fibrils of human IAPP. An increasing list of substances, like urea crystals and asbestos, have been shown to trigger the NLRP3 inflammasome, and human IAPP has recently been added. Inflammasome development and IL-1 release are induced in bone marrow-derived macrophages by oligomeric human IAPP coupled with LPS or slightly oxidized low-density lipoproteins. Fibrils were less efficient than oligomers in these macrophages. It was also reported that mice expressing human IAPP had increased IL-1 expression in their islets, demonstrating that IAPP may stimulate the synthesis of IL-1 in the body.[53,54]

Sensors and mediators of inflammation in T2DM

Despite the importance of subclinical inflammation in the pathogenesis of T2DM, the processes that cause it are yet unknown. The possible mechanisms include

i. Activation of the NF-kB and JNK pathways
ii. Generation of cytokines and chemokines
iii. Recruitment of immune cells

NLRP3 inflammasome

The concept of T2DM as an inflammatory disease has recently emerged and seems to be confirmed by accumulating evidences. A number of studies have suggested the role of inflammasomes.[7] Inflammasome is an intracellular multiprotein complex formed through the interaction of the nucleotide-binding oligomerization domain like receptor (NLR) family, the adaptor protein apoptosis associated speck- like protein containing a caspase recruitment domain (ASC), and the pro-caspase-1.[4,56] Among several NLRs that form inflammasome, major role appears to be played by NLR family pyrin domain-containing3 (NLRP3) inflammasome in the progression of T2DM.[4] Recent evidence also implicates NLRP3 to the pathogenesis of several inflammatory disorders like Alzheimer's disease, gout, autoinflammatory disease and atherosclerosis.[7] The NLRP3 inflammasome , a multiprotein complex is formed through the interaction of NLRP3, the protein adaptor ASC and the pro-caspase-1, leading to casapase-1 activation. Upon activation, caspase-1 cleaves the inactive precursor of IL-1β (pro- IL-1β) into its biological active form IL-1β which is secreted.[4] NLRP3 activation requires two step process. First or "priming" signal acts on TLR (toll like receptor) or cytokine receptor leading to activation of NF-kB pathway and transcriptional expression of NLRP3 and pro- IL-1β. The second or "activating" signal includes various PAMPs (pathogen-associated molecular patterns) and DAMPs (danger-associated molecular pattern) by innate pattern recognition receptors (PRRs), that directly induces NLRP3 inflammasome formation and instigates the caspase-1 activation which further leads to secretion of IL-1β.[4] IL-1β, one of the major pro-inflammatory cytokine produced by macrophages is a key contributor to the pathogenesis of T2DM.[4]

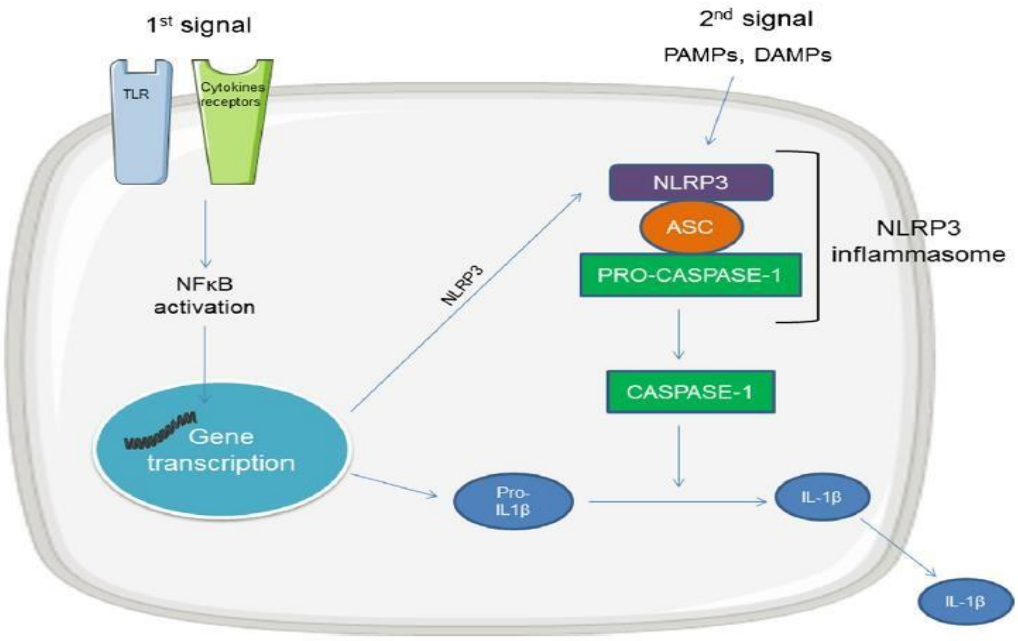


Figure 10 : The NLRP3 inflammasome[47]

β -cells of the pancreas and islet-infiltrating macrophages were the first to demonstrate activation of the NLRP3 inflammasome and the subsequent production of IL-1β thereby, increasing apoptosis and decreasing insulin synthesis by -cells. Additional evidence suggests that the islet amyloid polypeptide (IAPP), causes amyloid deposits to be formed in the islets, contributing to the production of islet IL-1β through activation of the NLRP3 inflammasome.[53] The NLRP3 inflammasome is also linked to insulin resistance induced by obesity.

NLRP3 inflammasome components, caspase-1 action, and interleukin-1beta are all reported to be elevated in the adipose tissue of obese mice models and diabetics, and these changes are associated with insulin resistance, metabolic syndrome, and severity of Type 2 Diabetes. The adverse consequences of visceral adipose tissue are probably due to higher expression and activation of the NLRP3 inflammasome compared to subcutaneous adipose tissue in obese people. Obesity appears to activate the NLRP3 inflammasome, which is a sensor for metabolic danger signals, such as high glucose, saturated free fatty acids (SFFA), and the presence of lipid intermediates such as ceramides, and activates the production of IL-1β and the induction of a wide range of pro-inflammatory mediators. Its inhibition has been shown to boost insulin signaling in all insulin-sensitive tissues, as well as pancreatic insulin production.[56]

A new study examined many aspects of NLRP3 inflammasome activation in a different subpopulation of obese individuals who don't show classical metabolic problems associated with obesity and had a decreased risk of T2DM and cardiovascular disease. According to the research, this phenotype is known as "metabolically healthy obesity" (MHO) and up to 30 percent of the entire obese population may be accounted for by this phenotype. Additionally, the MHO is associated with less inflammation and lower levels of systemic markers of inflammation than unhealthy obesity phenotypes. Compared to unhealthy obese visceral adipose tissue, MHO visceral adipose tissue has decreased NLRP3 inflammasome activation and a better inflammatory and immunological profile.[47]

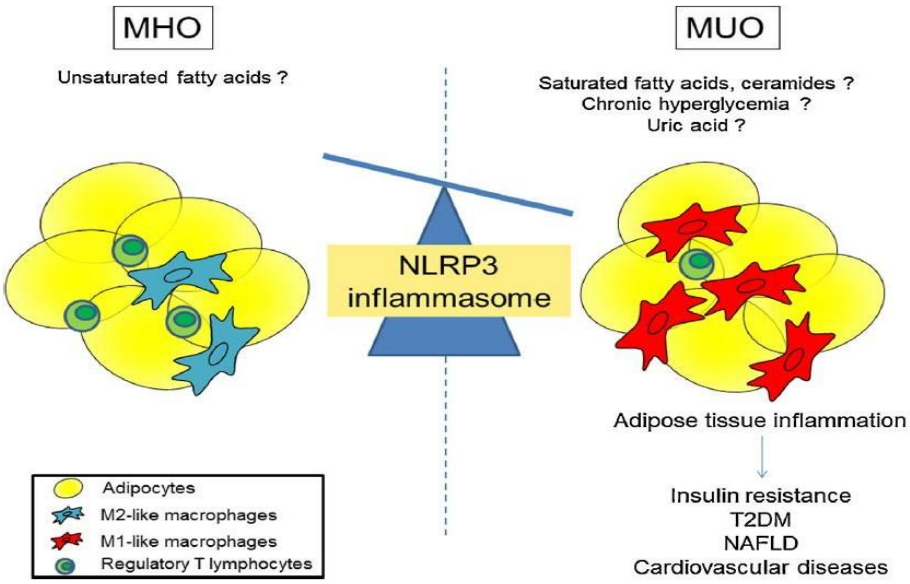


Figure 11: Adipose tissue inflammatory profile imbalance between metabolically healthy and unhealthy obese[47]

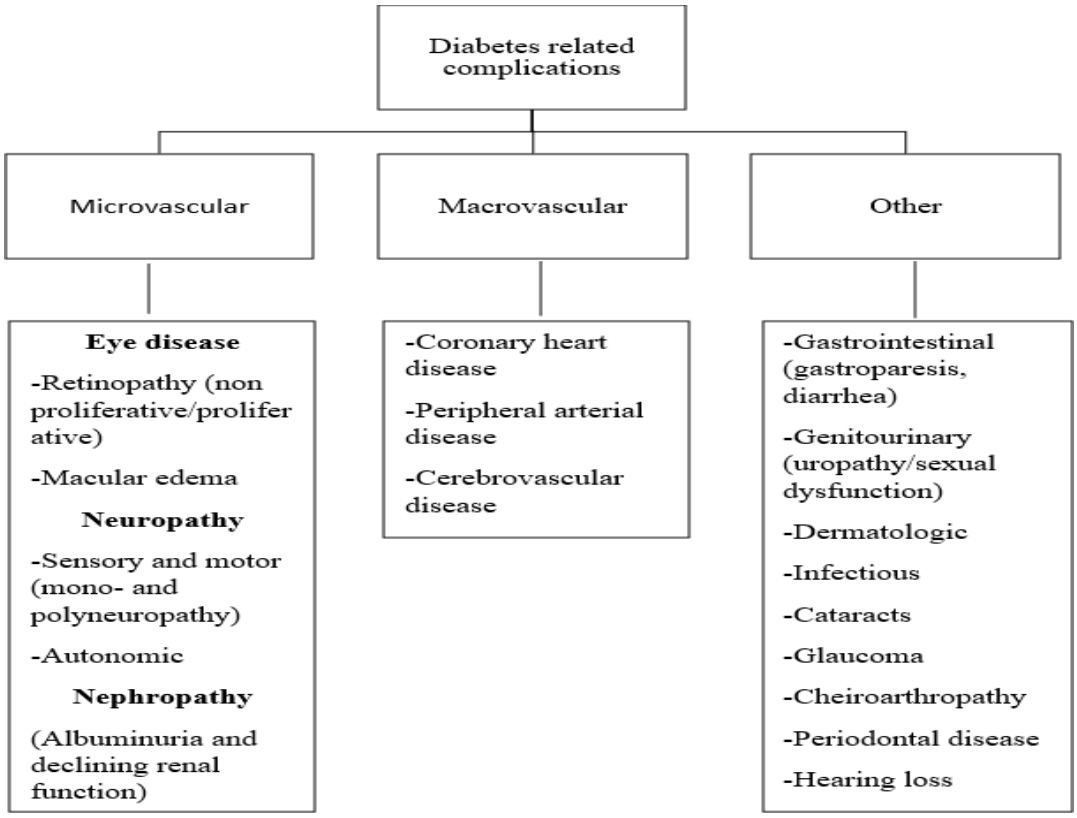
We can better comprehend obesity-related metabolic diseases if we can identify the components that contribute to differential inflammasome activation. Unsaturated fatty acids have powerful anti-inflammatory action and enhance insulin sensitivity in obese and Type 2 diabetic patients, making fatty acids a good candidate for the study of the pathologic basis of T2DM. It is also worth noting that unsaturated and omega-3 fats don't trigger the NLRP3 inflammasome and can block its activation by other stimuli.[57] The findings of yet another recent study, which demonstrated a different fatty acid profile across MHO and unhealthy obese persons, notably for saturated fatty acids, emphasized the importance of unsaturated fatty acids in the mediation of inflammation.[58] Therefore, to develop preventive and therapeutic solutions for obesity-related insulin resistance and inflammation, more research between these two groups is required.

Endoplasmic reticulum stress

The endoplasmic reticulum, a crucial location for protein folding, maturation, and trafficking, may also be involved in macrophage NLRP3 activation. Chronic nutritional overload promotes endoplasmic reticulum stress, triggering pro-inflammatory signal transduction pathways. ER stress has been found in the adipose tissue of obese insulin-resistant people, suggesting that it may play a role in insulin resistance and inflammation.[59]

Diabetic Complications:

Complications of Diabetes affect a variety of organ systems. Diabetes has been identified as the major cause of renal failure, new blindness in adults, and nontraumatic lower-extremity amputation. Diabetes has recently emerged as a major risk factor for coronary heart disease (CHD). Insulin resistance and hyperglycemia are the two most common causes of diabetic complications. In most cases, Diabetes-related problems related to hyperglycemia do not manifest themselves after a decade. Diabetes-associated cardiovascular disease (CVD) risk, on the other hand, might develop before hyperglycemia has been established in the patient. Because of the long asymptomatic period of hyperglycemia before diagnosis, many individuals with Type 2 Diabetes have both hyperglycemia and insulin resistance-related problems when they are diagnosed. These complications are classified as vascular or nonvascular, and they affect people with both major types of Diabetes. Microvascular and macrovascular problems are subsets of vascular complications. It’s important to note that Diabetes-related microvascular problems are present. Some of the pathophysiological characteristics of macrovascular problems are shared by both diabetics and the normal population, unlike microvascular complicationse.[1]



Diagnosis of T2DM

In recent years, the diagnostic criteria for Diabetes have undergone a substantial shift. The American Diabetes Association (ADA) and World Health Organization criteria for the diagnosis of diabetes have been widely accepted by the medical community.

