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EVALUATION OF TOLL-LIKE RECEPTORS 2 AND 4 IN STREPTOZOTOCIN INDUCED DIABETIC WISTAR RATS

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BY

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DEDICATION

First and foremost, I thank Almighty ALLAH for making this research work a success. I dedicate this project to Almighty Allah my creator, my strong pillar, my source of inspiration, wisdom, knowledge and understanding. HE has been the source of my strength throughout this program. I also dedicate this work to my Husband, Arc Ahmed Tijjani Tukur who has encouraged me all the way and whose encouragement has made sure that I give it all it takes to finish that which I have started. To my kids Aliyu and Aisha who have been affected in every way possible by this quest. Thank you. My love for you all can never be quantified. May Allah shower you with his blessings. Amin

CERTIFICATION

This dissertation report entitled *EVALUATION OF TOLL LIKE RECEPTORS 2 AND 4 IN STREPTOZOTOCIN INDUCED DIABETIC WISTAR RATS* by FATIMA ALIYU CHIROMA (**14211226010**) meets the regulations governing the award of Master in Chemical Pathology of the Usmanu Danfodiyo University, Sokoto and is approved for its contribution and literary presentation.

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ACRONYMS/DEFINITION OF TERMS

2HPG	2-hour plasma glucose
A1C	Glycated Hemoglobin
ADA	American Diabetes Association
AGE	Advanced Glycation End Product
Ang II	Angiotensin II
APCs	Antigen Presenting Cells
ARBs	Angiotensin Receptors Blockers
ASON	Antisense Oligonucleotide
AT1R	Angiotensin type 1 receptors
BB	Bio breeding
CD4	Cluster of differentiation 4
CEP	ω-(2-carboxyethyle) pyrrole
CPG	$5^{|}$-C-Phosphate-G-$3^{|}$
CREB	Cyclic response element binding protein
CVD	Cardiovascular Disease
DAMPs	Danger Associated Molecular Patterns

DC	Dendritic Cells
DCCT	Diabetes Control and Complication Trial
DHA	Decosahexaenoic acid
DM	Diabetes mellitus
DNA	Deoxyribonucleic acid
EDTA	Ethylenediamine Tetraacetic Acid
EPA	Ecosapentanoic acid
FFAs	Free Fatty Acids
FPG	Fasting Plasma Glucose
GDM	Gestational diabetes mellitus
GLUT	Glucose transporter
GOD	Glucose Oxidase
GPI	Glycosyl phosphatidyl inositol
H_2O_2	Hydrogen Peroxide
HbA1c	Glycated Haemoglobin Levels
HG	High Glucose
HIV/AIDS	Human immunodeficiency virus/ Acquired immunodeficiency syndrome
HLA	Human leucocyte antigen
HMGB1	High Mobility Group Box1

HMG-CoA	(3-hydroxy-3-methlyglutary CoA
HNF	Hepatocyte Nuclear Factor
HRP	Horseradish peroxidase
HSP	Heat Shock Protein
HSV	Herpes simplex virus
IDDM	Insulin dependent diabetes mellitus
IL	Interleukin
IPF	Insulin Promoter Factor
IRF	Interferon regulatory factors
LBP	Lipopolysaccharide binding protein
LDL	Low Density Lipoprotein
LK B1	Liver Kinase B1
LPS	Lipopolysaccharide
LRR	Leucine Rich Repeat
MAMPs	Microorganisms Associated Molecular Pattern
MANOVA	Multivariate analysis of variance
MAPK	Mitogen activated protein kinase
MCP	Monocyte chemoattractant protein
MD2	Myeloid differentiation factor 2

MHC	Major Histocompatibility Complex
MiRNA	MicroRNA
MMTV	Mouse mammary tumour virus
MODY	Maturity –Onset Diabetes of the Young
mRNA	Messenger ribonucleic acid
MYD88	Myeloid differentiation 88
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NEFAS	Non Esterified Fatty Acids
NFKB	Nuclear factor kappa light chain enhancer of activated B cells
NGSP	National Glycohemoglobin Standard Program
NHANES	National Health and Nutrition Examination Survey
NIDDM	Non-insulin dependent diabetes mellitus
NOD	Non-obese Diabetes Mice
OD	Optical density
OGTT	Oral Glucose Tolerance Test
PAMPK	Phosphorylated activated protein kinase
PAMPs	Pathogen Associated Molecular Patterns
PKC	Protein kinase C
PLNs	Pancreatic lymph Nodes

POD	Peroxidase
PP cells	Poly-peptide
PPAR	Peroxisome Proliferators Activator Receptors
PRRs	Pattern Recognition Receptors
PU. I	Transcription factor
PUFAs	Polyunsaturated Fatty Acids
RAGE	Receptors for Advance Glycation End Product
RAS	Family of related protein
ROS	Reactive oxygen specie
RSV	Respiratory Syncytial Virus
RT-PCR	Real time polymerase chain reaction
SPSS	Statistical Package for Social Sciences
STZ	Streptozotocin
T1DM	Type1 DM
T2DM	Type2 DM
THI	Transient hypergamma globunaemia of infancy
TIR	Toll Interleukin-1 Receptors
TLRs	Toll Like Receptors
TNF	Tumor necrosis factor

TORC2	Transducer of Regulated CREB Protein 2
TZD	Thiazolidinedione

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ABSTRACT

Diabetes is a multifactorial metabolic disorder that leads to complications. Hyperglycaemia induced inflammation is central in diabetes complications. TLR2 and TLR4 initiate and propagate persistent inflammation in diabetes. This study evaluated TLR2, TLR4, FBG and HBA1C levels in streptozotocin-induced DM, assessed the effect of metformin treatment and gender differences on TLR2, TLR4, FBG and HBA1C levels. Thirty (30) adult male and female albino wistar rats grouped into 3 with 10 rats in each group were used. Group 1 (non-diabetic rats as control), group 2 (streptozotocin-induced diabetic rats untreated) and group 3 (streptozotocin-induced diabetic rats treated with metformin). Serum TLR2, TLR4, FBG and HBA1C were analysed using standard techniques. TLR2 levels was significantly increased in non-treated and treated diabetic rats compared to control ($p<0.05$), TLR4 not significantly increased ($p>0.05$). Metformin treatment had no effect on TLR2, TLR4 and FBG levels but significant on HBA1C levels. No gender difference in TLR2, TLR4 and HBA1C levels, but significant difference ($P<0.05$) on FBG levels. Hyperglycaemia, the hallmark of DM elevated TLR2 levels. TLR2 and TLR4 sense hyperglycaemia as danger signals and mediate inflammatory cascade promoting disease progression. These results suggest the involvement of TLR2 in DM may contribute to proinflammatory state and disease progression.

CHAPTER ONE

1.0 INTRODUCTION

1.1 BACKGROUND OF THE STUDY

Diabetes mellitus (DM) is a group of metabolic disorders of carbohydrate, protein and fat metabolism in which glucose is under-utilized, producing hyperglycaemia (Orchard, 1996). It occurs either because of a lack of insulin or because of the presence of factors that oppose the action of insulin (Orchard, 1996). The result of insufficient action of insulin is an increase in blood glucose concentration (hyperglycaemia) leading to different complications such as, diabetic retinopathy, microangiopathy, nephropathy etc (Orchard, 1996). DM cannot be cured, it can only be controlled (Orchard, 1996). Symptoms of marked hyperglycemia include polyuria, polydipsia, weight loss, sometimes with polyphagia, and blurred vision. Impairment of growth and susceptibility to certain infections may also accompany chronic hyperglycemia (Metzger *et al.,* 2010).

DM is a life style disease affecting 8.3% of the adult population of the world and increasing at an alarming rate, is one of the most common non-communicable diseases of current era (IDF, 2013). The burden of this disease is immense owing to transition in lifestyle and dietary habits, ageing of the population and urbanization in the setting of a genetically predisposed environment (Nolan *et al*., 2011). The fact that the number of subjects with DM has doubled over the past three decades has made this disease a global challenge (Shaw *et al*., 2010). The number of DM patients is projected to increase from 282 million in 2013 to 592 million by 2025, denoting a net increase of 55% (IDF, 2013).