Table 2: Diagnostic criteria for Type 2 Diabetes Mellitus[60]

Diagnostic criteria for Diabetes Mellitus		
Parameter	**Values**	**Comments**
Fasting Plasma Glucose (FPG)*	≥ 126 mg/dl (7.0 mmol/l)	Fasting → no caloric intake for at least 8 h
or		
2- hour plasma glucose during OGTT*	≥ 200 mg/dL (11.1 mmol/L)	OGTT is done as per WHO description → by giving a glucose load equivalent of 75-g anhydrous glucose in water.
or		
Glycosylated Hb (HbA1C)*	≥6.5% (48 mmol/mol)	Performed in a laboratory using a that is NGSP certified method and standardized to the DCCT assay
or		
Random Plasma Glucose	≥200 mg/dL (11.1 mmol/L).	In patients with classic symptoms of hyperglycemia or hyperglycemic crisis

**In the absence of unequivocal hyperglycemia, repeat testing should be done on a different day to confirm the values*

Pharmacological therapy for T2DM

Table 3: Summary of drugs available for Type 2 Diabetes Mellitus[1,61]

Drugs	Mechanism of action and effects	Efficacy	Advantages	Disadvantages
Sulfonylureas Glibenclamide Glimepiride Glipizide	Bind to SURI on β cell resulting in ↑ Insulin secretion	High	Long term safety data	Weight gain Hypoglycemia
Biguanides Metformin	↑ Hepatic glucose production AMPK activation	High	Long term safety data Weight neutral	GI adverse events Contraindicated in renal disease
Thiazolidinediones **Pioglitazone** **Rosiglitazone**	PPAR-γ agonists ↑ Insulin sensitivity	High	Low risk of hypoglycemia	Edema High risk of heart failure
GLP-1 receptor agonists **Exenatide** **Liraglutide** **Dulaglutide** **Albiglutide**	↑ Insulin ↓ Glucagon	High	Weight loss Minimal hypoglycemia	Injectable GI adverse events
Insulin **1.Rapid acting** (Lispro, Aspart) **2. Short acting** (Actrapid) **3. Intermediate acting** (Humulin-I, Insulatard)	↑ Glucose utilization ↓ Hepatic glucose production	High	Injectable sustained glycemic improvement	Weight gain hypoglycemia

4. Long acting (Detemir, Glargine, Degludec) **Biphasic premixed**				
Meglitinides Nateglinide Repaglinide	Inhibit SGLT-2 transportes leading to increased renal glucose excretion	Intermedi-ate to high	Weight loss Low risk of hypoglycemia	Genital infection and UTI Possible increased risk of fractures
DPP-4 inhibitors **Sitagliptin** **Vildagliptin** **Saxagliptin** **Linagliptin**	Prolong endogenous GLP-1 action ↑ Insulin ↓ Glucagon	Intermedi-ate	Weight neutral hypoglycemia	Increased risk of pancreatitis
α- glucosidase inhibitors	↓ Glucose absorpion in GI tract	Modest	Weight neutral	GI adverse effects
Dopamine-2 agonist **Bromocriptine**	Activates hypothalamic dopamine receptors leads to suprression of hepatic glucose output	Modest	Weight neutral	Dizziness Nausea Fatigue
Bile acid sequestrant **Colesevlam**	Activates liver farnesoid receptors ↑ GLP-1 secretion	Modest	Weight neutral Decrease LDL Increase HDL	Constipation ↑ Triglyceride

AMPK, 5′ AMP-activated protein kinase; DPP-4, dipeptidyl peptidase 4; GLP-1, glucagon-like peptide 1; PPAR-γ, peroxisome proliferator-activated receptor γ; SGLT2, sodium/glucose cotransporter 2; SMBG, self-monitoring of blood glucose; SUR1, sulfonylurea receptor 1; GI, gastrointestinal.

B. TARGETTING INFLAMMATION IN DIABETES

Anti-inflammatory properties of current anti-diabetic drugs

Diabetes medications and lifestyle changes have anti-inflammatory benefits as well. Also, Diabetes medicines and lifestyle changes have anti-inflammatory qualities. The Diabetes Prevention Program (DPP) found that weight loss reduced CRP by 31% whereas Metformin reduced CRP by 13%.[62] Surgical weight loss methods have similar outcomes. Lifestyle adjustments can reduce insulin resistance, decrease the advancement of pre-Diabetes to Type 2 DM and delay the evolution of Diabetes mellitus and its consequences even without pharmacological therapy. For the same level of glucose reduction, thiazolidinedione has been proven to lower inflammation markers more than other regimens.[63] This could be due to PPAR-mediated trans repression of inflammatory response genes. A decrement in inflammation amplifies the advantages of these medications, regardless of the impact on glucose levels.[2]

In pancreas, prolonged exposure of pancreatic islet β-cells to elevated concentration of glucose and free fatty acids increases islet β-cell metabolic activity which leads to elevated reactive oxygen species (ROS) formation. This promotes the activation of NLRP3 inflammasome and caspase-1, thus enabling production of mature IL-1β and IL-1β auto-stimulation further amplifies the inflammation by inducing secretions of cytokines and chemokines like IL-6, TNF-α IL-8 etc. These cytokines promote the immune cells in the islet cells that result in insulin resistance and further leads to progression of T2DM.[1] Considering the central role of NLRP3 inflammasome and IL-1β in the pathogenesis of T2DM, it is not surprising that the blockade of IL-1β activity has shown improvement in glucose control in prediabetic or T2DM populations.[4]

Insulin therapy alone has been linked to a short-term reduction in inflammation. A reduction in the activity of nuclear factor-B (NF-B), the main transcriptional regulator of the inflammatory response, is responsible for this phenomenon.[64] However, this effect is temporary and/or requires greater insulin doses provided intravenously. Another advantage of starting insulin therapy early is that it may postpone the advancement of the disease and the development of its consequences. Statins are a class of medications commonly used in Type 2 DM with additional anti-inflammatory properties. Statins lower cholesterol by blocking hydroxymethylglutaryl-CoA reductase (HMG-CoA reductase). Statins also lower CRP by 25%-30%. This is a class effect and not dose-dependent. The reduction in CRP levels does not correspond to a reduction in cholesterol, implying that this is a statin-induced impact. Cardiovascular events can be predicted by CRP alone. An

interventional trial looked at Rosuvastatin's influence on primary cardiovascular events in those with high CRP but no hyperlipidemia. In this study, CRP was lowered by 37% but LDL was lowered by 50%, leaving the question of whether statins are actually anti-inflammatory or only lowering lipids.[65]

Table 4: Anti-inflammatory effects of glucose-lowering agents used in the treatment of Type 2 Diabetes[66]

Drug	Main findings	Remarks & limitation
Biguanides	• Reduced or unchanged CRP • Reduced markers of endothelial dysfunction	May have beneficial effects on chronic inflammatory disorders and cancer
Sulfonylureas	• Reduced or unchanged CRP • Reduced markers of endothelial dysfunction	Conflicting data
Thiazolidinediones	• Substantial reduction in CRP	Consistent anti-inflammatory effect
DPP-V inhibitors	• Reduced inflammatory cytokines • Reduced CRP	Moderate effect Requires further study
GLP-1 receptor Agonists	• Reduced markers of endothelial dysfunction • Reduced inflammatory cytokines	Moderate effect Requires further study
SGLT-2 inhibitors	• Unknown	Future studies needed
Insulins	• Reduced or unchanged inflammatory cytokines and immune mediators • Reduced or unchanged CRP	Moderate effect Although data conflicting

Unmet needs in Type 2 Diabetes therapy

The present diabetic medications do not offer disease-modifying attributes, which would reduce the deterioration in insulin secretion. Furthermore, Type 2 Diabetes is connected with dyslipidemia and hypertension, and it is a substantial risk factor for cardiovascular disease, nephropathy, retinopathy, and neuropathy. Most individuals with T2DM are administered a variety of drugs to prevent and manage these complications. Multi-drug regimens tend to reduce patient compliance. Several anti-diabetic drugs have also been associated with side effects. The most significant side effects are gastrointestinal

symptoms with Metformin, hypoglycemia and weight gain with Sulphonylureas or insulin, weight gain and bone fractures with Thiazolidinediones, and urinary tract infections with SGLT-2 inhibitors. Ideal diabetic treatment should thus manage hyperglycemia, prevent disease progression, handle comorbidities and provide long-lasting results with minimal side effects.[48]

Immunometabolism as a therapeutic target

Recent research shows a direct link between immune system function and metabolic alterations. This research has led to the creation of a new field called 'immunometabolism.' This is contrary to popular belief, that metabolism handles nutrition while immunity is responsible for host defense. But there is a striking similarity between these two functions: they both aim to restore homeostasis in the face of stress. Some of the most prevalent stresses of the immune system include pathogenic, mechanical, and chemical assaults, Over-nutrition also has joined this list because of the abundance of food available today. This could explain why the immunological response to pathogens and the metabolic stress response is so similar. Genetic predispositions may worsen chronic immunological hyperactivation caused by metabolic stress. This could eventually lead to chronic inflammatory disorders, such as T2DM An immunological treatment for T2DM should therefore either tweak or steer the immune system to work more effectively for them.[48]

Metabolic Effects of Anti-inflammatory Drugs

Targeted anti-inflammatory therapy has been suggested for both prevention and treatment of Diabetes.

Anti–TNF-α

TNF- α was the first proinflammatory cytokine implicated in the etiology of insulin resistance and Type 2 Diabetes. TNF- α antagonism has yet to show obvious benefit in human Type 2 Diabetes.[67] These clinical studies were all underpowered and short-lived, according to a thorough review[51]. No diabetic or inflammatory disorders such as rheumatoid arthritis or Crohn's disease have been shown to benefit from TNF- α antagonist treatment in observational studies. While most of these trials are not prospective, and the benefit is not necessarily due to improved glucose metabolism. Further studies are needed to evaluate the role of such treatments.[66]

Anti–IL-1β

The effect of IL-1β blockage in insulin resistance and T2DM has been studied extensively since its discovery. Using an IL-1 receptor antagonist (Anakinra) or an IL-1β–specific antibody (Gevokizumab, Canakinumab, and LY21891020) has been shown to improve metabolic parameters such as HbA1c, insulin sensitivity, and β-cell secretory function while decreasing inflammatory indicators.[68] Data from a recent study suggest an IL-1β blocking involvement in Diabetes-associated inflammation and metabolic abnormalities. Regarding safety, the drugs were tolerated well however Anakinra injection had to be given daily leading to frequent injection site responses. The humanized anti-IL-1β antibodies allow monthly injections, reducing localized responses.[66]

Salicylate and salsalate

Inhibition of NF-kB activity by sodium salicylate and Aspirin has been shown to promote glycaemic control in T2DM. Salsalate, a prodrug of salicylate, can increase insulin sensitivity and glucose management in prediabetic and Type 2 DM patients with a low risk of bleeding. In particular, the TINSAL-T2D trial indicated that salsalate improves glycemic control in Type 2 DM patients by lowering fasting glucose and HbA1C levels and improving the lipid profile.[69] NF-kB pathways, according to these findings, may constitute a unique treatment option to prevent and treat T2DM. Future studies are required to investigate whether the benefits of these medications may be sustained over time.

Table 5: Summary of clinical trials demonstrating metabolic effects of anti-inflammatory drugs[66-69]

Drug	Mechanism of action	Main findings	Remarks and limitation
Anti TNF-α antibody	TNF α antagonism	↑ Insulin secretions ↓ CRP	Studies underpowered and of short duration
IL-1 receptor antagonist	IL-1β antagonism	↑ Insulin secretions ↑ Insulin sensitivity ↓ CRP ↓ HbA1C	Effect persisted several weeks after treatment cessation, Long term studies ongoing
Salsalate	IKK-β, NFK- β inhibition	↑ Insulin secretions ↑ Insulin sensitivity ↓ CRP ↓ HbA1C ↓ FBG	↑ LDL cholesterol and urine albumin levels

Chloroquine/ HCQ	Unknown	↑ Insulin secretions ↓ Insulin degradation ↓ HbA1C ↓ FBG	Observational study or small scale prospective RCT

FBG, fasting blood glucose; IKK-b, inhibitory kB kinase-b; RCT, randomized controlled trials.

C. DRUGS USED IN THE PRESENT STUDY:

Ticagrelor

Ticagrelor is an orally administered directly acting P2Y12-receptor antagonist.[70] Ticagrelor, an oral anti-platelet drug belongs to the class of organic compounds known as triazolo pyrimidines. Ticagrelor was first approved in 2011 by US Food and Drug Administration for the management of CAD (coronary artery disease). In vitro studies have demonstrated that Ticagrelor binds reversibly and noncompetitively to the P2Y12 receptor at a site distinct from that of the endogenous agonist adenosine diphosphate (ADP).[70,71]

The development of Ticagrelor began by leveraging the structure of adenosine triphosphate, which is an endogenous antagonist of the P2Y12 receptor. Prior to the development of Ticagrelor, Cangrelor was identified as a potent and selective P2Y12-receptor antagonist (Figure 2). Currently, Cangrelor is being developed for intravenous administration. To identify orally active derivatives, the structure of Cangrelor was altered by replacing the purine with a triazolopyrimidine heterocycle as well as substitutions at other key locations.[71]

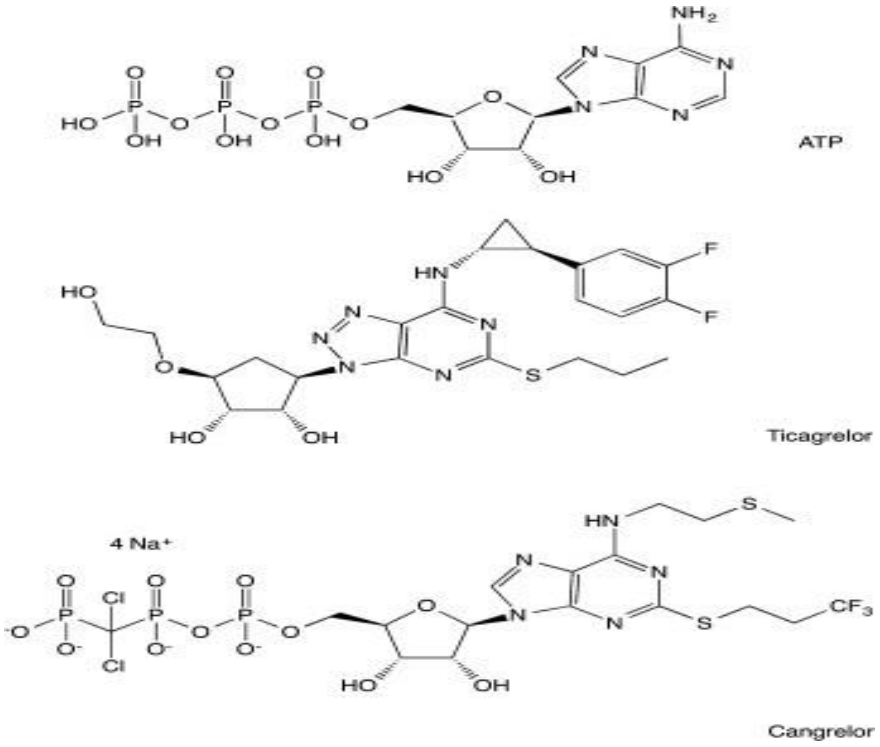


Figure 12: Chemical structures of ATP, Ticagrelor, and Cangrelor[71]

Mechanism of action:

The NLRP3 inflammasome that mediates the secretion of IL-1β and other inflammatory cytokines to initiate and promote DM, is the primary targets of Ticagrelor.[72] It works as a potent inhibitor of the NLRP3 inflammasome, which has been shown to affect IL-1β activation.[73] This leads to altered sensitivity to IL-1β. Because Ticagrelor has been demonstrated to suppress the activation of the transcription factor NF-κB, which is activated by IL-1β the generation of IL-1β may be also altered.[74,75]

Pharmacokinetics and Pharmacodynamics

Ticagrelor is initiated as a onetime loading dose of 180 mg (two 90-mg tablets), followed by continued treatment with 90 mg twice daily. Ticagrelor is rapidly absorbed, with a median time to peak concentration (Tmax) of 2–3 hours after multiple twice daily oral dosing. After absorption, Ticagrelor is highly bound to plasma proteins (more than 99.8%) and largely restricted to plasma. The absolute bioavailability of Ticagrelor is estimated at 36%, and the steady-state volume of distribution is about 88 L. Unlike Clopidogrel and Prasugrel, Ticagrelor is not a prodrug and does not require metabolic activation for antiplatelet activity. Still, ticagrelor is extensively metabolized, with Ticagrelor and its

active and approximately equipotent metabolite composing the major circulating components in the plasma.[71,76]

Many promising pharmacological benefits have been demonstrated by numerous researches on Ticagrelor's additional potential uses and it has been in widespread use worldwide.

Safety profile of Ticagrelor

Bleeding Events

Ticagrelor, like other antiplatelet drugs, can cause significant, sometimes fatal bleeding. Ticagrelor should not be used in patients with active pathological bleeding or a history of intracranial hemorrhage. Clinicians should not prescribe Ticagrelor for patients who are expected to undergo urgent CABG surgery. If possible, Ticagrelor should be discontinued for at least 5 days before any surgery. Clinicians should suspect bleeding in any patient who is hypotensive and has recently undergone coronary angiography, PCI, CABG, or other surgical procedures during Ticagrelor therapy. If possible, clinicians should manage bleeding without discontinuing Ticagrelor, because stopping therapy increases the risk of subsequent cardiovascular events.[76,78]

Table 6: Other Non-Hemorrhagic adverse events of Ticagrelor[76,77,78]

Adverse event	Frequency (%)
Dyspnea	13.8
Headache	6.5
Cough	4.9
Dizziness	4.5
Nausea	4.3
Hypertension	3.8
Diarrhea	3.7
Hypotension	3.2
Fatigue	3.2
Chest pain	3.1
Bradycardia	1.7%

REVIEW OF STUDIES

In recent years, there has been increasing evidence that Ticagrelor has the potential to become a novel therapeutic agent for the treatment of Diabetes. In diabetic nephropathy animal model of unilateral nephrectomy followed by STZ injections for 5 days, ticagrelor treatment not only prevented diabetes induced mesangial matrix expansion, podocyte effacement and glomerular endothelial cell injury but also, significantly reduced plasma as well as kidney m-RNA expressions and levels of TNF-α and CASPASE 3.[6] Huang B, et al demonstrated that Ticagrelor inhibits activation of NLRP3 inflammasome which is responsible for pathogenesis of complex diseases like ACS, DM etc. Ticagrelor has been reported to reduce the inflammation in various models that are known to induce NLRP3. In nigericin stimulated LPS primed mouse bone marrow derived macrophages, Ticagrelor significantly decreased the maturation of CASPASE-I and reduced IL-1β in a dose dependent manner, and decreased LPS induced expression of pro-IL-1β and TNF-α. In Alum induced peritonitis mouse model, Ticagrelor treatment remarkably decreased IL-1β levels and numbers of peritoneal exudate cells and neutrophils in the peritoneal lavage fluid. Similarly, in LPS induced sepsis model, Ticagrelor markedly reduced serum levels of IL-1β and IL-18. Ticagrelor treatment increased the survival of both wild type of mice and $P2Y_{12}$ knockout ($P2Y_{12}^{-}$) mice, suggesting that its effect on NLRP3 was independent of $P2Y_{12}$ action.[5]

Similarly, in humans, Ticagrelor blocked CASPASE-I maturation and production of IL-1β and TNF-α in LPS primed PBMCS (Peripheral blood mononuclear cells) obtained from ACS patients, thus indicating that it rapidly mitigates NLRP3 associated severity of inflammation in ACS patients.[5] Ticagrelor therefore could prove beneficial in patients of DM with other CVS co-morbid states. However, there is scarcity of information regarding the effect of Ticagrelor on the glycemic parameters in DM perse. Therefore, the present study was planned to investigate the effect of commonly used anti-platelet drug Ticagrelor in an animal model of T2DM.

D. Selection of rodent model

Diabetes is a major public health emergency of the 21st century. Globally, 415 million people were affected by diabetes in 2015; and this prevalence is projected to rise 642 million by the year 2040.

Researchers use a variety of animal models and scientific methodologies to establish diabetic features in rodents. In general non-mammalian models have a short life cycle, cheap maintenance costs, and a wide range of gene-editing techniques. However translational relevance is restricted because of their different anatomy and physiology. Nevertheless, large animals like dogs, pigs, and non-human primates share much of our physiology. Nonetheless, these species have significant maintenance expenses and extended lifespans. Rats and mice are suitable models for balancing throughput and translational physiology. In particular, small rodents, and particularly the laboratory mouse, provide an excellent compromise between throughput and translational physiology. Rodents are preferred over non-mammalian models due to their near physiology to humans, small size, short life cycle, high fertility, and simplicity of modifying their genome.[84]

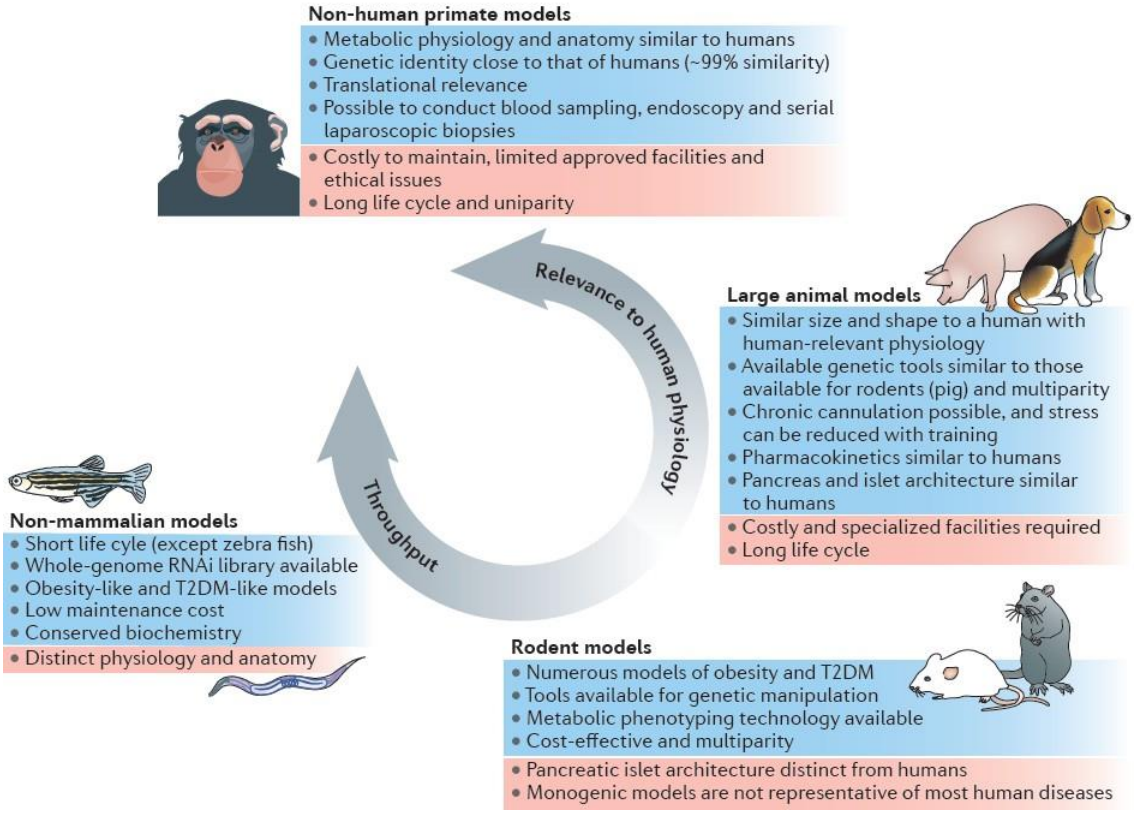


Figure 13: Key advantages and disadvantages of different classes of animal models used in Diabetes research[84]

The experimental animals used to study Diabetes can be classified into three distinct types.

Table 7: The experimental animals used to study Diabetes[85-89]

1. Chemically induced Models	• Adult STZ/Alloxan Models • Neonatal STZ/Alloxan Models • High Fat-diet Fed Models • Fat-Fed STZ Models • Nicotinamide STZ Models • Fructose Fed Models
2. Experimentally induced models	• Partial Pancreatectomised Models • Intrauterine Growth Retardation Models
3. Genetically derived diabetic animals	• $Lep^{ob/ob}$ mouse • db/db mice • Lepp mice • Zucker Fatty rats • Zucker Diabetic Fatty rats • OLETF rat • NZO mice • TallyHo/Jng mice • Nile grass rat • GK rat • hIAPP mice • AKITA mice
4. Miscellaneous	• Steroid hormone induced • Drug induced

Table 8: Characteristics of T2DM animal models[85-89]

Model	Features
Adult alloxan model	• Alloxan kills pancreatic beta cells, it has recognized side effects such as liver and kidney damage • Alloxan given i.p at 40-200 mg/kg BW in rats
Adult STZ model	• STZ is naturally occurring antibiotic produced by *streptomyces achromogens* bacterium. • It acts as a strong alkylating agent, disrupting glucose transport and glucokinase activity while also causing DNA strands to breakdown. • STZ given i.p. at different dose (35-65 mg/kg BW) in rats
Neonatal STZ model	• Since mid-1970's, these models have been used • STZ administered i.p. to new born rats after two days of birth. Until the 4th week , FBG levels are mild hyperglycemic. When rats reach adulthood, it results in adult-onset T2DM.
High fat diet fed model	• This approach was used in 1980 • The principle of this model was that, because of obesity is a major contributor to the development of T2DM • C57BL/6 J mice used for this model • HFD (40-60 % of total calories) for atleast 10 weeks necessary to produce the primary pathology of T2DM
High Fat-Fed STZ model	• This model was originally developed in 1947 • Animals fed with High fat diet to establish insulin resistance, followed by a low dose of STZ injection to produce partial pancreatic beta cell failure. • The advantage of this model is that it replicates natural pathophysiology of T2DM.

Nicotinamide STZ model	• The rationale behind this model is that STZ causes DNA damage while nicotinamide protects pancreatic beta cells from damage caused by STZ. • The initial model used 230 mg/kg BW nicotinamide (i.p) 15 minutes before administering 65 mg/kg BW STZ (i.p) in to 3 month old wistar rats
Partial pancreatectomized model	• This model inducemild to moderate hyperglycemia after 4 days of surgery that can lasts up to 6 weeks • It mimics T2DM by having decreased pancreatic beta cell mass
Intrauterine growth retardation (IUGR) model	• IUGR has been linked to onset of disease later in life, including obesity, hypertension and T2DM • In newborns, IUGR causes significant loses in pancreatic beta cell mass, which does not recover in adulthood and results in impaired glucose tolerance and development of T2DM. • This is caused by bilateral uterine artery ligation, which results in partial reduction in blood flow to the fetus resulting in IUGR.
Zucker fatty (ZF)/ Zucker diabetic fatty rats (ZDF)	• ZF rats were created in 1961 as a result of cross between Merck M and Sherman rats • They have faulty leptin receptor which resulted in hyperglycemia and formation of obese rats at 4 weeks of age. • The ZDF strain was discovered after mutation in ZF strain which are less obese but have increased insulin resistance
Otsuka long-Evans Tokushima Fat (OLETF) rats	• This rat was created after 18 weeks of selective breeding at Tokushima Research Institute from naturally diabetic rat discovered in an outbred colony of Long Evans rats in 1984
Nile Grass rat	• The Nile Grass rat *(Arvicanthis Niloticus)* has been recommended as a model for metabolic syndrome.

	• When fed a standard chow diet in captivity, these rats develop obesity, dyslipidemia and hyperglycemia.
Goto-Kakizaki (GK) rats	• A Japanese group developed GK rats by repeatedly breeding wistar rats with the lowest glucose tolerance. • As a result, slim model of T2DM with glucose intolerance and poor glucose induced insulin production was created.
Corticosteroid induced	• When the adrenal cortex in rodent is stimulated by corticotrophin, it secretes large levels of steroids and ultimately results in steroid induced diabetes. • Dexamethasone and Prednisolone are the most prevalent glucocorticoid that cause steroid diabetes. • They enhance gluconeogenesis and inhibit insulin action which results in increased hepatic glucose production and insulin resistance.
Atypical antipsychotic induced diabetic model	• When it is given for 60 days, atypical antipsychotic such as olanzapine (10 mg/kg i.p) induce significant increase in blood glucose levels, LDL and cholesterol in rats

Table 9: Advantages and disadvantages of various experimental models for Diabetes[86]

Animal model	Advantages	Disadvantages
Spontaneous diabetic animals	• Involves genetic factors • Animals exhibit similar characteristics of human T2DM • Minimum variability of results and hence small sample size is enough	• Limited availability • Expensive • High mortality due to ketosis and requires insulin for survival
Diet/ Nutrition induced	• Developed diabetes with obesity which is similar to some humans • Chemical toxicity on organs can be avoided	• Long period of dietary required • Frank hyperglycemia does not develop and not suitable for screening
Chemically induced	• Selective loss of β- cells • Ketosis and mortality are low • Cheaper, easier to induce and maintain • Animals live long without exogenous insulin	• High variability of results • Hyperglycemia primarily due to insulin deficiency after than insulin resistance
Surgically induced	• Chemical toxicity on organs can be avoided • Resembles human diabetes in terms of reduced beta cell mass	• Cumbersome technical procedure and post operative care • Higher mortality • Chances of occurrence of other digestive problems
Genetically modified (transgenic/ knockout)	• Effect of single gene mutation can be studied	• Highly sophisticated and expensive

Type 2 Diabetes in rats using high fat diet and Streptozotocin

The current HFD/STZ rat model was initially reported by Reed et al. Rats with Type 2 Diabetes were bred to mimic the progression from prediabetes and insulin resistance to full-blown Diabetes in a way that mimics the natural pathology of diabetes. Reed et al. fed a diet containing 40% kcal of fat to seven-week-old Sprague–Dawley rats for two weeks.[110] Then the animals were fasted overnight and administered a single dose of STZ (50 mg/kg). Subsequently, eligible rats were given Metformin three days after receiving the STZ treatment, and the response was assessed. [87] Later, a comparable model utilizing a modest dose of STZ (35 mg/kg) was developed. [88] This was altered by Zhang et al. who employed a multiple low dose regimen of STZ instead of a single dose.[89] Numerous variants of HFD/STZ rats have been published since. A high-fat diet in rats induces obesity, insulin resistance, and/or glucose intolerance which is a natural pathology in humans as well. Both the duration and composition of the special diet affect the weight gain and fat distribution in rats. The duration is highly variable ranging from a couple of weeks to more than three months. The diets used also vary in both sources of nutrients and composition. Some studies have used a high carbohydrate diet, but the most typical method is to use a diet rich in fat and low in sugar.[90,111]

β-cell failure is a distinguishing feature of T1DM; nevertheless, it is also the ultimate event in the progression of T2DM, which occurs after the appearance of insulin resistance. In both T1DM as well as T2DM animal models, STZ has been employed as a β-cell toxin. Evidence shows that at the time of diagnosis of type 1 Diabetes around 60 to 80 percent of fully functioning β-cell mass is destroyed.[90] In contrast the percentage reduction of β-cell in patients with less than five years of T2DM is around 24%.[91] For rat models, the amount of STZ will be the most important factor in determining how many β -cells are destroyed. Though there is some debate over the optimal STZ dose, most researchers agree that a single low dose or multiple low doses of STZ in high fat-fed rats is a good model for Type 2 Diabetes.[92]

Basic properties of Streptozotocin

Streptozotocin (2-deoxy-2-(3-(methyl-3-nitrosoureido)-D-glucopyranose) is a fungal antibiotic derived from *Streptomycetes achromogenes.* Streptozotocin represents a nitrosourea analog in which the *N*-methyl-*N*-nitrosourea moiety is linked to the carbon-2 of a hexose. Unlike other nitrosoureas, STZ is hydrophilic due to the hexose substitution. Courtesy of glucose-like structure STZ similarly enters beta cells as glucose.[93]

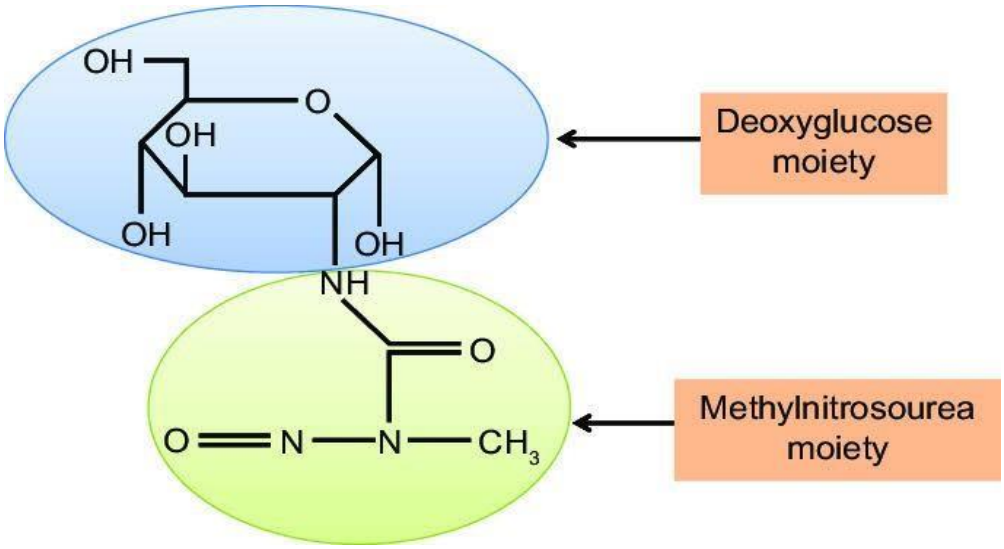


Figure 14: Chemical structure of Streptozotocin[93]

The mechanism of Streptozotocin-induced Diabetes

Streptozotocin is a hydrophilic molecule that uses GLUT2 transporters to enter the cells. It causes DNA alkylation and over activation of poly-ADP ribose polymerase (PARP), resulting in NAD+ depletion, reduced cellular ATP and compromising insulin.[93–96]

After the administration of STZ diabetes develops through at least three different mechanisms and the result of all these mechanisms is DNA destruction.