Complications of diabetes are common at the time of presentation in Nigeria for example, neuropathy 56%, erectile dysfunction 36%, nephropathy 9%, and retinopathy 7%. This is partly because DM is a progressive illness with an initial asymptomatic phase associated with on-going tissue damage and decline in pancreatic beta cell mass and function (Oputa and Chinenye, 2015).

Toll-like receptors (TLRs) are a family of pattern recognition receptors (PRRs) expressed by a variety of cell types, especially cells of the innate immune system (Aisling *et al*., 2011). A primary function is to sense danger or damage and mediate innate immune responses to pathogens and tumors (Aisling *et al*., 2011). Like other PRRs, TLRs bind exogenous pathogen associated molecular patterns (PAMPs), conserved structures expressed by pathogens such as lipopolysaccharide (LPS), flagellin, viral and bacterial nucleic acids, but also endogenous danger associated molecular patterns (DAMPs), such as High Mobility Group Box1 (HMGB1) and fatty acids (Lu *et al*., 2015). Binding of agonists to surface or intracellular TLRs results in the activation of a number of downstream signaling pathways, including Mitogen-Activated Protein kinase, Nuclear Factor-kB and Interferon Regulatory Factor pathways, which leads to the production of type I interferons, chemokines and cytokines, especially though not exclusively those that mediate inflammatory responses (Aisling *et al*., 2011). Consequently, activation of TLRs can not only promote innate inflammatory responses but can also direct the induction of adaptive immunity. This has resulted in the exploitation of TLR agonists as adjuvants for infectious disease and cancer vaccines and immunotherapeutics for tumors (Aisling *et al*., 2011). Conversely, because of their key role in inflammation, molecules that antagonize TLRs or inhibit TLR signalling pathways have considerable potential as immune therapeutics against a

variety of inflammatory conditions, including autoimmune disease, atherosclerosis, type 2 diabetes, osteoarthritis and reperfusion injury (Higgins and Mills, 2010).

It has been reported that TLRs sense high glucose (HG) and high free fatty acids (FFAs) as danger signals and subsequently mediate the inflammatory cascade to promote disease progression (Dasu *et al*., 2008; Dasu and Jialal, 2011).

Among the TLRs, TLR2 and TLR4 play a critical role in the pathogenesis of insulin resistance, diabetes, and atherosclerosis in both clinical and experimental conditions (DeFronzo and Ferrannini, 1991; (a) Hoffman *et al*., 1999; Wong and Weng, 2008; Olefsky and Glass, 2010). TLRs also interact with endogenous ligands which are also elevated in diabetes (Tsan and Gao, 2004; Taylor *et al*., 2004; Wagner, 2006; Osterloh and Breloer, 2008; Chiu *et al*., 2009; Hreggvidsdottir *et al*., 2009). Ligands for TLR2 and TLR4 include high-mobility group B1 protein (HMGB1), heat shock protein (HSP) 60, HSP70, endotoxin, hyaluronan, advanced glycation end-products, and extracellular matrix components (Takeda *et al*., 2003). TLR2 and TLR4 bind to components of the gram-positive and -negative bacteria, respectively (Takeda *et al*., 2003). Consequently, different TLRs are amenable to targeting by different types of agents.

1.2 STATEMENT OF THE RESEARCH PROBLEM

DM is now one of the most common non-communicable diseases, the fourth or fifth leading cause of death in most high income countries (IDF, 2013). Complications from DM such as coronary artery and peripheral vascular disease, stroke, diabetic nephropathy, renal failure, and blindness results in increasing disability, reduce life expectancy and enormous health cost for diabetic patients (IDF, 2013).

DM leads to both microvascular and macrovascular complication and its undeniable that TLRs are emerging as major factors in many disease conditions (Kenia *et al*.,

2015). Furthermore, TLR 2 and 4 play a pivotal role in initiating and propagating persistent inflammation in DM (Mohammed *et al*., 2006).

1.3 JUSTIFICATION OF THE STUDY

Evidence from experimental animal models as well as humans have indicated that systemic inflammation plays a role in the pathophysiological processes of DM and is facilitated by innate immune responses (Pino *et al*., 2010). TLRs are key innate immune receptors that recognize conserved PAMPs, induce inflammatory responses essential for host defenses and initiate an adaptive immune response. These observations suggest a potential role for TLR2 and TLR4 in the pathology of DM.

Although TLR expression is increased in a plethora of inflammatory disorders, the effects of metabolic aberrations on TLRs and their role in DM and its complications is not completely understood. From available literature on TLRs in DM and its complications, there is high optimism that TLR may prove to be a potential therapeutic option in the management of diabetes patients.

Taken together, levels of TLR2 and TLR4 have never been reported in the North western region of Nigeria, Sokoto in focus. The current study could open up TLR research in our localities so as to better manage our diabetes patients.

1.4 AIM AND OBJECTIVES OF THE STUDY

1.4.1 Aim

The aim of this study was to evaluate the pattern of toll-like receptors 2 and 4 in streptozotocin-induced diabetic wistar rats.

1.4.2 Specific Objectives

The specific objectives of this study were as follows:

i. To determine the effect of streptozotocin-induced DM on TLR2 and TLR4 expression in Wistar rats.

ii. To evaluate the effect of metformin treatment on TLR2 and TLR4 expression in streptozotocin-induced diabetic rats.

iii. To evaluate the effect of sex on the concentrations of TLR2 and TLR4 in streptozotocin-induced diabetic rats treated with metformin.

iv. To evaluate the effect of metformin treatment on fasting blood glucose glycated haemoglobin levels (HbA1c) in streptozotocin-induced diabetic wistar rats.

1.5 RESEARCH HYPOTHESIS

H01 Diabetes does not affect the expression of TLR2 and TLR4 in wistar rats.

HA1 Diabetes affect the expression of TLR2 and TLR4 in wistar rats.

H02 Expression of TLR2 and TLR4 are not different between male and female diabetic wistar rats.

HA2 Expression of TLR2 and TLR4 are different between male and female diabetic wistar rats.

H03 Metformin treatment does not affect the fasting blood glucose concentration in diabetic wistar rats.

HA3 Metformin treatment affect the fasting blood glucose concentration in diabetic wistar rats.

H04 Metformin treatment does not affect the haemoglobin concentration of diabetic wistar rats.

HA4 Metformin treatment affect the haemoglobin concentration of diabetic wistar rats.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 DIABETES MELLITUS

Diabetes mellitus (DM) is a chronic disorder that can alter carbohydrate, protein, and fat metabolism. It is caused by the absence of insulin secretion due to either the progressive or marked inability of the β-Langerhans islet cells of the pancreas to produce insulin, or due to defects in insulin uptake in the peripheral tissue (Scheen, 2003). Diabetes mellitus is the commonest endocrine-metabolic disorder characterized by chronic hyperglycaemia giving rise to the risk of microvascular (retinopathy, nephropathy, and neuropathy) and macrovascular (ischaemic heart disease, stroke and peripheral vascular disease) damage, with associated reduced life expectancy and diminished quality of life (Sonny and Ekene, 2016). Deficient insulin action results from inadequate insulin secretion and/or diminished tissue responses to insulin at one or more points in the complex pathways of hormone action. Impairement of insulin secretion and defects in insulin action frequently coexist in the same patient, and it is often unclear which abnormality, if either alone, is the primary cause of the hyperglycaemia.

2.1.1 Economic Burden of Diabetes Mellitus

Economic aspects of diabetes and diabetes care currently attract considerable attention as the world diabetes epidemic takes hold and the healthcare activities of countries come under pressure to accomplish more within constrained resources (Sobngwi *et al*., 2001). Diabetes mellitus is a very expensive disease and has profound implications in terms of long-term microvascular and macrovascular complications and their associated cost. These complications reduce both life expectancy and quality of life (Piero *et al*., 2014).

According to Kirigia *et al.* (2009), diabetes mellitus poses a big economic burden with regards to health system costs, indirect costs arising from losses occasioned by patient disability and premature mortality, time spent by family members accompanying patients when seeking care, and intangible costs in terms of psychological pain to the family and loved ones.