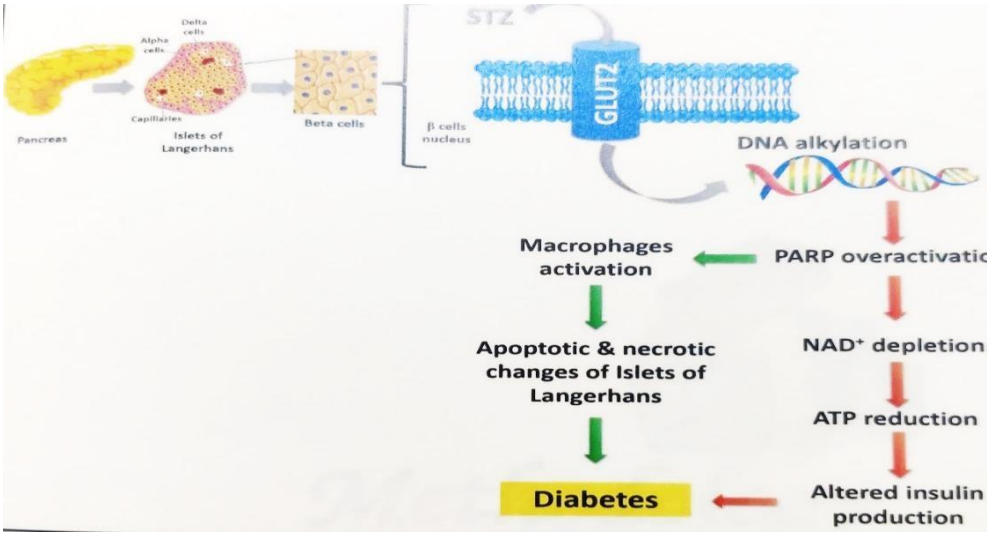


Figure 15: Mechanism of action of STZ induced Diabetes Mellitus[97]

Table 10: Summary of Mechanism of action of STZ action[93–96]

Mechanism	Comments
DNA Alkylation	• Most important and most likely mechanism of action [84,85] • The first step is the transfer of methyl group from STZ to the beta cell DNA molecule, which is followed by cascade of events resulting in fragmentation and destruction of DNA • This further activates PARP-1 and results in diminution of NAD+ and ATP stores [83] • The reduction in ATP of beta cells results in necrosis and NAD+ depletion results in inhibition of insulin synthesis and secretion[83]
Nitric Oxide (NO) release	• Considered mostly as an alternative mechanism • NO is released as a result of STZ metabolism, independent of NO synthase enzyme [86]
ROS generation	• Superoxide and hydroxide radicals are the main ROS generated and are produced during the hypoxanthine metabolism

After 48 hours of STZ injection, a state of permanent hyperglycemia develops in the rats and is considered to be the beginning of the disease. In the first 48 hours, Insulin and glucose levels undergo significant transformations. After an hour of STZ injection, there is a significant rise in glucose level, which is followed by a drop in glycemic levels in the next 4-8 hours. This hypoglycemia can persist for several hours and can prove fatal to the animals and is considered to be the most crucial moment during the process of induction. The final stage of this triphasic response is the establishment of persistent hyperglycemia at approximately 72 hours post-injection. Hence this is considered as the best time point for measuring the blood glucose level to confirm the diagnosis of diabetes.

METHODOLOGY

The present study was conducted over the period of one year, from March 2021 to February 2022.

Study design:

This was an experimental study involving adult healthy male Wistar rats. The total sample size was 38. Animals were divided into 5 groups with 8 animals in each group except for the normal control group which had 6 rats. The number of rats in the diabetic groups was higher to compensate for the expected mortality

Ethical committee approval:

The study was approved by the IAEC (Institutional Animal Ethics Committee) (Annexure-I). The study was conducted as per the guidelines of CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals), New Delhi.

Experimental Animals:

3–4-month-old adult healthy male Wistar rats with a mean weight of 180 ± 20g were procured from the central animal house of J.N. Medical College, KAHER, Belagavi. Animals were housed in polypropylene cages with stainless steel top grills having facilities for providing food and water. The rats had free access to water and food ad libitum. The food was administered in the form of pellets. Paddy husk was used as the bedding in the cages. The animals were acclimatized to a 12:12 hour light-dark cycle for 10 days, prior to the day of experimentation.

Study drug and kits:

- Metformin, Ticagrelor and Thiopentone sodium were purchased from the hospital pharmacy
- Streptozotocin (Cayman Chemical Company, catalog no. 13104) was procured from Everon Lifesciences, New Delhi.
- High-fat diet was purchased from VRK Nutritional Solutions, Pune, India
- Gluco One Glucometer was purchased from the hospital pharmacy.
- Rat IL-6 GENLISATM ELISA (Cat. No. KLR0135), Rat TNF-alpha GENLISA (Cat. No. KB3145), and Rat Interleukin 1β, IL-1β GENLISA (Cat. No. KLR0119) were purchased from Krishgen Biosystems.

Study Methodology

- The model of HFD- STZ induced T2DM was previously standardized in the department by a pilot study.
- The rats of the required weight range were kept fasting overnight in the cages, a day prior to initiation of the study.
- On day 1, blood (2 ml) was collected from all rats from the tail veins using a 30G insulin needle and syringe. The sample was used for baseline estimation of fasting blood glucose (FBG), HbA1C. These were labelled as baseline values.
- 6 rats were randomly selected and assigned to the normal control group. The rest of the 32 rats were fed a high-fat diet for 14 days. The normal control rats were fed with the standard chow pellet (Amrut Brand).

Table 11: Composition of High Fat Diet [HFD][108]

Ingredients	**Diet (g/kg)**
Powdered Normal Pellet diet (NPD)	365
Lard	300
Casein	280
Soya oil	50
Vitamin & Mineral Mix	50
Starch	260
Yeast Powder	01
Cholesterol	10

- On day 15, blood glucose levels were measured for all rats using tail veins with a 30G insulin needle.
- On day 15 the experimental induction of Diabetes was carried out in 32 rats as described below.

Experimental induction of Diabetes:

T2DM was induced in rats as per the existing literature and based on pilot study.[98,99]

Preparation of STZ[100]

40 mg STZ was weighed in a glass beaker and the beaker was covered with aluminium foil. Fresh citrate buffer of 0.05M at a pH of 4.5 was prepared immediately prior to injection. The 0.05M citrate buffer was prepared by mixing 0.05M sodium citrate & 0.05M citric acid in a ratio of 2:3 and the pH was adjusted to 4.5). Immediately prior to injection, STZ was dissolved in the sodium citrate buffer to a final concentration of 40 mg in 7 ml.

Administration of STZ

The STZ solution was prepared immediately before injection and administered within 5 min of dissolution. Using a 1ml syringe and 23G needle, STZ was injected intraperitoneally (i.p.) into the rats belonging to various experimental groups at 30 mg/kg (5.25 ml/kg). An equal volume of citrate buffer was injected intraperitoneally into the normal control rats. After injection, STZ-treated rats were given 5% glucose instead of water for 24 hours to minimize hypoglycemic shock-related mortality.

Confirmation of the Diabetes

After 72 hours of STZ administration, the fasting blood glucose levels of all rats were measured using a glucometer from tail vein blood samples. Diabetic rats were defined as those with fasting blood glucose levels of more than 200 mg/dL and were included in further study.[99,101]. Out of a total of 32 rats, one died in the first 48 hours after receiving STZ and one rat's blood glucose level was less than 200 mg/dl. and they were excluded from study. The remaining 30 rats were randomized into various treatment groups.

Randomization:

As mentioned earlier, 6 rats were randomly selected and assigned to the normal control group before starting the dietary intervention. Following the successful induction of Diabetes, 30 surviving rats were randomly assigned to one of four groups. (Diabetic control = 8 rats, Metformin = 7 rats, Ticagrelor(16.2 mg/kg) = 8 rats, Ticagrelor(35 mg/kg) = 7 rats). Blinding was introduced to eliminate observer bias. It was achieved by coding

and masking of all the drugs used in the experiment by the guide before the start of the experiment.

Treatment Schedule

The confirmation day of Diabetes was considered as day one of Diabetes. The rats were divided into various groups and received treatment according to the following table.

Table 12: Number of rats per group with treatment schedule

Groups	**Treatment**	**Dose**
Group I: Normal Control (NC) (n=6)	Vehicle only	1 ml
Group II: Diabetic Control (DC) (n=8)	Vehicle only	1 ml
Group III: Diabetic Rats + Metformin (MF) (n=7)	Metformin(standard)	180mg/kg (around 1 ml)
Group IV: Diabetic Rats + Ticagrelor -16.2 mg/kg (TCG-16.2) (n=8)	Ticagrelor	16.2 mg/kg (around 1ml)
Group V: Diabetic Rats Ticagrelor -35 mg/kg (TCG-35) (n=7)	Ticagrelor	35 mg/kg (around 1 ml)

The doses of the drugs used have been calculated using the multiplication factor proposed by Paget and Barnes.[115]
Accordingly,
1) 2 gm/day maximum human dose of Metformin is equivalent to 180 mg/kg of rat.
2) 180 mg/day maximum human dose of Ticagrelor is equivalent to 16.2 mg/kg of rat.
3) 35 mg/kg/day dose of Ticagrelor in rats is the minimum dose reported to exhibit inhibitory effect on NLRP3 inflammasome.[5]

All the drugs were administered orally as a single daily dose for a period of 6 weeks.

Metformin was dissolved in appropriate quantity of water. Ticagrelor was dissolved in water after mixing and triturating with ethanol. After calculating, appropriate quantities of these drug solutions were fed to respective rats by oral gavage.

Outcome measures:

The study variables, their time and method of measurement is depicted in the following table.

Table 13: Details of study parameters

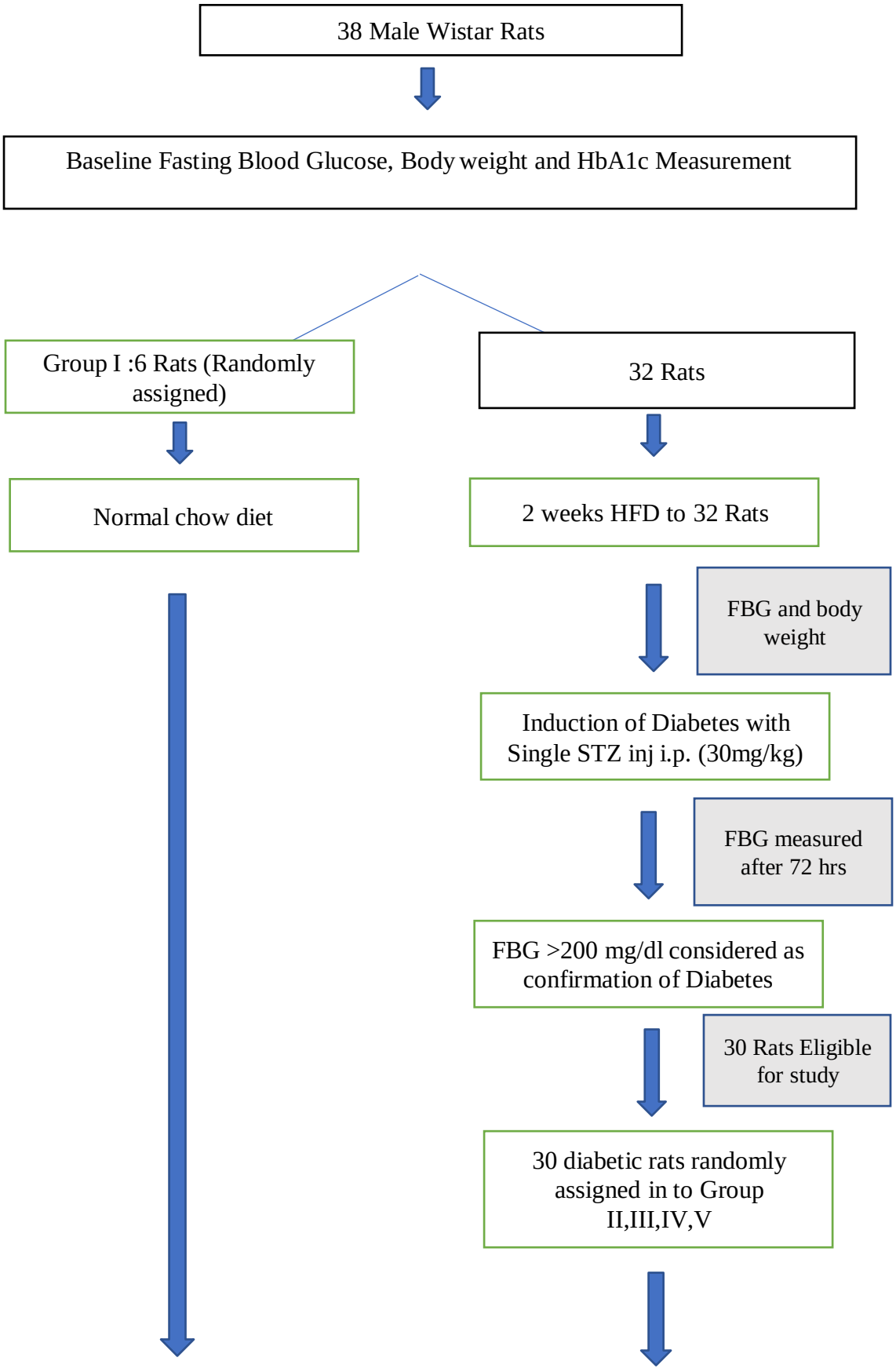
Parameter	Timing of measurement	Method
1. Body weight	• Baseline • After 14 days of HFD • 21 days after DM induction, • End of the study	Weighing balance
2. Fasting blood glucose	• Baseline • After 14 days of HFD • 72-hours after STZ administration • 21 days after DM induction • End of the study	Rat tail vein samples were tested after overnight fasting using a glucometer
3. HbA1C	• Baseline • End of the study	Fasting samples from rat tail vein were tested at a professional laboratory
4. IL -1β	• End of the study	Blood sample collected from cardiac puncture was analyzed using ELISA kits
5. IL- 6		
6. TNF- α		

Euthanasia

Animals were sacrificed using an overdose of anaesthesia as per the CPCSEA guidelines. On day 43 of the study, thiopentone sodium at a dose of 120 mg/kg was given as an intraperitoneal injection and the animals were sacrificed.[114]

Statistical analysis

All the results were expressed as Mean ± Standard Error of Mean (SEM). The data was analysed using SPSS. Level of significance was set at $p < 0.05$. The study variables were analysed using one-way ANOVA followed by post hoc Dunnett's and Bonferroni's tests wherever appropriate. Paired data were analysed using Paired-t test.


38 Male Wistar Rats
Baseline Fasting Blood Glucose, Body weight and HbA1c Measurement
Group I :6 Rats (Randomly assigned)
32 Rats
Normal chow diet
2 weeks HFD to 32 Rats
FBG and body weight
Induction of Diabetes with Single STZ inj i.p. (30mg/kg)
FBG measured after 72 hrs
FBG >200 mg/dl considered as confirmation of Diabetes
30 Rats Eligible for study
30 diabetic rats randomly assigned in to Group II,III,IV,V

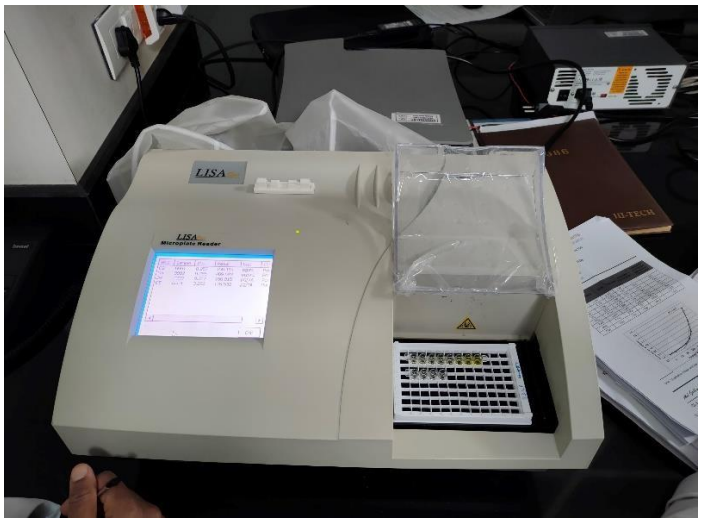
Groups	Group I (Normal control) NC	Group II (Diabetic control) DC	Group III (Diabetic Rats + Metformin) MF	Group IV (Diabetic Rats + Ticagrelor) TCG-16.2	Group V (Diabetic Rats + Ticagrelor) TCG-35
No.of Rats	6	8	7	8	7
Treatment for 36 days	Vehicle 1 ml	Vehicle 1 ml	Metformin 180mg/kg	Ticagrelor 16.2 mg/kg	Ticagrelor 35 mg/kg

Bodyweight and FBG measured on day 21 and at the end of study

Day 43	Cardiac puncture to obtain blood from all groups to estimate FBG, HbA1C, IL -1β, IL -6 and TNF- α, followed by euthanasia with overdose of anesthesia

Figure 16: Schematic representation of the study design

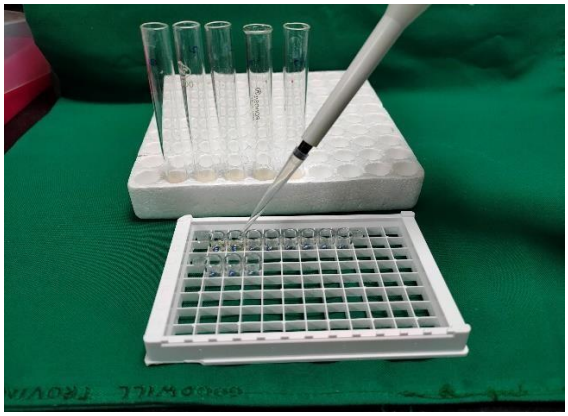
a) Rat TNF-alpha GENLISA (Cat. No. KB3145, Krishgen biosystems)



b) Elisa microplate reader machine

Figure 17: Sample images of test kits, instruments and selected procedures

c) Creating serial dilutions of the stock solution



d) Transferring diluted stock to microtiter coated plate wells

e) Incubating microtiter plates after adding conjugation antibody

Figure 17: Sample images of test kits, instruments and selected procedures

RESULTS

Data generated in the study were compiled into an excel sheet and were analyzed using appropriate statistical tests. The results of the study are described using graphs and tables. Data is expressed as Mean ± SEM.

1. Body weight

Body weight was measured at baseline, following 14 days of the high-fat diet, 21 days after treatment, and at the end of the study.

The mean body weights of all the groups were comparable at baseline. A one-way ANOVA revealed that there was no significant difference between various groups at baseline. After 14 days of the high-fat diet, the mean body weights (in grams) of NC, DC, MF, TCG-16.2 and TCG-35 groups were 206.67 ± 1.82, 211.50 ± 1.31, 214.29 ± 1.86, 217.14 ± 1.58 and 211.0 ± 2.57 respectively. One way ANOVA followed by post-hoc Dunnett test showed that the body weight in DC, MF, TCG-16.2 and TCG-35 groups were higher compared to the normal control group but not statistically significant. However among these groups post-hoc Bonferroni's analysis did not detect any statistically significant difference.

After 21 days of treatment, the mean body weights (in grams) of NC, DC, MF, TCG-16.2 and TCG-35 groups were 247.67 ± 3.59, 204.50 ± 6.13, 237.86± 2.80, 234.57 ± 2.33 and 252.33 ± 4.67 respectively. A one-way ANOVA and the post-hoc Dunnett test revealed a statistically significant weight reduction in the DC group in comparison with all other groups ($p < 0.001$). According to the post-hoc Bonferroni's analysis, there was no statistical difference among the treatment groups.

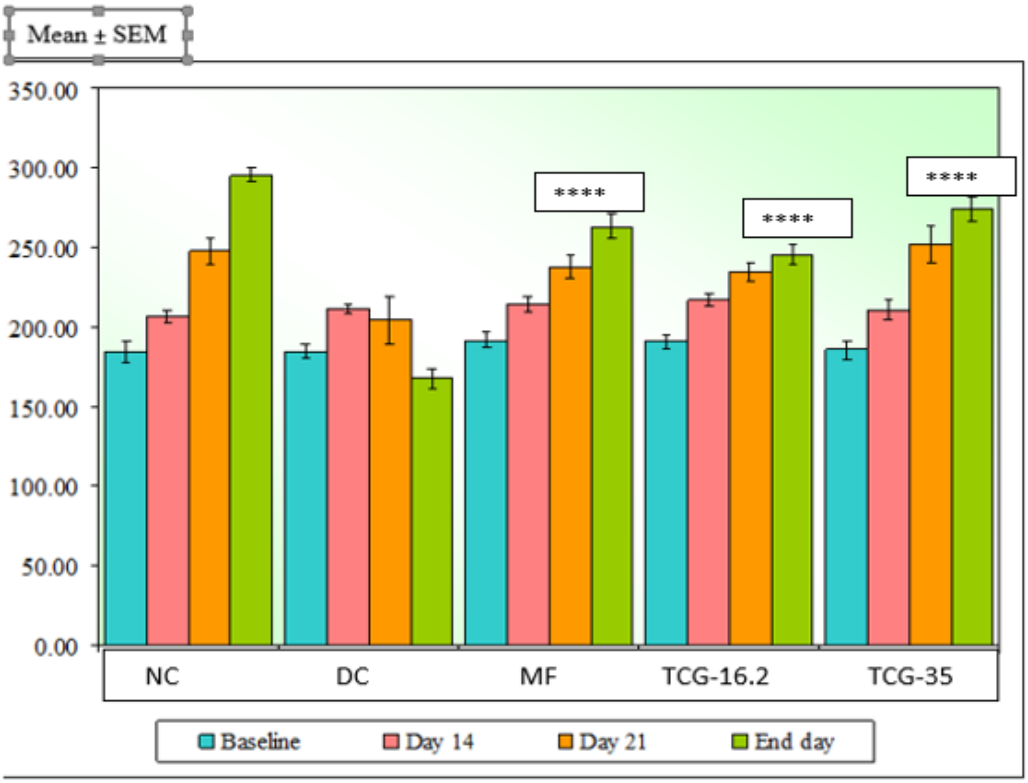
At the end of the study mean body weights (in grams) of NC, DC, MF, TCG-16.2 and TCG-35 groups were 295.67 ± 1.74, 167.33 ± 2.67, 263.43 ± 2.95, 245.57 ± 2.33 and 274.17 ± 3.09 respectively. According to the post-hoc Dunnett test, there was astatistically significant weight reduction in the DC group in comparison with all other groups. ($p < 0.0001$). Post-hoc Bonferroni's analysis did not reveal statistical difference among the treatment groups.

Table 14: Effect of various treatments on body weight at various time intervals

Day of study	Body weight in grams (Mean ±SEM)					ANOVA Result	
	Normal Control (NC)	Diabetic Control (DC)	Metformin (MF)	Ticagrelor (16.2) TCG-16.2	Ticagrelor (35) TCG -35	F	P value
Baseline	184.5 ± 2.68	184.83 ± 1.72	191.86 ± 1.81	190.86 ± 1.64	185.83 ± 2.46	2.7276	0.06
After 14 Days of HFD	206.67 ± 1.82	211.50± 1.31 ns	214.29 ± 1.86 ns	217.14 ± 1.58 ns	211.0 ± 2.57 ns	4.4627	0.0067
After 21 Days of induction	247.67 ± 3.59	204.50 ± 6.13	237.86 ± 2.80 ****	234.57 ± 2.03 ****	252.33 ± 4.67 ****	21.227	<0.0001
End of Study	295.67 ± 1.74	167.33 ± 2.67	263.43 ± 2.95 ****	245.57 ± 2.33 ****	274.17 ± 3.09 ****	332.75	<0.0001

Values are expressed as Mean ± SEM, n=6, *p < 0.05, **p< 0.01, ****p< 0.0001indicates the significant difference between diabetic control and treatment groups by using ANOVA followed by Dunnett's multiple comparison test.

Graph 1: Effect of various treatments on body weight at various time intervals



Values are expressed as Mean ± SEM, n=6, ****p< 0.0001 indicates the significant difference between diabetic control and treatment groups by using ANOVA followed by Dunnett's multiple comparison test. NC – Normal control, DC- Diabetic control, MF- Metformin group, TCG-16.2- Ticagrelor 16.2 mg/kg BW group and TCG-35 – Ticagrelor 35 mg/kg BW group.

2. Fasting Blood Glucose (FBS) at various intervals

Fasting blood glucose was measured at baseline, following 14 days of the high-fat diet, 72 hours after Streptozotocin injection, 21 days after treatment, and at the end of the study.

The mean FBS of all the groups were comparable at baseline. A one-way ANOVA revealed that there was no significant difference between various groups at baseline. One-way ANOVA followed by Dunnett's test performed on FBS values after 14 days of HFD demonstrated a significant increase in FBS in all groups compared to the NC group ($p< 0.001$). Following 72 hours of STZ administration the mean FBS values (mg/dL) of DC, MF, TCG-16.2, and TCG-35 groups were 307.33 ± 13.80, 327.71 ± 14.31, 313.71± 16.57, and 327.17 ± 25.34 respectively. The FBS values after 72 hours of STZ showed significant increased as compared to respective baseline values suggesting hyperglycemia and confirming Diabetes Mellitus. However, there was no statistical difference among the groups.

After 21 days of treatment the mean FBS values (mg/dL) of DC, MF, TCG-16.2 and TCG-35 group were 104.00 ± 1.69, 372.33 ± 14.67, 299.71 ± 10.88, 314.29 ± 14.15 and 308.00± 28.38 respectively. There was a significant reduction in the FBS value of MF group ($p <0.05$) in comparison with the diabetic control group according to the post-hoc Dunnett's analysis. Although TCG-16.2 and TCG-35 groups also showed the reduction in FBS level as compared to DC group but reduction was not statistically significant.

At the end of the study, the mean FBS values (mg/dL) of NC, DC, MF, TCG-16.2, and TCG-35 group were 103.50 ± 2.45, 390.17 ± 8.88, 256.57 ± 9.30, 283.71 ± 6.36, and 262.83 ± 12.52 respectively. According to the post-hoc Dunnett's test, the FBS value of the DC group was significantly high in comparison with all other groups ($p< 0.001$). Bonferroni's multiple comparison test indicated that the FBS of the MF group and TCG-35 was significantly lower as compared to the TCG-16.2 group ($p<0.05$). The FBS of the MF group and TCG-35 group were comparable.

Within each group, a Student's Paired t-test was used to compare FBS levels before and after therapy/intervention. After 21 days of treatment, the TCG-35 group demonstrated a statistically significant reduction in FBS value when compared to the FBS value after induction of diabetes ($p<0.05$). FBS level compared after induction and at the end of study and there was statistically significant reduction in MF, TCG-16.2 and TCG-35 group ($p <$

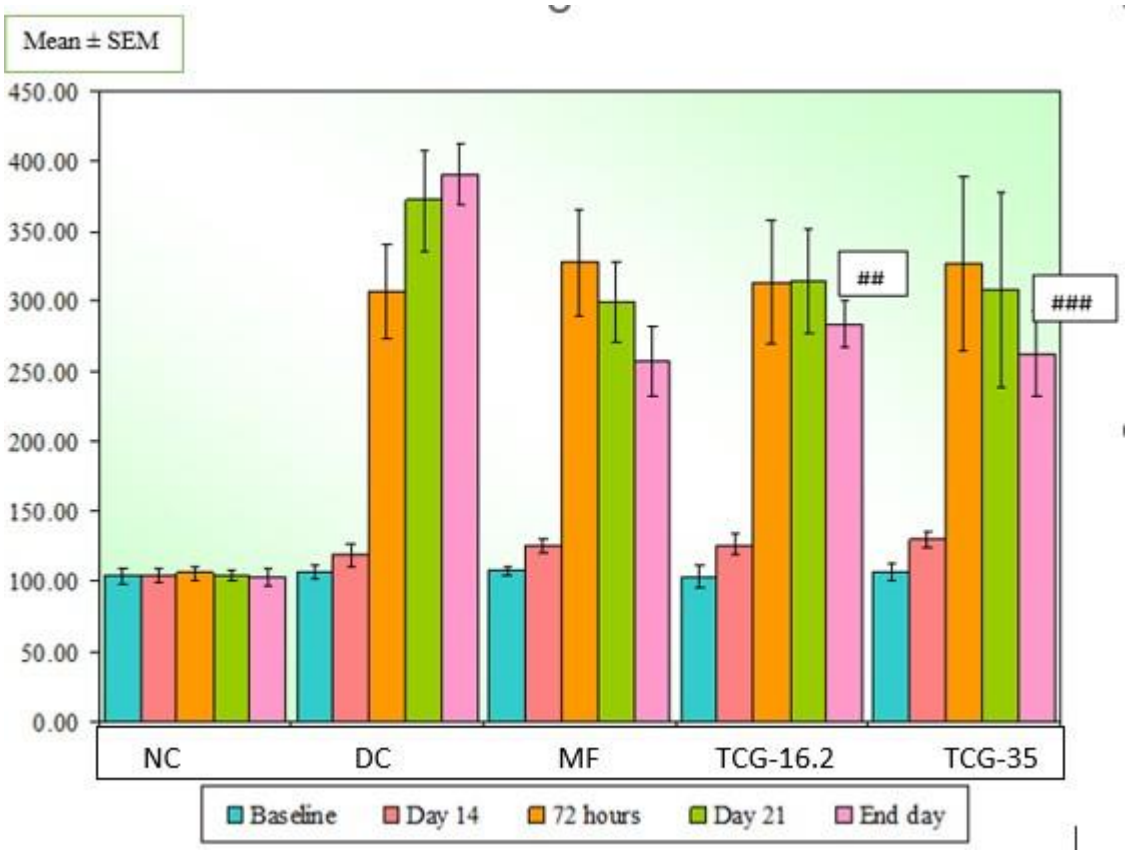
0.0001). At 21 days after treatment and the end of therapy, the FBS values in the TCG-16.2 ($p < 0.01$) and TCG-35 ($p < 0.001$) groups were also significantly lower than after induction of diabetes.