2.1.2 Prevalence of Diabetes Mellitus

The prevalence of diabetes is increasing rapidly worldwide and the World Health organization (2014) has predicted that by 2030 the number of adults with diabetes would have almost doubled worldwide, from 177 million in 2000 to 370 million. Experts project that the incidence of diabetes is set to soar by 64% by 2025, meaning that a staggering 53.1 million citizens will be affected by the disease (Rowley and Bezold, 2012). The estimated worldwide prevalence of diabetes among adults in 2010 was 285 million (6.4 %) and this value is predicted to rise to around 439 million (7.7 %) by 2030 (Shaw *et al.*, 2010).

The prevalence of diabetes in the WHO African Region was estimated in 2000 to be at 7.02 million people. Of these, about 0.702 million had type 1 diabetes and 6.318 million had type 2 diabetes. In addition, close to 113,100 people died from diabetes related causes, 561,600 were permanently disabled, and 6,458,400 experienced temporary disabilities (WHO, 2008; Kirigia *et al.*, 2009). As far back as the beginning of the twentieth century, DM was described as being an uncommon disorder in the African. There is however, compelling data to show an increasing incidence and prevalence of DM in the continent (Kengne *et al.*, 2005). The estimated prevalence of diabetes in Africa is 1% in rural areas, and ranges from 5% to 7% in urban Sub Saharan Africa (Kengne *et al.*, 2005).

Nigeria, with a population of 158 million people, is the most populous country in Africa and accounts for one sixth of Africa's population. According to World Population Prospects (WPP), approximately 50% of Nigerians are urban dwellers and the country has cultural diversity and 398 documented ethnic groups (WPP, 2012).

2.1.3 Risk Factors of Diabetes Mellitus

Guyton and Hall (2006) summarized the following as some of the causes of insulin resistance; Obesity/overweight (especially excess visceral adiposity), excess glucocorticoids (Cushing's syndrome or steroid therapy), excess growth hormone (acromegaly), pregnancy (gestational diabetes), polycystic ovary disease, lipodystrophy (acquired or genetic, associated with lipid accumulation in liver), autoantibodies to the insulin receptor, mutations of insulin receptor, mutations of the peroxisome proliferators' activator receptor γ (PPAR γ), mutations that cause genetic obesity (e.g., melanocortin receptor mutations), hemochromatosis (a hereditary disease that causes tissue iron accumulation) (Guyton and Hall, 2006).

2.2 Classification of Diabetes Mellitus

Four major classes of diabetes have been defined by the American Diabetes Association, ADA (2014) and the World Health Organization (WHO, 2014).

Diabetes can be classified into the following general categories:

2.0 Type 1 diabetes (due to b-cell destruction, usually leading to absolute insulin deficiency)

2. Type 2 diabetes (due to a progressive insulin secretory defect on the background of insulin resistance).

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

3.0 Specific types of diabetes due to other causes, e.g., monogenic diabetes syndromes (such as neonatal diabetes and maturity-onset diabetes of the young [MODY]), diseases of the exocrine pancreas (such as cystic fibrosis), and drug or chemical induced diabetes (such as in the treatment of HIV/AIDS or after organ transplantation).

2.3 Diagnosis of Diabetes Mellitus

DM may be diagnosed based on glycated hemoglobin (A1C) criteria or plasma glucose criteria, either the fasting plasma glucose (FPG) or the 2-h plasma glucose (2-h PG) value after a 75-g oral glucose tolerance test (OGTT) (ADA, 2014). The same tests are used to both screen for and diagnose diabetes. Diabetes may be identified anywhere along the spectrum of clinical scenarios: in seemingly low risk individuals who happen to have glucose testing, in symptomatic patients, and in higher-risk individuals whom the provider tests because of a suspicion of diabetes. The same tests will also detect individuals with prediabetes (ADA, 2014)

2.3.1 Glycated Hemoglobin (A1C)

The A1C test should be performed using a method that is certified by the National Glycohemoglobin Standard Program (NGSP) and standardized or traceable to the Diabetes Control and Complications Trial (DCCT) reference assay (ADA, 2014).

The A1C has several advantages to the FPG and OGTT, including greater convenience (fasting not required), greater preanalytical stability, and less day-to-day perturbations during stress and illness. These advantages must be balanced by greater cost, the limited availability of A1C testing in certain regions of the developing world, and the incomplete correlation between A1C and average glucose in certain individuals (ADA, 2014).

2.3.2 Hemoglobinopathies/Anemias

Interpreting A1C levels in the presence of certain hemoglobinopathies and anemia may be problematic. For patients with an abnormal hemoglobin but normal red cell turnover, such as those with the sickle cell trait, an A1C assay without interference from abnormal hemoglobins should be used. In conditions associated with increased red cell turnover, such as pregnancy (second and third trimesters), recent blood loss or transfusion, erythropoietin therapy, or hemolysis, only blood glucose criteria should be used to diagnose diabetes (ADA, 2014).

2.3.3 Fasting and 2-Hour Plasma Glucose

In addition to the A1C test, the FPG and 2-h PG may also be used to diagnose diabetes. The concordance between the FPG and 2-h PG tests is imperfect, as is the concordance between A1C and either glucose-based test. National Health and Nutrition Examination Survey (NHANES) data indicate that an A1C cut point of ≥ 6.5 % identifies one-third fewer cases of undiagnosed diabetes than a fasting glucose cut point of ≥ 126 mg/dL (7.0 mmol/L) (Pickup J, 2004). Numerous studies have confirmed that, compared with these A1C and FPG cut points, the 2-h PG value diagnoses more people with diabetes.

2.4 Type 1 Diabetes Mellitus

Type 1 diabetes is an immune mediated diabetes that represents around 10 % of all cases of diabetes, affecting approximately 20 million people worldwide (ADA, 2011).

Although type 1 diabetes affects all age groups, the majority of individuals are diagnosed either at around the age of 4 to 5 years, or in their teens and early adulthood (Blood *et al*., 1975). Type 1 diabetes is the result of an autoimmune reaction to proteins of the islets cells of the pancreas (Holt, 2004). There is a strong association between IDDM and other endocrine autoimmunity (for example, Addison disease) and an

increased incidence of autoimmune diseases are seen in family members of IDDM patients.

2.4.1 Pathogenesis of Type 1 Diabetes Mellitus

Type 1 diabetes mellitus is a chronic autoimmune disease associated with selective destruction of insulin producing pancreatic β-cells. The onset of clinical disease represents the end stage of β-cell destruction leading to type 1 diabetes mellitus. Al Homsi and Lukic (1992) explained that several features characterize type 1 diabetes mellitus as an autoimmune disease:

i. Presence of immuno-competent and accessory cells in infiltrated pancreatic islets,

ii. Association of susceptibility to disease with the class II (immune response) genes of the major histocompatibility complex (MHC), human leucocyte antigens (HLA),

iii. Presence of islet cell specific autoantibodies; Alterations of T cell mediated immunoregulation, in particular in CD4+ T cell compartment,

iv The involvement of monokines and TH1 cells producing interleukins in the disease process,

v. Response to immunotherapy,

vi. Frequent occurrence of other organ specific auto-immune diseases in affected individuals or in their family members.

The pathogenesis of selective β-cell destruction within the islet in type 1 DM is difficult to follow due to marked heterogeneity of the pancreatic lesions. At the onset of overt hyperglycemia, a mixture of pseudoatrophic islets with cells producing glycogen (a

cells), somatostatin (d cells) and pancreatic poly-peptide (PP cells), normal islets, and islets containing both b-cells and infiltrating lymphocytes and monocytes may be seen (Al-Homsi and Lukic, 1992). Lymphocytic infiltration is found only in the islet containing residual β cells and is likely that the chronicity with which type 1 DM develops reflects this heterogeneity of islet lesions (Al-Homsi and Lukic, 1992). In contrast to this chronicity in the natural history of the disease, β-cells are rapidly destroyed when pancreas is transplanted from identical twin donors into their long term diabetic twin mates in the absence of immunosuppression. In these cases, massive insulitis develops rapidly with infiltrating T lympocytes indicating an anamnestic autoimmune reaction (Al Homsi and Lukic, 1992). In addition, this observation also indicates that the chronic time course in type 1 DM (but not in a transplanted pancreas) is a consequence of down regulatory phenomena taking part in immune pathogenesis of the disease (Al Homsi and Lukic, 1992).