Table 15: Effect of various treatments on Fasting Blood Glucose at various time intervals

Day of study	Fasting Blood Glucose (mg/dL) (Mean ±SEM)					ANOVA Result	
	Normal Control (NC)	Diabetic Control (DC)	Metformin (MF)	Ticagrelor (16.2) TCG-16.2	Ticagrelor (35) TCG -35	F	P value
Baseline	104.00 ± 2.25	107.17 ± 2.01	107.57 ± 1.48	103.43 ± 3.14	106.67 ± 2.67	0.6562	0.6276 ns
After 14 Days of HFD	104.17 ± 1.97	119.00 ± 3.21 ##	125.43 ± 1.60 ####	126.14 ± 2.78 ####	129.50 ± 2.49 ####	16.0162	<0.0001
After 72 Hours of STZ	--------	307.33 ± 13.80	327.71 ± 14.31	313.71 ± 16.57	327.17 ± 25.34	33.3089	0.9899 ns
After 21 Days of induction	104.00 ± 1.69	372.33 ± 14.67	299.71±10.88 *	314.29 ±14.15	308.00 ±28.38	38.6738	<0.0001
End of Study	103.50 ±2.45	390.17 ± 8.88	256.57 ±9.30 ****	283.71 ±6.36 ****	262.83 ±12.52 ****	135.5981	<0.0001

Values are expressed as Mean ± SEM, n=6, #### $p< 0.0001$indicates the significant difference between Normal control and other groups by using ANOVA followed by Bonferroni's multiple comparison test. ****$p< 0.0001$indicates the significant difference between diabetic control and treatment groups by using ANOVA followed by Dunnett's multiple comparison test.

Graph 2: Effect of various treatments on Fasting Blood Glucose at various time intervals



Values are expressed as Mean ± SEM, n=6, ## $p<0.01$ and ### $p<0.001$ indicates significant difference between the TCG-16.2 group and TCG-35 group respectively at 21 days of treatment and at end of the study vs after Diabetes induction by using Paired t test.

3. HbA1C

HbA1C was measured at baseline and at the end of the study.

The mean HbA1C (%) of all the groups was comparable at baseline. At Baseline mean HbA1C values of NC, DC, MF, TCG-16.2, and TCG-35 groups were 4.68 ± 0.35, 4.36 ± 0.28, 4.35 ± 0.20, 4.32 ± 0.24, and 4.57 ± 0.21 respectively. The one-way ANOVA test showed no significant difference among the groups.

At the end of the study the mean HbA1C values (%) of NC, DC, MF, TCG-16.2, and TCG-35 groups were 4.67 ± 0.31, 9.81 ± 0.14, 7.20 ± 0.16, 8.61 ± 0.17, and 7.46 ± 0.15 respectively. One-way ANOVA followed by Dunnett's test revealed that the HbA1C value in the DC group was significantly high compared to NC ($p< 0.0001$), MF ($p< 0.0001$), TCG-16.2 ($p < 0.001$), and TCG-35 group ($p< 0.0001$). According to the post-hoc Bonferroni analysis, HbA1C in the MF group was significantly lower than in the TCG-16.2 group ($p< 0.001$). However, the HbA1C levels of MF and TCG-35 groups were comparable.

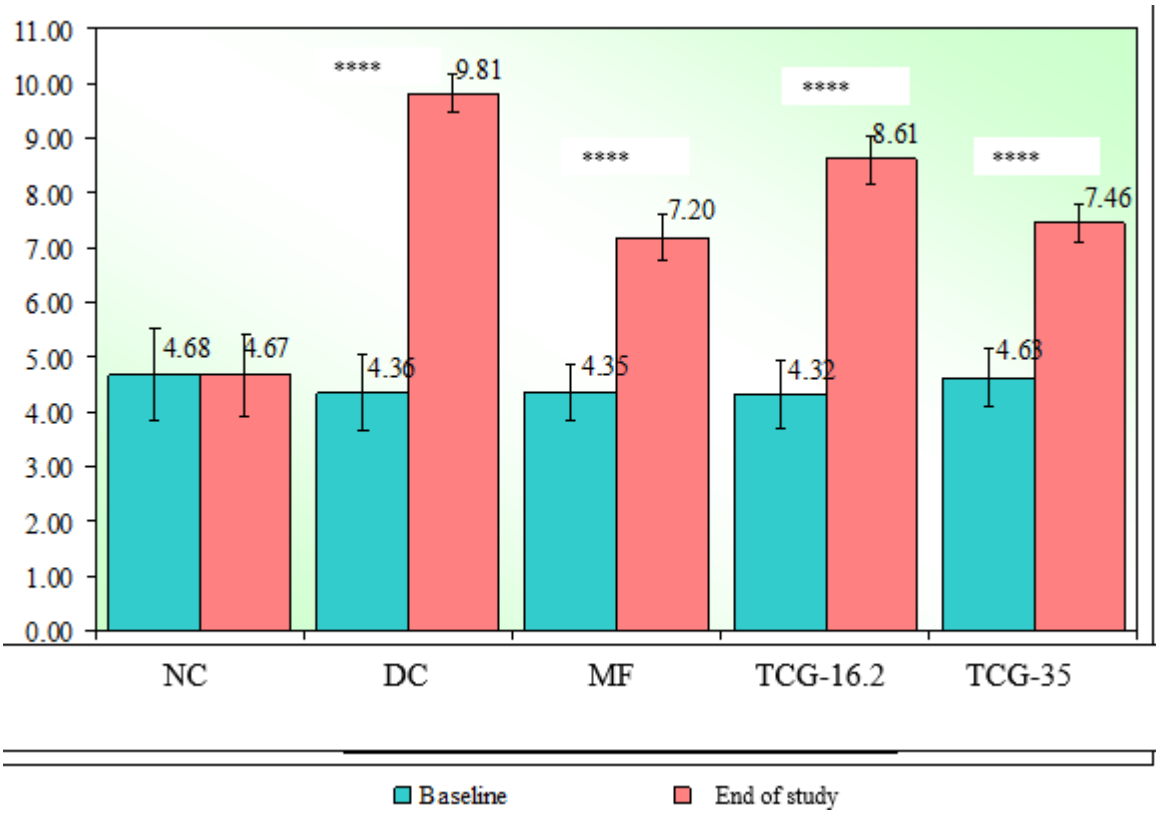
A Student's Paired t-test was performed to compare HbA1C levels before and after therapy/intervention in each group. At the end of the study, the DC group showed a statistically significant rise in HbA1C compared to baseline ($p < 0.0001$). The values in MF ($p < 0.0001$), TCG-16.2 ($p < 0.0001$) and TCG-35 group ($p < 0.0001$) at the end of the study were high compared to the corresponding values at baseline and were statistically significant.

Table 16: Effect of various treatments on HbA1C at various time intervals

Day of study	HbA1C (%) (Mean ±SEM)					ANOVA Result	
	Normal Control	Diabetic Control	Metformin	TCG-16.2	TCG-35	F	P value
Baseline	4.68 ± 0.35	4.36 ± 0.28	4.35 ± 0.20	4.32 ± 0.24	4.57 ± 0.21	0.4522	0.7699 ns
End of Study	4.67 ± 0.31	9.81 ± 0.14	7.20 ± 0.16 ****	8.61 ± 0.17 *** ##	7.46 ± 0.15 **** ns	73.56	<0.0001

Values are expressed as Mean ± SEM, n=6, *p < 0.05, ****p< 0.0001indicates the significant difference between diabetic control and treatment groups by using ANOVA followed by Dunnett's multiple comparison test. ##p< 0.001 indicates the significant difference between TCG-16.2 group vs the MF group by using Bonferroni's test.

Graph 3: Effect of various treatments on HbA1C at various time intervals



Values are expressed as Mean ± SEM, n=6. ****p < 0.0001, ***p<0.001 indicates a significant difference between HbA1C values at the end baseline using Paired t-test.

4. Inflammatory markers

Serum Il-1β, TNF- α and IL-6 levels were measured at the end of the study.

i. IL -1β

At the end of the study the mean IL-1β values of NC, DC, MF, TCG-16.2, and TCG-35 groups were 0.31 ± 0.01, 2.22 ± 0.13, 0.68 ± 0.02, 1.04 ± 0.08, and 0.68 ± 0.04 respectively. The post-hoc Dunnett's test showed that the IL-1β value of the DC group was significantly high compared to NC ($p < 0.0001$), MF ($p = 0.0001$), TCG-16.2 ($p < 0.0001$), and TCG-35 group ($p < 0.0001$). According to Bonferroni's analysis the TCG-16.2 group showed significantly higher IL-1β compared to MF ($P<0.01$) and TCG -35 ($p < 0.01$) There was no statistical difference between MF and TCG-35 group.

ii. TNF-α

At the end of the study the mean TNF-α values of NC, DC, MF, TCG-16.2, and TCG-35 groups were 29.83 ± 3.03, 93.32 ± 3.59, 66.47 ± 2.25, 73.50 ± 2.60, and 61.98 ± 3.41 respectively. According to the post-hoc Dunnett's test TNF-α in the DC group was significantly high compared to NC ($p < 0.0001$), MF ($p= 0.0001$), TCG-16.2 ($p < 0.01$) and TCG-35 group ($p < 0.0001$). Bonferroni's analysis revealed that the TNF-α level in TCG-35 group had low TNF-α as compared MF group and TCG-16.2 group though not statistically significant.

iii. IL-6

At the end of the study the mean IL-6 values of NC, DC, MF, TCG-16.2, and TCG-35 groups were 27.02 ± 1.43, 113.82 ± 1.61, 55.86 ± 2.40, 96.23 ± 1.24, and 64.53 ± 1.42 respectively. The post-hoc Dunnett's test demonstrated that the IL-6 value of the DC group was significantly high compared to NC ($p < 0.0001$), MF ($p = 0.0001$), TCG-16.2 ($p < 0.0001$), and TCG-35 group. ($p < 0.0001$). Post hoc Bonferroni's analysis revealed that the

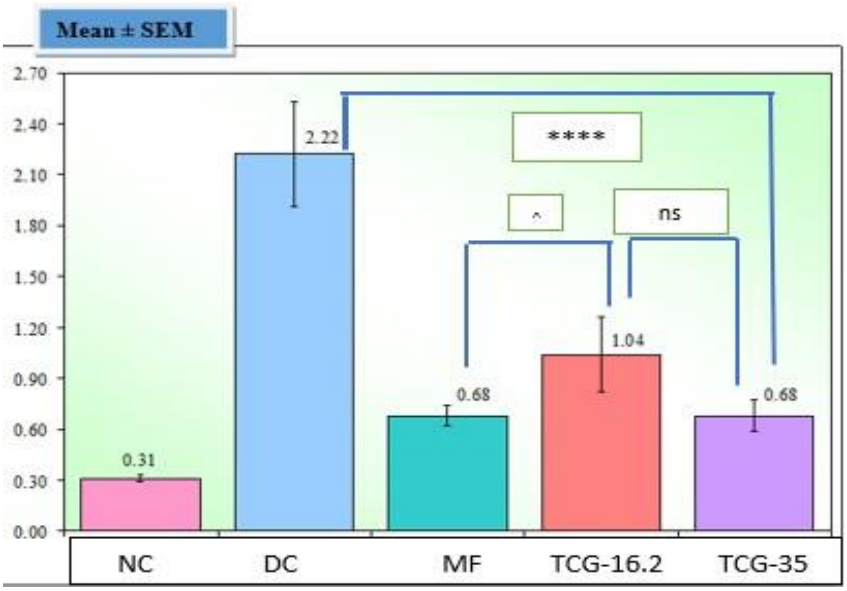
IL-6 level was significantly low in the MF group compared to TCG-16.2 ($p < 0.0001$) and TCG-35 group ($p < 0.01$). Moreover, the TCG-35 group showed a significantly lower IL-6 level compared to the TCG-16.2 group. ($p = 0.0001$)

Table 17: Effect of various treatments on serum inflammatory markers

	Normal control	Diabetic control	Metformin	Ticagrelor 16.2 mg/kg	Ticagrelor 35 mg/kg
IL-1β (pg/ml)	0.31 ± 0.01 ****	2.22 ± 0.13	0.68 ± 0.02 ****	1.04 ± 0.08 **** ^	0.68 ± 0.04 **** ns
TNF– α (pg/ml)	29.83 ± 3.03 ****	93.32 ± 3.59	66.47 ± 2.25 ****	73.50 ± 2.60 ** ns	61.98 ± 3.41 **** ns
IL-6 (pg/ml)	27.02 ± 1.43 ****	113.82 ± 1.61	55.86 ± 2.40 ****	96.23 ± 1.24 **** ^^ ####	64.53 ± 1.42 **** ^

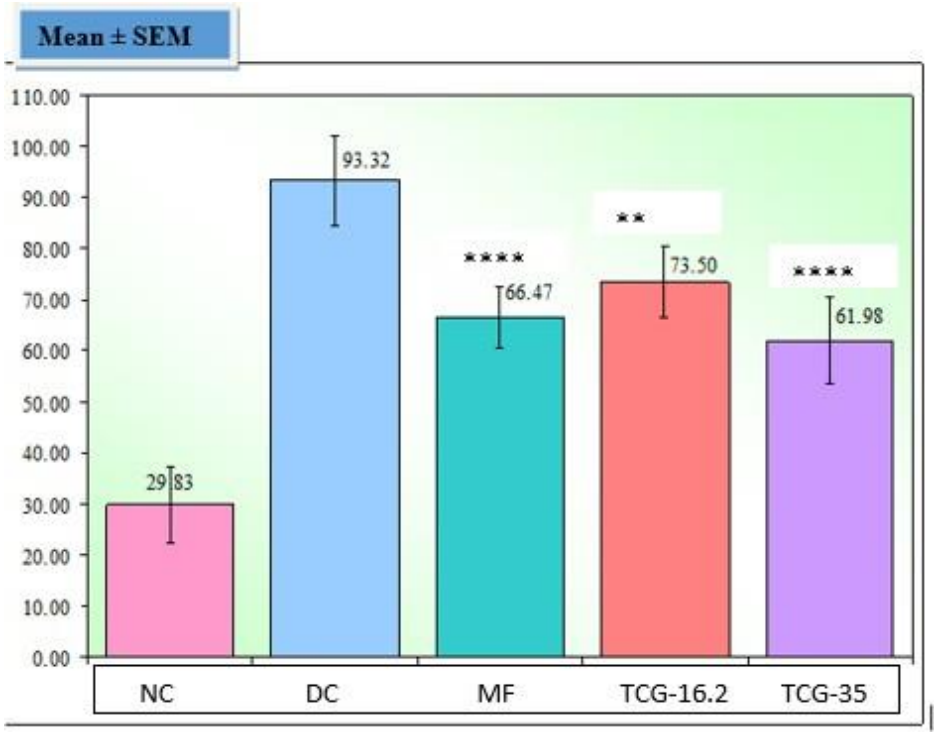
Values are expressed as Mean ± SEM, n=6, *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$ indicates the significant difference of various groups compared to diabetic control by using ANOVA followed by Dunnett's multiple comparison test. ^^$p < 0.0001$, ^$p < 0.01$ indicates the significant difference between various groups compared to the Metformin group. ####$p < 0.0001$ indicates the significant difference between TCG-16.2 group and TCG-35 group.