2.5 Type 2 Diabetes

Type 2 diabetes is the predominant form of diabetes and accounts for at least 90% of all cases of diabetes mellitus (Gonzalez *et al.*, 2009). The rise in prevalence is predicted to be much greater in developing than in developed countries (69% versus 20%) (Shaw *et al.*, 2010). In developing countries, people aged 40-60 years (that is, working age) are affected most, compared with those older than 60 years in developed countries (Shaw *et al*., 2010). This increase in type 2 diabetes is inextricably linked to changes towards a Western lifestyle (high diet with reduced physical activity) in developing countries and the rise in prevalence of overweight and obesity (Chan *et al.*, 2009; Colagiuri, 2010). The incidence of diabetes increases with age, with most cases being diagnosed after the age of 40 years. This equates to a lifetime risk of developing diabetes of 1 in 10 (Neil *et al.*, 1987). Type 2 diabetes is a heterogeneous disorder caused by a combination of

genetic factors related to impaired insulin secretion, insulin resistance and environmental factors such as obesity, over eating, lack of exercise, and stress as well as aging (Kaku, 2010). It is typically a multifactorial disease involving multiple genes and environmental factors to varying extents (Holt, 2004). Type 2 diabetes is the common form of idiopathic diabetes and is characterized by a lack of the need for insulin to prevent ketoacidosis. It is not an autoimmune disorder and the susceptible genes that predispose to NIDDM have not been identified in most patients. This could be due to the heterogeneity of the genes responsible for the susceptibility to NIDDM.

2.5.1 Obesity and Type 2 Diabetes Mellitus

Obesity has genetic as well as environmental causes. It has a strong effect on the development of type 2 DM (Bjorntorp, 1992; Haffner *et al*., 1992). Obesity is more than just a risk factor; it has a causal effect in the development of type 2 DM against a genetic background. The evolution from obesity to type 2 DM results from a succession of pathophysiological events:

(a) Augmentation of the adipose tissue mass, leading to increased lipid oxidation,
(b) Insulin resistance noted early in obesity, revealed by euglycemic clamp, as a resistance to insulin mediated glucose storage and oxidation, blocking the function of the glycogen cycle,
(c) Despite maintained insulin secretion, unused glycogen prevents further glucose storage leading to type 2 DM,
(d) Complete β-cell exhaustion appears later (Felber, 1992).

Type 2 DM patients have a characteristic shoulder, girdle-truncal obesity. Nutrient composition has also been found to be a risk factor for developing type 2 DM, where increased fat and decreased carbohydrate consumption have contributed to

hyperinsulinemia of obesity. Dietary fibers, both soluble and insoluble, improve type 2 DM. It is also found that simple sugars do not directly cause diabetes. Deficiency of micronutrients, such as chromium and copper, is found to be an important cause of type 2 DM in a minority of cases. Stress has also been thought to induce type 2 DM. Actually obesity and over availability of food rather than stress are the contributing factors to type 2 DM. Therefore, when permanent change in dietary habits is established, some people should be allowed to escape the "life-long" diagnosis of type 2 DM (Akinmokun *et al.,* 1992).

2.5.2 Obesity, T2DM, Immune Responses and Inflammation

Although insulin resistance seems to be a central abnormality, the origin of the impaired insulin action and the many other related complications of T2DM is still an enigma. Increased circulatory acute-phase response markers and pro-inflammatory cytokines in type 2 diabetic patients are first reported in 1997 (Pickup *et al.,* 1997). Since then, emerging evidences show that T2DM is a chronic inflammatory disease in which increased levels of cytokines are produced under various stimuli such as over nutrition, increasing age, genetic or fetal metbolic preprogramming (Dandona *et al*., 2004; Lumeng *et al*., 2009). This chronic inflammation will result in glucose intolerance, diabetes, and eventually, the diabetic complications.

Not only T2DM, obesity is also reported to be associated with a systemic but chronic inflammatory response characterized by altered cytokine production and activation of inflammatory signaling pathways. Many reports demonstrate the linkage between increased production of inflammatory cytokines, such as TNF-α, IL-6, and certain adipokines during the inflammatory process in obesity, to the development of insulin resistance (Anderson *et al*., 2008; Gustafson *et al*., 2007; Maury and Brichard, 2010).

2.6 Gestational Diabetes Mellitus (GDM)

For many years, GDM was defined as any degree of glucose intolerance that was first recognized during pregnancy (Ethridge *et al.,* 2014), regardless of whether the condition may have predated the pregnancy or persisted after the pregnancy. This definition facilitated a uniform strategy for detection and classification of GDM, but it was limited by imprecision. The ongoing epidemic of obesity and diabetes has led to more type 2 diabetes in women of childbearing age, resulting in an increase in the number of pregnant women with undiagnosed type 2 diabetes (Lawrence *et al.*, 2008). Because of the number of pregnant women with undiagnosed type 2 diabetes, it is reasonable to test women with risk factors for type 2 diabetes as (outlined above) at their initial prenatal visit, using standard diagnostic criteria.

Women with diabetes in the first trimester would be classified as having type 2 diabetes. GDM is diabetes diagnosed in the second or third trimester of pregnancy that is not clearly overt diabetes.

2.6.1 Maturity-Onset Diabetes of the Young (MODY)

MODY is characterized by impaired insulin secretion with minimal or no defects in insulin action (Stride *et al.*, 2002). It is inherited in an autosomal dominant pattern. Abnormalities at six genetic loci on different chromosomes have been identified to date. The most common form is associated with mutations on chromosome 12 in a hepatic transcription factor referred to as hepatocyte nuclear factor (HNF)-1a. A second form is associated with mutations in the glucokinase gene on chromosome 7p and results in a defective glucokinase molecule (Stride *et al.,* 2002). Glucokinase converts glucose to glucose-6-phosphate, the metabolism of which, in turn, stimulates insulin secretion by the b-cell. The less common forms of MODY result from mutations in other

transcription factors, including HNF-4a, HNF-1b, insulin promoter factor (IPF)-1, and NeuroD1 (Pearson *et al.,* 2003).

2.7 Animal Models for Diabetes Study

Rats and mice are animals commonly used for studying the effects of diabetes. Type 2 DM can be induced in animal models through dietary modification such as the administration of sucrose, fructose, high fat diet and glucose infusion or through genetic manipulation such as db/db mice, ob/ob mice, Goto-Kakizaki rats, Zucker diabetic rats and BHE rats (Kikutani and Makino, 1992).

On the other hand, type 1 diabetes can be replicated in animal models through genetic modifications i.e. non-obese diabetic mice (NOD), which spontaneously develop type 1 diabetes in a manner similar to humans (Kikutani and Makino, 1992). Other animal models genetically selected are the Bio Breeding rats (BB), in which the pancreatic islets are under the attack of immune T cells, B cells, macrophages and natural killer cells. At approximately 12 weeks of age, these diabetic rats presented weight loss, polyuria, polyphagia, hyperglycemia and insulinopenia. As in humans, if these rats are not treated with exogenous insulin, ketoacidosis is severe and fatal (Rees and Alcolado, 2005). Another way to obtain experimental animals with type 1 diabetes involves the administration of diabetogenic chemicals such as alloxan or streptozotocin (Mordes *et al*., 2004). Successful induction of diabetes was defined as a blood glucose level >13.2 mmol/l.