Graph 4: IL-1β - End of study (One-way ANOVA followed by Bonferroni's multiple comparison test)



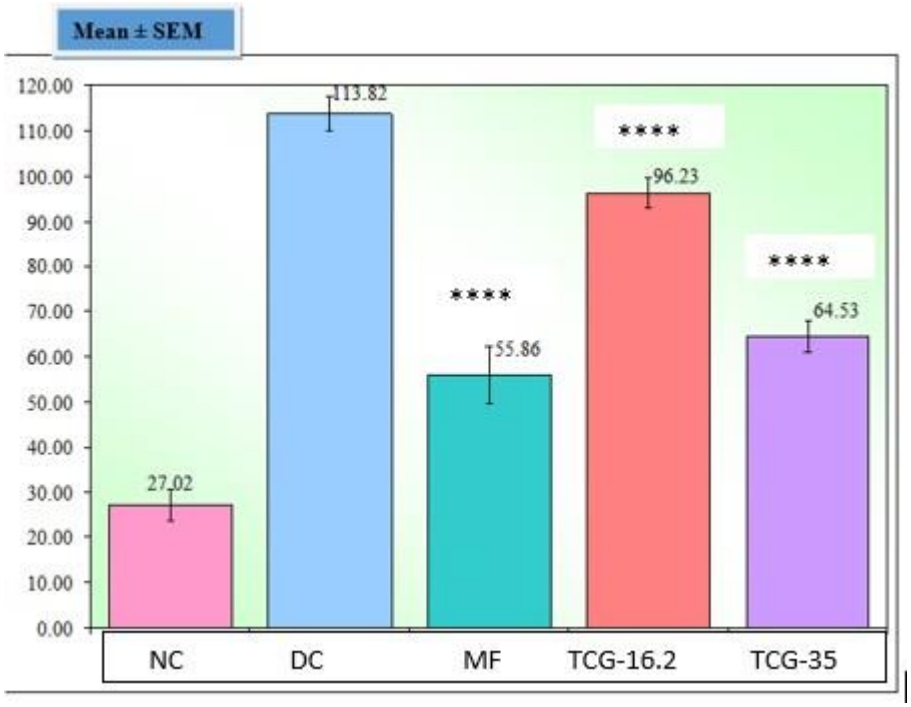
Values are expressed as Mean ± SEM, n=6, P<0.05: statistically significant; **p < 0.01, ****p < 0.0001 indicates the significant difference of various groups compared to diabetic control and ^p<0.01 indicates the significant difference between various groups compared to the Metformin group.

Graph 5: TNF-α - End of study (One-way ANOVA followed by Bonferroni's multiple comparison test)



Values are expressed as Mean ± SEM, n=6, P<0.05: statistically significant; **p < 0.01, ****p < 0.0001 indicates the significant difference of various groups compared to DC.

Graph 6: IL-6 - End of study (One-way ANOVA followed by Bonferroni's multiple comparison test)



Values are expressed as Mean ± SEM, n=6, statistically significant; **p < 0.01, ****p < 0.0001 indicates the significant difference of various groups compared to diabetic control.

DISCUSSION

The present study was conducted to evaluate the effect of anti platelet drug, Ticagrelor on glycemic parameters in a high-fat diet and Streptozotocin-induced diabetic model in male Wistar rats. The anti-inflammatory effects of Ticagrelor are well established. However, the evidence on the effects of Ticagrelor on blood glucose and HbA1c level is inconclusive which has been the objective of the present study.

The high-fat diet induces insulin resistance, and/or glucose intolerance in rats, in a way similar to humans. The β-cell failure is brought about by Streptozotocin by DNA alkylation, NO release, and ROS generation. STZ is considered to be a better chemical for diabetes induction compared to alloxan due to its selective action via GLUT-2 receptors, lesser toxicity, and lower mortality rate. [101,102] As the HFD-STZ model mimics the natural pathology of human diabetes, it was used for induction of diabetes in the current study. A state of permanent hyperglycemia develops in the rats 48 hours after STZ injection, and this is considered the start of the disease. Also, 72 hours post-injection is considered as the best time point for measuring the blood glucose level to confirm the diagnosis of diabetes.

Effect on body weight

In our study, the body weight of all the groups was high compared to the normal control group at the end of 14 days of the high-fat diet but weight gain was not statistically significant. A study by Marques, Cláudia et al suggested that weight gain with high fat diet in Wistar rats were statistically significant only after 4 weeks of high fat diet, although weight gain was reported after 2 weeks of High fat diet but weight gain was not statistically significant which supports our study [103] Wistar rats fed with HF diet had higher amounts of calorie intake compared to the normal control rats fed with chow diet. As a result, weight growth was greater in these animals, owing primarily to an increase in adipose tissue mass.[118-121]

With the progression of diabetes, there was significant weight reduction in the untreated diabetic rats compared to the non-diabetic animals. Such a drastic weight loss was also observed in studies done by Cheng, Daye et al.[104] and Mestry, Snehal Nitin et al.[105]. This can be explained on the basis that STZ-induced diabetes is accompanied by a substantial decrease in body weight as a result of hyperglycemia, hypoinsulinemia, muscle wasting, and protein loss.[104,106]. Treatment of diabetic rats with Metformin, Ticagrelor (16.2 mg/kg BW) and Ticagrelor (35mg/kg BW) significantly improved body weight, indicating that muscle tissue damage caused by hyperglycemia was prevented. It is known that Metformin's weight-neutral or weight-sparing effects provide a therapeutic benefit in

diabetes care, while other first-line oral antidiabetic medications frequently cause clinically substantial weight gain.[107] The energy expenditure during lactate–glucose metabolism may contribute to this effect of Metformin.[61]

Chen H et al concluded that Ticagrelor has no effect on the body weight of mice.[79] However, The current investigation found that Ticagrelor (16.2 mg/kg BW) and Ticagrelor (35 mg/kg BW) were effective at preventing diabetes-induced dramatic weight loss. The effect of Ticagrelor (35 mg/kg BW) on body weight was comparable to Metformin therapy and showed a statistically significant reduction in body weight when compared to Ticagrelor (16.2 mg/kg BW) group.

Effect on Fasting Blood Glucose

In our experiment, all of the rats fed with high-fat diet had a significant rise in fasting blood glucose at the end of 14 days when compared to rats fed with regular chow diet. Our findings are in accordance with previous research which showed that rats fed with a high-fat diet have higher blood glucose levels. Fat metabolites and FFAs that enter the liver are known to impair insulin sensitivity of liver cells and disrupt glucose metabolism leading to elevated glucose levels.[108,109]

A state of hyperglycemia was successfully generated following a single-dose of Streptozotocin in this study. The fasting blood glucose levels of untreated diabetic rats were consistently raised throughout the trial. The FBS of the rats treated with Metformin was substantially lower than that of the untreated rats halfway through the study. Ticagrelor (16.2 mg/kg BW) and Ticagrelor (35 mg/kg BW) was also successful in bringing down the glucose levels, though the difference was not statistically significant. At the end of 6 weeks, Ticagrelor (35 mg/kg BW) significantly reduced the FBS in comparison with the control diabetic rats. Moreover, the results of Ticagrelor (35 mg/kg BW) were comparable with Metformin therapy.

This anti-hyperglycemic effect of Ticagrelor is consistent with findings of previous studies by Ahmed Y et al.[80] An Experimental animal study on mice by Chen H et al reported that addition of Ticagrelor to Dapagliflozin improved glycemic control in diabetic mice. Though Ticagrelor alone did not have any beneficial effects on blood glucose. The study concluded that both Dapagliflozin and Ticagrelor had

cardioprotective effects by attenuating the progression of diabetic cardiomyopathy in BTBR mice with T2DM by virtue of inhibiting the NLRP3 inflammasome and their further pathway.[79]

Effects on HbA1C

The HbA1C levels of rats at baseline levels were comparable in all groups. HbA1C level was not measured after 14 days of HFD in our study because of two reason. One, The HFD was given for at least 8 weeks in earlier studies that demonstrated a significant increase in HbA1C. Second, given the modest increase in FBS levels over 14 days and the fact that Wistar rats' RBCs have an average life span of 59.8 days, there may not have been any changes in HbA1C at that time.[112]

At the end of the study, the HbA1C of the untreated diabetic rats was significantly higher than that of the non-diabetic rats. All three treatment groups (Metformin, Ticagrelor-16.2 and Ticagrelor -35) lowered HbA1C significantly which indicates that all three treatments were able to control the rise in HbA1C. The beneficial effect of Ticagrelor on HbA1C seen in our investigation is consistent with prior animal and human studies. THEMIS-PCI trial found that Ticagrelor produced favourable net clinical benefit across diabetes duration and HbA1C level.[81] A substudy from the PLATelet inhibition and patient Outcomes (PLATO) trial by James S et al. suggested that Ticagrelor reduced ischaemic events in ACS patients and decreased HbA1C level without an increase in major bleeding events.[7] Recently, an experimental animal study by Chen H et al postulated that Ticagrelor lowered pancreatic cell inflammation and increased insulin production.[79] However there is no evidence that improvement in HbA1C is based on interference with inflammation, and emphasizes the importance of filling gap with Ticagrelor as a promising anti-inflammatory agent for further studies. This gap has been filled by the present study, which evaluated the effect of Ticagrelor on inflammatory markers like IL-1, IL-6, TNF-alpha to confirm our findings.

In our study, HbA1C was reduced by Metformin and Ticagrelor both the doses, although the absolute values were significantly higher in Ticagrelor at the dose of 16.2 mg/kg BW than the value of Metformin and Ticagrelor at the dose of 35 mg/kg BW. Furthermore, the HbA1C levels of the Ticagrelor (16.2 mg/kg BW) group was not in a range that is compatible with ADA recommendations for target HbA1C.[116] However, the Ticagrelor at the dose of 35 mg/kg BW was effective in maintaining the HbA1C level within the recommended range. In cases of inadequate control of HbA1C, the American Diabetes Association recommends adding a second-line medication to Metformin therapy.[117] Based on the findings of our study and other existing evidence, Ticagrelor may be a favourable candidate for use as a second-line anti-diabetic drug. In Addition, Ticagrelor might be promising agent for treating ACS with DM.

Effects on Inflammatory markers

In our study, all three treatment groups were able to significantly lower serum IL-1, IL-6, and TNF- levels in diabetic rats when compared to untreated diabetic rats. Metformin has previously been reported to lower these inflammatory markers in animal models of diabetes. In a rodent study by Mohamed-I Kotb El-Sayed et al., it was observed that Metformin treatment significantly reduced the serum TNF and IL-6 compared to the diabetic control rats.[122] In a 2018 study by Ling Kou et al., Metformin significantly reduced serum IL-6, TNF-, and IFN- when compared to untreated diabetic rats.[123] In a similar study which used STZ induced diabetic rats Metformin reduced the IL-1 levels significantly compared to the untreated rats.[124] Our study also confirmed the beneficial effect of Metformin on these inflammatory markers.

We found that Ticagrelor, either at platelet inhibitory dose (16.2 mg/kg) or Anti-inflammatory dose (35 mg/kg), was effective in reducing these inflammatory parameters. But inhibitory effect by Ticagrelor (35 mg/kg) was statistically significant than Ticagrelor (16.2 mg/kg), which suggests that the superiority seen in this study could be likely due to potent anti-inflammatory action at higher dose of Ticagrelor leading to better glycemic control by improving insulin resistance. The anti-inflammatory characteristics of Ticagrelor are well documented; it is a powerful inhibitor of IL-6, IL-1, and TNF-alpha, NF-kβ, and caspase 3 [113,125–128] According to a recent 2020 review, Ticagrelor has a potential function in reducing the risk of numerous diseases based on its pharmacological efficacy, which is mediated through anti-inflammatory mechanisms.[75] Studies in the past have attempted to connect Ticagrelor's anti-inflammatory capabilities to its potential benefit in hyperglycemic conditions. According to Uil M et al., Ticagrelor attenuates diabetic nephropathy and reduces inflammation, endothelial function and fibrosis by downregulating proinflammatory cytokines such as TNF-α, NF-kβ, and caspase 3.[6]

An extensive animal study by Huang B et al. found Ticagrelor to be effective in improving insulin signaling in the liver and adipose tissue while reducing the NLRP3 inflammasome in macrophages. It also reduced ER stress and reduced macrophage infiltration in adipocytes.[5] In another study, Chen H et al. concluded that Ticagrelor was effective in reversing chronic inflammation indicating that Ticagrelor may be an alternative treatment for insulin resistance.[79] Wang et al has suggested that although P2Y12 is a known platelet receptor, it also exists in endothelial cells and plays an important pathological pro-

inflammatory role which justify the effect of Ticagrelor on reducing endothelial Ang-II-mediated ROS generation and eNOS phosphorylation and ultimately endothelium-stabilizing effects and anti-inflammatory effects.[83]

In another RCT by Husted S et al. found that Ticagrelor administration reduced levels of the inflammatory biomarkers namely CRP, IL-6, and MPO significantly.[80] Published evidence suggests that drugs targeting inflammatory mediators like IL-1β and TNF-α increase insulin sensitivity and exerts beneficial effects on glycemic parameters.[80-83]

The finding of present study thus support that Ticagrelor exert beneficial effects on glycemic parameters of diabetes that could be due to its effect on inflammatory markers. However, further studies are required to establish the effect of Ticagrelor in combination with standard anti-diabetic drugs like Metformin to supports its addition in the treatment of diabetes with co-morbid conditions like ACS.

Limitations and future recommendations:

Two doses of Ticagrelor have been employed in the present study, a lower dose based on the clinically used human dose for anti-platelet effect and a minimum dose reported to exhibit anti-inflammatory effect. Our study has reported the effects of Ticagrelor alone on glycemic parameters of diabetes. Further studies can be conducted utilizing anti-inflammatory dose of Ticagrelor in combination with Metformin (Standard dug for diabetes) for maximum benefit in treatment of diabetes with co-morbid condition like ACS.