2.8 Toll like Receptors

The Toll-like receptors (TLRs) are part of a family of pattern-recognition receptors that recognize the molecular signatures of a variety of microbes (Akira *et al*., 2006). This group of proteins is growing in size and currently there are at least 13 TLRs identified

in mice and 11 in humans. Most of these TLRs are extracellular, except for TLRs 3, 7, 8, and 9, which recognize intracellular signals. The TLR family is known to consist of 10 members (TLR1-TLR10), and no doubt more will be found in the future (Medzhitov *et al*., 1997; Rock *et al*., 1998; Takeuchi *et al*., 1999; Chuang *et al*., 2000). The chromosomal location of each human TLR gene has been determined. TLR family members are characterized structurally by the presence of a leucine rich repeat (LRR) domain in their extracellular domain and a TIR domain in their intracellular domain. A comparison of the amino acid sequences of the human TLRs reveals that members of the TLR family can be divided into five subfamilies: the TLR3, TLR4, TLR5, TLR2 and TLR9 subfamilies. The TLR2 subfamily is composed of TLR1, TLR2, TLR6, and TLR10; the TLR9 subfamily is composed of TLR7, TLR8, and TLR9 (Takaeda *et al*., 2003). In the TLR2 subfamily TLR1 and TLR6 are highly similar proteins and exhibit 69.3% identity in overall amino acid sequence, but the TIR domains of both receptors are highly conserved, with over 90% identity (Takeuchi *et al*., 1999).

The cytoplasmic portion of a TLR is similar to that of the interleukin (IL)-1 receptor family. It is therefore called the Toll/IL-1 receptor (TIR) domain. A TIR domain is required for initiating intracellular signaling. The extracellular region of TLRs and IL-1R are markedly different. Whereas IL-1R possesses an Ig-like domain, TLRs contain LRRs in the extracellular domain. LRRs are responsible for the recognition of PAMPs (Akira *et al*., 2006).

Each LRR consists of a β-strand and an α-helix connected by loops. The LRR domain of TLRs was supposed to form a horseshoe structure with the ligand binding to the concave surface. However, the three-dimensional structure of the human TLR3 LRR motifs suggested that negatively charged dsRNA is more likely to bind to the outside convex surface of TLR3. It is uncertain whether this model fits the other TLR family

members. Future crystallographic analysis of other TLRs will be necessary for elucidating the ligand/receptor binding mechanism. The TLR family is an important group of receptors through which innate immunity recognizes invasive microorganisms (Satoshi and Shizuo, 2008). TLRs are key molecules for microbial elimination, such as the recruitment of phagocytes to infected tissues and subsequent microbial killing. Recent gene targeting studies have revealed that TLRs sense organisms ranging from bacteria to fungi, protozoa, and viruses (Satoshi and Shizuo, 2008).

2.8.1 Innate Immunity

In mammals, host defenses sense pathogen invasion through PRRs (Satoshi and Shizuo, 2008). Toll-like receptors are evolutionally conserved transmembrane proteins and play crucial roles as PRRs. Recent molecular biological studies (Satoshi and Shizuo, 2008), have clarified the function of TLRs in microbial infection. TLRs recognize specific components of microorganisms including fungi, protozoa, and viruses, and they induce innate immune responses (Satoshi and Shizuo, 2008).

The mammalian immune system is divided into two types of immunity: *innate* and *adaptive* (Satoshi and Shizuo, 2008). Adaptive immunity is characterized by specificity and develops by clonal selection from a vast repertoire of lymphocytes bearing antigen-specific receptors that are generated by gene rearrangement. This mechanism allows the host to generate immunological memory. However, it takes time for specific clones to expand and differentiate into effector cells before they can serve for host defense. Therefore, the primary adaptive immune system cannot induce immediate responses to invasive pathogens. To induce immediate responses when it encounters a pathogen, a host is equipped with innate, non-adaptive defenses that form pre emptive barriers against infectious diseases. Although the innate immune system was first described by

Elie Metchnikoff over a century ago, it has long been ignored: viewed as merely a nonspecific response to simple phagocytose pathogens and as something that presents antigens to the cells involved in acquired immunity (Satoshi and Shizuo, 2008).

The immune system detects and eliminates invading pathogenic microorganisms by discriminating between self and non-self. In mammals the immune system can be divided into two branches: “innate immunity” and “adaptive immunity.” Adaptive immunity detects nonself through recognition of peptide antigens using antigen receptors expressed on the surface of B and T cells. In order to respond to a wide range of potential antigens, B and T cells rearrange their immunoglobulin and T cell receptor genes to generate over 1011 different species of antigen receptors. Engagement of antigen receptors by the cognate antigen triggers clonal expansion of the lymphocyte and further production of antigen-specific antibodies. This highly sophisticated system is observed only in vertebrates and is a potent defense against microbial infection. In contrast, the innate immune system, which was first described over a century ago, is phylogenetically conserved and is present in almost all multicellular organisms (Kiyoshi *et al*., 2003). However, in 1996, Hoffmann and colleagues demonstrated that the *Drosophila* protein Toll is required for flies to induce effective immune responses to *Aspergillus fumigatus* (Lemaitre *et al*., 1996). This study made us aware that the innate immune system functions as a pathogen detector. The targets of innate immune recognition are conserved molecular patterns of microorganisms. Therefore, the receptors involved in innate immunity are called pattern recognition receptors (Medzhitov and Janeway, 1997). These molecular structures were originally called pathogen associated molecular patterns (PAMPs). However, it is more appropriate to designate them as microorganism-associated molecular patterns (MAMPs) since they are found not only in pathogenic but also in nonpathogenic microorganisms. MAMPs

are generated by microbes and not by the host, suggesting that MAMPs are good targets for innate immunity to discriminate between self and non-self. Furthermore, MAMPs are essential for microbial survival and are conserved structures among a given class, which allows innate immunity to respond to microorganisms with limited numbers of PRRs. There are many PRRs associated with opsonization, phagocytosis, complement and coagulation cascades, proinflammatory signaling pathways, apoptosis, and so on. Among them, Toll receptors and the associated signaling pathways represent the most ancient host defense mechanism found in insects, plants, and mammals (Akira *et al*., 2006). Studies of the fruit fly have shown that the Toll family is one of the most crucial signaling receptors in innate immunity.

2.9 Toll-Like Receptor 2

TLR2 (TIL4, CD282) was identified and characterized in 1998. It is an innate immune cell receptor that recognizes several pathogen-associated molecular patterns (PAMPs) and DAMPs and subsequently activates MYD88-dependent intracellular signaling. Chromosome 4 (4p32) is the location of the TLR2 gene. This molecule is a type I transmembrane protein that has a similar structure to other TLRs, consisting of the following domains from N-terminal to C terminal: extracellular leucine-rich repeat (LRR) domains; a transmembrane domain; and a toll/interleukin-1 receptor (TIR) domain. TLR2 is expressed in several immune cell types, including macrophages and dendritic cells, and non-immune cell types, including endothelial cells, epithelial cell lines and hepatocytes (Sepehri *et al*., 2016).

TLR2 recognizes its ligands in both homodimer and heterodimer (with TLR1 or 6) forms. In its homodimer form, it recognizes lipopolysacharide (LPS), porins, lipoprotein, lipoteichoic acid, bacterial peptidoglycan, viral hemagglutinin and

glycoproteins. In its TLR2/1 heterodimer form, it recognizes bacterial triacylated lipopeptides and synthetic triacylated lipopeptide (Pam3CSK4), and in its TLR2/6 heterodimer, it recognizes bacterial diacylated lipopeptides and lipoteichoic acid. As mentioned previously, TLR2 also recognizes some DAMPs as endogenous ligands, including human glycosaminoglycan hyaluronan, β-defensin3, heat shock proteins and high mobility group box 1 protein, some of which are released during inflammatory diseases like type 2 diabetes.

TLR2–ligand interactions lead to the activation of MAPK and MYD88-dependent signaling pathways. Although MYD88-dependent signaling pathway activation results in the phosphorylation and activation of pro-inflammatory transcription factors, such as IRF3, IRF7.

Therefore, it appears that TLR2-ligand interaction leads to either activation or suppression of immune responses. The principal mechanisms causing the activation or suppression of immune responses by TLR2 are unclear, but it has been hypothesized that the TLR2 ligand concentration may be the determining factor (Sepehri *et al*., 2016).

2.9.1 Pathogen Recognition by TLR

2.9.2 Bacteria

Lipopolysaccharide is a cell wall component of Gram-negative bacteria and a strong immunostimulant. TLR4 is essential for recognition of LPS, which is composed of lipid A (endotoxin), core oligosaccharide, and O-antigen. TLR4 recognizes lipid A of LPS. For LPS recognition, a complex formation of TLR4, MD2, and CD14 on various cells, such as macrophages and dendritic cells, is necessary (Shimazu *et al*., 1999). LPS is associated with an accessory protein, LPS-binding protein (LBP) in serum, which converts oligomeric micelles of LPS to monomers for delivery to CD14, which is a

glycosyl phosphatidyl inositol (GPI) anchored, high-affinity membrane protein. CD14 concentrates LPS for binding to the TLR4/MD2 complex (Takeda *et al*., 2003).

TLR2 recognizes various bacterial components, such as lipoproteins/lipopeptides and peptidoglycans from Gram-positive and Gram-negative bacteria, and lipoteichoic acid from Gram-positive bacteria, a phenol-soluble modulin from *Staphylococcus aureus*, and glycolipids from *Treponema maltophilum* (Takeda *et al*., 2003). TLR2 is also reported to be involved in the recognition of LPS from non-enterobacteria, including *Leptospira interrogans, Porphyromonas gingivalis*, and *Helicobacter pylori* (Takeda and Akira, 2005). These are atypical LPSs whose structures are different from typical LPSs of Gram-negative bacteria (Netea *et al*., 2002b). However, a recent report has indicated that lipoproteins contaminated in LPS preparation from *P. gingivalis* stimulated TLR2 and that LPS from *P. gingivalis* itself had poor TLR4 stimulation activity (Hashimoto *et al*., 2004).

2.9.3 Fungi

TLRs have been implicated in the recognition of the fungal pathogens such as *Candida albicans, Aspergillus fumigatus, Cryptococcus neoformans* and *Pneumocystis carinii* (Takeda *et al*., 2003). Several components located in the cell wall or cell surface of fungi have been identified as potential ligands. Yeast zymosan, derived from *Saccharomyces cerevisiae*, activates TLR2/TLR6 heterodimers, whereas mannan, derived from *S. cerevisiae* and *C. albicans*, are detected by TLR4. TLR4-deficient mice show increased susceptibility to disseminated candidiasis due to the decreased release of chemokines and the impaired recruitment of nutrophils to infected sites (Netea *et al*., 2002a). Phospholipomannan, present on the cell surface of *C. albicans*, is also recognized by TLR2, while TLR4 mainly interacts with glucuronoxylomannan, the major capsular polysaccharide of *C. neoformans* (Netea *et al*., 2004). Dectin-1 is a

lectin family receptor for the fungal cell wall component, β-glucan, which is a major component of zymosan (Brown *et al*., 2002). Dectin-1 has been reported to functionally collaborate with TLR2 in response to yeast (Netea *et al*., 2004). The Dectin-1mediated signaling pathway uses spleen tyrosine kinase (Syk), and interactions with Syk directly induce cellular responses such as the respiratory burst and IL-10 production. Destin-1 is also reported to collaborate with TLR2 and to induce proinflammatory responses such as the induction of TNF-α and IL- 12 (Rogers *et al*., 2005).

2.9.4 Virus

TLR4 recognizes not only bacterial components but also viral envelope proteins. The fusion (F) protein from respiratory syncytial virus (RSV) is sensed by TLR4 (Kurt-Jones *et al*., 2000). C3H/HeJ mice were sensitive to RSV infection (Haynes *et al*., 2001). The envelope protein of mouse mammary tumor virus (MMTV) directly activates B cells via TLR4 (Rassa *et al*., 2002). TLR2 has also been reported to be involved in the recognition of envelope proteins of measles virus, human cytomegalovirus, and HSV-1 (Bieback *et al*., 2002; Compton *et al*., 2003; Kurt-Jones *et al*., 2004).

2.10 Toll like Receptors in Diabetes

Activation of the innate immune response and subsequent pro inflammatory reactions is mediated by mammalian TLRs which recognises microbial components. Among the TLRs, TLR2 and TLR4 play a critical role in the pathogenesis of insulin resistance, diabetes, and atherosclerosis in both clinical and experimental conditions (DeFronzo and Ferrannini, 1991; Hoffman *et al*., 1999; Wong and Weng, 2008; Olefsky and Glass, 2010). Ligands for TLR2 and TLR4 include high-mobility group B1 protein (HMGB1), heat shock protein (HSP) 60, HSP70, endotoxin, hyaluronan, advanced glycation end products, and extracellular matrix components. TLRs interact with these endogenous

ligands which are also elevated in diabetes (Tsan and Gao, 2004; Taylor *et al*., 2004; Wagner, 2006; Chiu *et al*., 2009; Hreggvidsdottir *et al*., 2009). Consequently, different TLRs are amenable to targeting by different types of agents. Several studies in both experimental and clinical conditions have demonstrated that, TLR2 and TLR4 among the TLRs play a critical role in the pathogenesis of insulin resistance, inflammation and diabetes due to their involvement in mechanisms that are elevated in diabetic condition and TLR mediated responses in diabetes (Mohammad *et al*., 2006; Song *et al*., 2006; Senn, 2006; Shi *et al*., 2006; Kim *et al*., 2007; Reyna *et al*., 2008; Creely *et al*., 2007; Dasu *et al*., 2010).

Apoptosis is a process of removing harmful or useless cells and is fundamental to the maintenance of mammalian homeostasis. Apoptotic cells are rapidly engulfed by phagocytes such as macrophages, and thereby the release of potentially cytotoxic or inflammatory cellular contents is prevented. Phagocytosis of apoptotic cells can also provide immunosuppressive effects. Type 1 diabetes (T1D) is an autoimmune disorder resulting from the specific destruction of pancreatic b-cells producing insulin. In pancreatic lymph nodes (PLNs), b-cell specific T cells are primed by dendritic cells (DCs) that ferry the debris from apoptotic b-cells, and then activated T cells invade islets thereafter. This event may be pronounced in non-obese diabetic (NOD) mice, an animal model of T1D, which might reflect the reported defect in the phagocytosis of apoptotic cells by macrophages in those mice (O'Brien *et al*., 2006). These studies suggest that apoptotic cells should be cleared at an early stage so that they could be prevented from undergoing secondary necrosis that would deliver endogenous stimuli that could activate the immune system. Kim *et al*. (2007) study investigated the mechanisms by which the interaction between phagocytes and apoptotic islet cells triggers immune responses and the development of autoimmune diabetes. They found

that apoptotic b-cells undergoing secondary necrosis could stimulate the priming of diabetogenic T cells through a toll-like receptor 2 (TLR2)-dependent activation of antigen-presenting cells (APCs). The relevance of this finding was further supported by marked inhibition of the development of autoimmune diabetes in Tlr2_/_ mice. They explored whether an increased cell death and accumulation of dead cells could promote autoimmune diabetes through a TLR2-dependent mechanism in vivo. For this purpose, they employed two types of autoimmune diabetes models—multiple low dose streptozotocin (STZ) model and non-obese diabetic mouse model. When administered in multiple low dose, STZ, a selective cytotoxic agent against pancreatic b-cells, induces accumulation of apoptotic b-cells and development of autoimmune diabetes in susceptible strains (Elias *et al.*, 1994). It was found that Tlr2_/_ mice of C57BL/6 background were significantly resistant to the STZ-induced autoimmune diabetes compared to Tlr2+/+ mice.

Fatty acids and their derivatives are essential biological molecules as the components of cellular membrane and the major components of stored fat in the form of triacylglycerols.

Fatty acids are known to affect the cellular physiological function (proliferation, differentiation, apoptosis, and metabolism) and also implicated in the modulation of a variety of pathological states including obesity, hyperlipidemia, diabetes, atherosclerosis, hypertension, inflammation, and cancer. Recent evidence suggests that non-microbial molecules can activate TLRs. Endogenous TLR agonists released from host in the body may cause sterile inflammatory responses and simultaneously unnecessary immune responses. Therefore, the activation of TLRs by microbial pathogens, tissue injury, and stress leads to the expression of mediators for both immune and inflammatory processes. The original purpose of TLR activation is to

initiate and to amplify immune responses as host defense system against microbial infection. However, the accompanying inflammatory responses are unavoidable. If the inflammatory responses are not appropriately controlled and are prolonged, tissue injury may be progressed and chronic inflammatory diseases can be further developed. It is well known that excessive activation of TLRs can lead to the development of severe systemic inflammation including septic shock with high mortality (Lee and Hwang, 2006).