Our study did not estimate effect of Ticagrelor on HOMA-IR (Homeostasis model assessment-insulin resistance), which is considered as a valid estimate of insulin sensitivity and further studies can be planned to study the same

.

CONCLUSION

The present study showed that the treatment of diabetic rats with oral Ticagrelor improved the HFD-STZ induced biochemical alterations in glycemic parameters and inflammatory markers. Furthermore, Metformin and Ticagrelor was found to be equally efficacious across all trial variables. Based on the findings of this study, it can be concluded that Ticagrelor may be a promising option for the management of Type 2 Diabetes with ACS like comorbid condition. Future research of Ticagrelor in combination with standard anti-diabetic drugs like Metformin in patients of diabetes with co-morbid condition like ACS, as well as clinical trials with larger sample sizes, are recommended.

SUMMARY

The present study was conducted to evaluate the effects of Ticagrelor in a High-fat diet Streptozotocin-induced rodent model of diabetes. In this study, the effect of Ticagrelor on glycaemic parameters and inflammatory markers was evaluated. We found that treatment of diabetic rats with oral Ticagrelor improved the HFD-STZ induced biochemical alterations in all these parameters.

Key findings of the study:

- Ticagrelor was effective at preventing Diabetes-induced dramatic weight loss.
- Ticagrelor was effective in reducing the Fasting Blood Glucose.
- Ticagrelor was effective in reducing the HbA1C levels.
- Ticagrelor significantly lowered serum IL-1β, IL-6, and TNF-α levels in comparison with the untreated rats.
- Ticagrelor was found to be more efficacious across all trial variables.
- Two doses of Ticagrelor have been employed in the present study, a lower dose (16.2 mg/kg BW) based on the clinically used human dose for anti-platelet effect and a higher dose which is minimum dose reported to exhibit anti-inflammatory effect (35 mg/kg BW). Ticagrelor (35mg/kg BW) is more effective than Ticagrelor (16.2 mg/kg BW) in reducing FBG, HbA1c and Inflammatory marker levels.

According to the findings of this study, Ticagrelor may be a viable choice in the management of Type 2 Diabetes with co-morbid condition like ACS. Clinical trials with bigger sample sizes, as well as further research of Ticagrelor in combination with standard anti-diabetic drugs like Metformin in patients of diabetes with co-morbid condition like ACS are recommended.

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ANNEXURE – I - IAEC APPROVAL CERTIFICATE

KLE ACADEMY OF HIGHER EDUCATION AND RESEARCH
(Deemed to be University)
JAWAHARLAL NEHRU MEDICAL COLLEGE,
NEHRU NAGAR, BELAGAVI - 590010, (KARNATAKA).
INSTITUTIONAL ANIMAL ETHICS COMMITTEE.
Phone No. JNMC (0831)- 2444040

Chairperson, IAEC.	Main Nominee - CPCSEA	Member - Secretary IAEC
Prof & Head Physiology,	Prof & Head of Pharmacology,	Asso Prof of Pharmacology
J.N.Medical College, Belagavi	USM-KLE, IMP, Belagavi	J.N.Medical College, Belagavi

CPCSEA Reg.No.: 627/PO/Re/S/02/CPCSEA

MEMBERS:

ger
Scientist-D, RMRC,
ICMR, Belagavi.

Non-scientific Social worker,
Nidasosi.

ldy.
Hon.Veternarian,
Belagavi.

de,
Officer Incharge,
Central Animal House,
JNMC, Belagavi.

alli,
Prof of Anatomy.
JNMC,Belagavi

AHM
Link Nominee CPCSEA.
Dept of Pharmacology &
Toxicology
KLE's Coll Of Pharmacy,
Hubballi

CERTIFICATE

This is to certify that the M.D/ M.D.S/ Ph.D/ Research project Entitled "Effect of Ticagrelor on High Fat Diet and Streptozotocin induced Diabetes Mellitus in male Wistar rats- An experimental study".

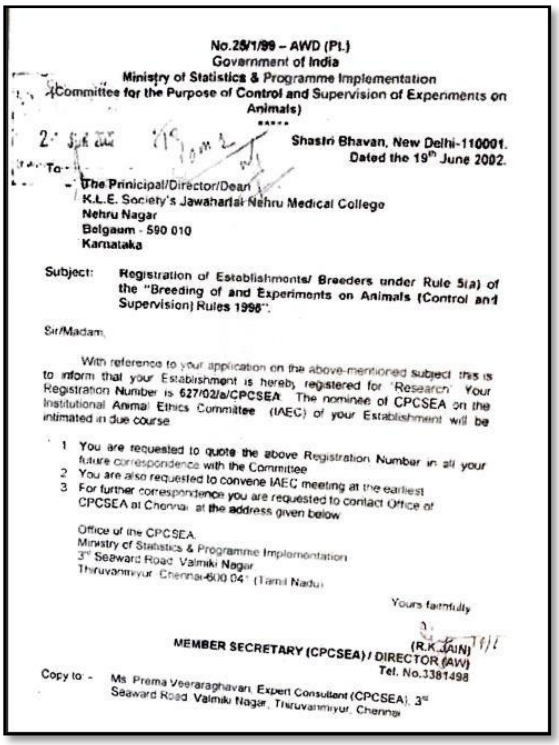
Submitted l,
PG Pharmacology, JNMC

Has been approved by the Institutional Animal Ethical Committee Meeting held on 5.2.2021 vide Resolution No. 14/4.
For sanction of 38 Male Wistar Rats.

(Dr. P.A. Patil)
Signature and Name:
CPCSEA-Main Nominee
Main Nominee CPCSEA
IAEC-JNMC, Belagavi.

Signature and Name:
Chairman/Mem.Secretary
Member Secretary
IAEC-JNMC, Belagavi.

ANNEXURE - II - CPCSEA REGISTRATION & RENEWAL



No.28/1/99 – AWD (Pt.)
Government of India
Ministry of Statistics & Programme Implementation
Committee for the Purpose of Control and Supervision of Experiments on Animals)

Shastri Bhavan, New Delhi-110001.
Dated the 19th June 2002.

To -
The Principal/Director/Dean
K.L.E. Society's Jawaharlal Nehru Medical College
Nehru Nagar
Belgaum - 590 010
Karnataka

Subject: Registration of Establishments/ Breeders under Rule 5(a) of the "Breeding of and Experiments on Animals (Control and Supervision) Rules 1998".

Sir/Madam,

With reference to your application on the above-mentioned subject this is to inform that your Establishment is hereb, registered for 'Research'. Your Registration Number is 627/02/a/CPCSEA. The nominee of CPCSEA on the Institutional Animal Ethics Committee (IAEC) of your Establishment will be intimated in due course.

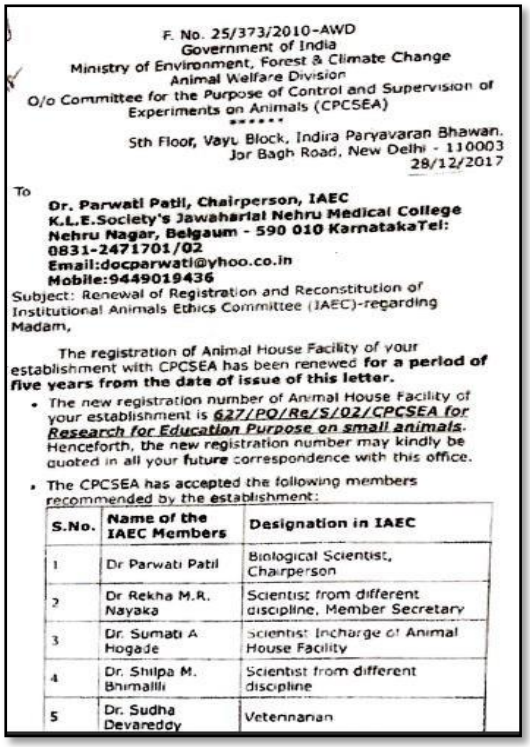
1. You are requested to quote the above Registration Number in all your future correspondence with the Committee
2. You are also requested to convene IAEC meeting at the earliest.
3. For further correspondence you are requested to contact Office of CPCSEA at Chennai at the address given below.

Office of the CPCSEA
Ministry of Statistics & Programme Implementation
3rd Seaward Road, Valmiki Nagar
Thiruvanmiyur, Chennai-600 041 (Tamil Nadu)

Yours faithfully

(R.K.JAIN)
MEMBER SECRETARY (CPCSEA) / DIRECTOR (AW)
Tel. No.3381498

Copy to: - Ms. Prema Veeraraghavan, Expert Consultant (CPCSEA), 3rd Seaward Road, Valmiki Nagar, Thiruvanmiyur, Chennai



F. No. 25/373/2010-AWD
Government of India
Ministry of Environment, Forest & Climate Change
Animal Welfare Division
O/o Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA)

5th Floor, Vayu Block, Indira Paryavaran Bhawan,
Jor Bagh Road, New Delhi - 110003
28/12/2017

To
Dr. Parwati Patil, Chairperson, IAEC
K.L.E.Society's Jawaharlal Nehru Medical College
Nehru Nagar, Belgaum - 590 010 KarnatakaTel: 0831-2471701/02
Email:docparwati@yhoo.co.in
Mobile:9449019436

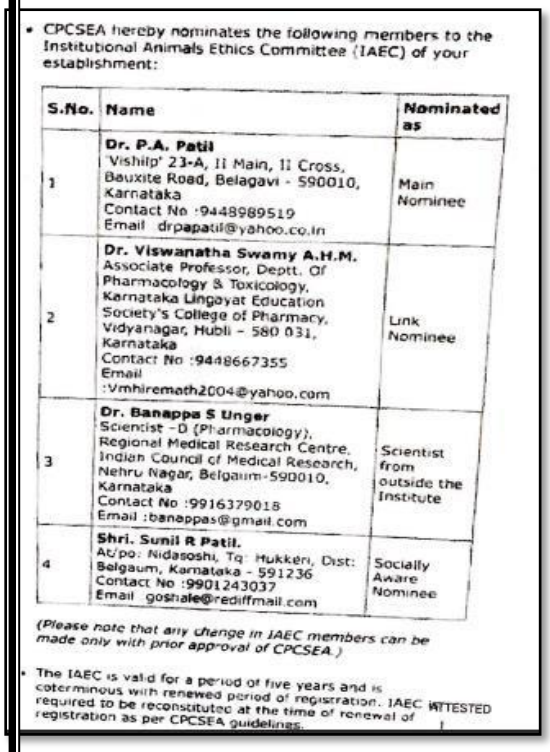
Subject: Renewal of Registration and Reconstitution of Institutional Animals Ethics Committee (IAEC)-regarding

Madam,

The registration of Animal House Facility of your establishment with CPCSEA has been renewed **for a period of five years from the date of issue of this letter.**

- The new registration number of Animal House Facility of your establishment is ***627/PO/Re/S/02/CPCSEA for Research for Education Purpose on small animals***. Henceforth, the new registration number may kindly be quoted in all your future correspondence with this office.
- The CPCSEA has accepted the following members recommended by the establishment:

S.No.	Name of the IAEC Members	Designation in IAEC
1	Dr Parwati Patil	Biological Scientist, Chairperson
2	Dr Rekha M.R. Nayaka	Scientist from different discipline, Member Secretary
3	Dr. Sumati A Hogade	Scientist Incharge of Animal House Facility
4	Dr. Shilpa M. Bhimalli	Scientist from different discipline
5	Dr. Sudha Devareddy	Veterinarian

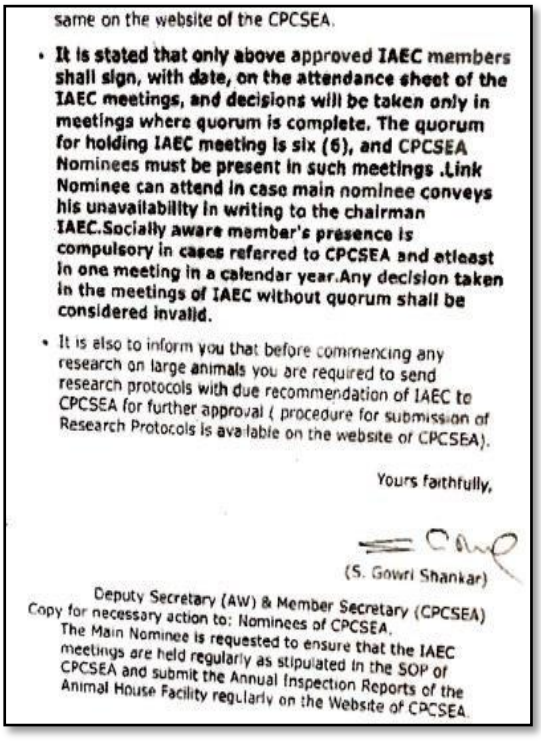


- CPCSEA hereby nominates the following members to the Institutional Animals Ethics Committee (IAEC) of your establishment:

S.No.	Name	Nominated as
1	**Dr. P.A. Patil** 'Vishilp' 23-A, II Main, II Cross, Bauxite Road, Belagavi - 590010, Karnataka Contact No :9448989519 Email :drpapatil@yahoo.co.in	Main Nominee
2	**Dr. Viswanatha Swamy A.H.M.** Associate Professor, Deptt. Of Pharmacology & Toxicology, Karnataka Lingayat Education Society's College of Pharmacy, Vidyanagar, Hubli - 580 031, Karnataka Contact No :9448667355 Email :Vmhiremath2004@yahoo.com	Link Nominee
3	**Dr. Banappa S Unger** Scientist - D (Pharmacology), Regional Medical Research Centre, Indian Council of Medical Research, Nehru Nagar, Belgaum-590010, Karnataka Contact No :9916379018 Email :banappas@gmail.com	Scientist from outside the Institute
4	**Shri. Sunil R Patil.** At/po: Nidasoshi, Tq: Hukkeri, Dist: Belgaum, Karnataka - 591236 Contact No :9901243037 Email :goshale@rediffmail.com	Socially Aware Nominee

(Please note that any change in IAEC members can be made only with prior approval of CPCSEA.)

- The IAEC is valid for a period of five years and is coterminous with renewed period of registration. IAEC ATTESTED required to be reconstituted at the time of renewal of registration as per CPCSEA guidelines.



same on the website of the CPCSEA.

- **It is stated that only above approved IAEC members shall sign, with date, on the attendance sheet of the IAEC meetings, and decisions will be taken only in meetings where quorum is complete. The quorum for holding IAEC meeting is six (6), and CPCSEA Nominees must be present in such meetings .Link Nominee can attend in case main nominee conveys his unavailability in writing to the chairman IAEC.Socially aware member's presence is compulsory in cases referred to CPCSEA and atleast in one meeting in a calendar year.Any decision taken in the meetings of IAEC without quorum shall be considered invalid.**
- It is also to inform you that before commencing any research on large animals you are required to send research protocols with due recommendation of IAEC to CPCSEA for further approval (procedure for submission of Research Protocols is available on the website of CPCSEA).

Yours faithfully,

(S. Gowri Shankar)
Deputy Secretary (AW) & Member Secretary (CPCSEA)

Copy for necessary action to: Nominees of CPCSEA.
The Main Nominee is requested to ensure that the IAEC meetings are held regularly as stipulated in the SOP of CPCSEA and submit the Annual Inspection Reports of the Animal House Facility regularly on the Website of CPCSEA.