Free fatty acids levels of which are elevated in obesity, due to increased release from expanded adipose tissue, have been hypothesized to cause insulin resistance. Indeed, FFA infusion in vivo has been shown to impair the ability of insulin to suppress hepatic glucose production and to stimulate glucose uptake into skeletal muscle (Dresner *et al*., 1999), which in turn leads to insulin resistance. In light of data indicating a pathophysiologic role for FFAs in inflammation and insulin resistance, it is intriguing that the lipid component of LPS is sufficient to trigger TLR4 signaling. In particular, a medium-chain fatty acid component of LPS, lauric acid, has been shown to initiate TLR4 signaling in a macrophage cell line (Lee *et al*., 2003).

A study conducted by Dasu *et al*. (2008) and Schwartz *et al.* (2010) in humans demonstrated that interactions among increased glucose levels, elevated NEFAs (non-esterified fatty acids) and resultant pro-inflammatory cytokines in diabetes have clear implications for the immune system. Studies in animal models as well as humans have suggested that diabetes might be associated with changes in the innate immune response (Pino *et al*., 2010). Mohammad *et al.* (2006) reported increased TLR2 and TLR4 expression in bone marrow-derived macrophages of Type 1 diabetic NOD mice, correlating with increased NF-κB (nuclear factor κB) activation in response to endotoxins and increased pro-inflammatory cytokines. Recently, Dasu *et al*. (2010)

have shown increased TLR2 and TLR4 expression, intracellular signalling and TLR mediated inflammation in monocytes with a significant correlation with HbA1c (glycated haemoglobin) levels in diabetic patients. Creely *et al.* (2007) have shown increased TLR2 expression in adipose tissue from Type 2 diabetic patients with strong correlations with plasma endotoxin levels. In addition, Song *et al.* (2006) reported increased TLR4 mRNA expression in differentiating adipose tissue of *db*/*db* mice. Furthermore, recent findings have shown increased TLR2/TLR4 expression, signalling, ligands and functional activation in diabetic subjects with and without complications (Dasu *et al*., 2010; Devaraj *et al*., 2010). Findings from above researches suggest a potential role for TLR2 and TLR4 in the pathology of diabetes.

2.11 Molecular Mechanisms that Link High Glucose, Non Esterified Fatty Acids, Cytokines and Reactive Oxygen Species with TLR-Mediated Innate Immune Responses in Diabetes

2.11.1 Hyperglycaemia

Hyperglycaemia which means high blood sugar levels is implicated in the development of diabetes and considered to be the major cause of diabetic complications (Danaei *et al*., 2011). Hyperglycaemia induces damage in tissues via: (i) increased polyol pathway flux; (ii) increased intracellular AGE (advanced glycation end products) formation; (iii) increased expression of receptors for advanced glycation end products (RAGE) and activating ligands in vascular cells; (iv) increased protein kinase C activation and (v) increased hexosamine pathway flux (Dasu *et al*., 2008; Yang and Seo, 2008). Mitochondrial superoxide production is the process that underlies the above hyperglycaemia induced pathogenic mechanisms (Dasu *et al*., 2012). Dasu *et al*. (2008)

showed that a high-glucose dose time-dependently induces a marked increase in TLR2 and TLR4 mRNA and protein expression in human monocytes.

2.11.2 Non Esterified Fatty Acids (NEFA)

Serum concentrations of fatty acids are well elevated in established cases of metabolic disorders such as dyslipidaemias, obesity and diabetes (Tripathy *et al*., 2003). Saturated fatty acids, inflammation and Insulin Resistance clearly have a link according to studies by (Lee *et al*., 2003; Shi *et al*., 2006; Lee and Hwang, 2006). Studies by Schwartz *et al*. (2010), have indicated that increased concentrations of SFAs lead to the activation of TLR2 and TLR4 potentially inducing inflammation, with supporting proof-of-concept *in vivo* studies (Himes and Smith, 2010; Ehses *et al*., 2010; Kuo *et al*., 2011). Thus it is generally thought that SFAs induce inflammation (Lee *et al*., 2004), whereas mono unsaturated fatty acids (MUFA) increase insulin sensitivity in diabetic patients (Lee *et al*., 2004), and healthy subjects (Vessby *et al*., 2001).

2.11.3 Cytokines

Hyperglycaemia and elevated NEFA levels induce inflammation, characterized by the increased expression of pro-inflammatory cytokines and activation of NF- κB linked with IR and diabetes (Wellen and Hotamisligil, 2005). Research carried out by Dasu *et al.* (2010), similar with Shanmugam *et al*. (2003), have shown that high-glucose treatment activates monocytes and induces an increase in TNFα, IL-1β and MCP (monocyte chemoattractant protein)- 1 gene expression. Findings from Dasu *et al.* (2010) showing increased levels of these cytokines in the monocytes under high glucose were similar to those observed in bovine retinal endothelial cells (Song *et al*., 2006), human macrophages (Shashkin *et al*., 2006) and human pancreatic islets (Maedler *et al*., 2002).

2.11.4 Reactive Oxygen Species

A pro-inflammatory state is caused by changes in the reduction–oxidation balance, typically seen in metabolic injury and diabetes. ROS include reactive products such as superoxide anions, H_2O_2 and hydroxyl radicals, which are formed as by-products of mitochondrial oxidative phosphorylation (Giacco and Brownlee, 2010; Gill *et al*., 2010), activation of phagagocyte NADPH oxidase and glycoxidation via interactions with RAGEs (Giacco and Brownlee, 2010) in diabetes. Mounting evidence indicates that TLRs may be involved in this response. The consequences of inflammation from oxidative stress injuries can lead to complications, death or disability (Dasu *et al*., 2012). ROS can also be produced during *β*-oxidation of fatty acids, especially as a by-product of peroxisomal acyl-CoA oxidase activity.

2.11.5 TLR2 and TLR4 in the Pathogenesis of Type 1 Diabetes

Recently, it has become evident (Kenia *et al*., 2015) that the dysregulation of the innate immune system can precipitate autoimmune diseases, including T1D. Given its essential role in orchestrating innate immune responses, the TLRs may be expected to play a significant role in the T1D development, progression, and its complications. The connections among inflammation, hyperglycemia, and diabetes have clear implications for the immune system (Kenia *et al*., 2015). In addition, TLRs activate two types of downstream signaling pathways that lead to the activation of NF-κB with concomitant increase in inflammatory cytokine secretion. Both pathways contribute significantly to the pathophysiology of inflammation in endothelial dysfunction and are relevant to diabetic microangiopathy. Therefore, the key point regarding the involvement of TLRs in T1D and its complications seems to be the inflammatory process (Kenia *et al*., 2015). The activation of the innate immune system via TLRs, in particular, TLR2 and TLR4, seems to play an important role in the development of T1D. Many authors proposed

the sensing of DAMPs released from damaged pancreatic β-cells by TLR2 to be first event in the development of T1D. The Increased expression of TLR2 and TLR4 in monocytes was described in patients with T1D compared to healthy patients. In addition, the expression of TLR2 is augmented in T1D in both rat and human kidneys and has been associated with vascular complications. Furthermore, T1D patients with microvascular complications showed increases in TLR2 and TLR4 activity in monocytes compared with matched controls (Devaraj *et al*., 2011). The higher expression of TLR2 and TLR4 is associated with poor glycemic control, while the knockdown of both TLR2 and TLR4 resulted in a 76% decrease in a high glucose-induced NF- κB response, suggesting an additive effect. Also, it has been demonstrated that deletion of TLR2 in mice significantly abrogates the proinflammatory state of T1D for up to 14 weeks in mice and improves the wound healing process, supporting a role for TLR2 in promoting inflammation in diabetes. There are ample data supporting an important role for inflammation associated with atherosclerosis in T1D and TLRs may be mediating this process. A recent study demonstrated that TLR2 and TLR4 mediate inflammatory pathways in endothelial cells exposed to high glucose (Kenia *et al*., 2015).

2.12 Type 2 Diabetes and TLR2 and TLR4

DAMPs are endogenous molecules that are produced and released by several cell systems during inflammation or infection (Sammy and Lim, 2015; Shin *et al*., 2015). They can also be released during type 2 diabetes (Singh *et al*., 2015). Both can be recognized by TLR2, leading to the either activation or suppression of immune cells. It has been documented that inflammation is a major cause of pancreatic beta cell dysfunction in type 2 diabetes (Nackiewicz *et al*., 2014). Moreover, inflammatory markers link the pathology of insulin resistance and type 2 diabetes. Activation of the

innate immune system via toll-like receptors (TLRs) is implicated in the pathogenesis of insulin resistance, diabetes, and atherosclerosis (Curtiss and Tobias, 2009). Complimentary genetic studies link TLR2 and TLR4 polymorphisms to type 2 diabetes, suggesting a casual relationship between TLR function and diabetes and its complications (Bagarolli *et al*., 2009). Therefore, the inflammatory effects of TLR2-ligand interaction may be an important factor in type 2 diabetes progression. Nackiewicz *et al*. (2014) showed that interaction between TLR2/6 and its related ligands results in the activation of macrophages and the production of IL-1 and IL-6 as pro-inflammatory cytokines that contribute to islet inflammation (Nackiewicz *et al*., 2014).

Several studies confirmed the important roles played by reactive oxygen species (ROS) in the pathogenesis of type 2 diabetes. Interestingly, activation of TLR2 by zymosan leads to ROS production by neutrophils in a manner dependent on TLR2 NADPH oxidase but not dependent on MAPK (Sepehri *et al*., 2016). Hyperglycemia and chronic periodontitis also lead to up regulation of TLR2 in the gingival tissue of type 2 diabetes patients (Promsudthi *et al*., 2014). Interestingly, it has been demonstrated that insulin suppresses the expression of TLR2 at the mRNA level, possibly via down regulation of PU.1 (Ghanim *et al*., 2008). Kuzmicki *et al*. (2013) revealed that the gestational diabetes patients had significantly higher TLR2 expression than pregnant women with normal glucose tolerance (Kuzmicki *et al*., 2013). Interestingly, TLR2 was found to be up regulated in women who exhibited normal glucose tolerance but later developed gestational diabetes when compared to the women who remained normoglycemic. Ahmad *et al*. (2012) demonstrated that the expression levels of TLR2 were up regulated in obese individuals (Ahmad *et al*., 2012). Additionally, they showed that obese type 2

diabetes patients had higher expressions of TLR2 in comparison to obese patients without type 2 diabetes (Ahmad *et al*., 2012).

Another study also identified that TLR2 not only participates in the development of type 2 diabetes but is also involved in the pathogenesis of related vascular complications (Jialal and Kaur, 2012). Moreover, via up regulation of IL-6 and osteopontin, TLR2 causes impaired insulin-mediated brain activities, which are an early step in the development toward type 2 diabetes (Sartorius *et al*., 2012). Duarte *et al*. (2012) revealed higher mRNA levels of TLR2 in gingival biopsies from type 2 diabetes patients with chronic periodontitis in comparison to periodontally healthy patients (Duarte *et al*., 2012). Thus, it appears that the up regulation of TLR2 is a marker of type 2 diabetes rather than a marker of periodontitis. Another study identified that free fatty acids and high glucose levels up regulate the expression of TLR2 and TLR6, which resulted in increased activity of monocytes and increased production of superoxides, which are released in an NF-kB-dependent manner (Dasu *et al*., 2012). Ehses *et al*. (2010) also reported that a high fat diet was unable to induce insulin resistance and beta cell dysfunction in TLR2-deficient mice. Free fatty acids also play important roles in the induction of inflammation in pancreatic beta cells via TLR2 (Boni-Schnetzler *et al*., 2009). Interestingly, another study showed that not only TLR2 has been more highly expressed on the immune cells of type 2 diabetes patients than on those of healthy subjects, but also the levels of TLR2 ligands, including hyaluronan, HSP60, HSP70, HMGB1 and endotoxin, were higher (Dasu *et al*., 2010). TLR2 inhibition using a TLR2 antisense oligonucleotide (ASON) leads to recovery of insulin sensitivity and signaling in muscle and white adipose tissue of mice that were fed a high-fat diet (Caricilli *et al*., 2008). It has also been documented that oxidized LDL, which is produced during type 2

diabetes, induced expression of TLR2 in macrophages (Holvoet, 2007) The expression of TLR2 on the monocytes of obese women is also higher (Ajuwon *et al*., 2009).

TLR2 also recognizes PAMPs such as exogenous microbial ligands, so it may be hypothesized that microbial infections could be important factors in the development of type 2 diabetes and may also participate in the pathogenesis of the disease. Interestingly, a study by Ajuwon *et al*. (2009) revealed that peptidoglycan derived from *Staphylococcus aureus* resulted in elevated TLR2 expression on the 3 T3-L1 adipocytes cell lines (Ajuwon *et al*., 2009). Chen *et al*. (2012) identified that treatments with agents that improve glycemic control are associated with decreased expressions of TLR2 and its related intracellular signaling molecules (Chen *et al*., 2012). Previous studies demonstrated that decreased methylation is associated with higher expression of the genes (Rozenberg *et al*., 2014). Accordingly, CpGs methylation in the promoter of the TLR2 gene was significantly decreased in type 2 diabetes patients in comparison to the levels for the controls (Remeley *et al*., 2014).

2.13 TLRs as Sensors for Metabolic Disorders

The ‘danger’ theory by Matzinger (2002), proposed that endogenous molecules, namely damage/danger-associated molecular patterns (DAMPs), can activate the immune system in a fashion analogous to pathogen-associated molecular patterns (PAMPs). The identification of endogenous ligands for TLRs has greatly extended our knowledge of the pathogenic role of TLRs in various noninfectious inflammatory conditions such as diabetes, atherosclerosis and chronic kidney disease (Dasu *et al*., 2010). In addition to the damage-associated endogenous ligands mentioned above, emerging evidence showed that TLRs might be the molecular link between inflammation and metabolic syndrome associated disorders such as hyperglycemia, dyslipidemia, hyperuricemia and

hemodynamic abnormalities: (i) Hyperglycemia: In vitro studies showed that high glucose (HG) induced TLR2 and TLR4 expression via PKC and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activation, and knocking down of TLR2 and TLR4 significantly down regulated HG-induced NF-κB activation (Dasu *et al*., 2008). One of the mechanisms involved in HG-induced TLR activation in tubular epithelial cells could be the secretion of HMGB1, which is a nuclear DNA binding protein that regulates transcription and promotes inflammatory response by binding to TLR2 and TLR4 (Kim *et al*., 2011). (ii) Dyslipidemia: Because saturated FFAs are essential components for LPS to activate TLR signaling, it is postulated that FFAs modulate insulin resistance via TLRs. In vitro, FFAs could activate TLR2/4 NF-κB signaling in macrophages (Nguyen *et al*., 2007) muscle cells, podocytes (Cha *et al*., 2013) and vascular endothelial cells (Kim *et al*., 2007), which is closely associated with the inflammatory mechanisms of insulin resistance. Fetuin-A could act as an endogenous ligand of TLR4 to promote FFAs-induced insulin resistance (Pal *et al*., 2012). Besides, FFAs also augment HG-induced proinflammatory effects in macrophages, adipocytes and podocytes via enhancing the expression of TLRs (Cha *et al*., 2013). In vivo, TLR4 deficiency may protect against diet-induced obesity and insulin resistance via inhibition IκB kinase and c-Jun NH2-terminal kinase pathway. Furthermore, atherogenic lipoproteins such as minimally modified low-density lipoprotein (Bae *et al*., 2009) and oxidized low-density lipoprotein (Miller *et al*., 2005) trigger inflammation in monocytes via interaction with TLR4/TLR2, while advanced glycation end products-modified LDL can induce IL-6 and IL-8 production via the TLR2/4-MyD88-dependent pathway in tubular epithelial cells (Cheng *et al*., 2013). (iii) Uric acid: UA induced endothelial cells to release nuclear HMGB1, which then activated NF-κB activity and angiopoietin-2 expression via TLR4 in an autocrine/paracrine manner. In vivo, TLR2/4-

deficient mice were protected from monosodium urate monohydrate crystal-induced inflammation or tubulointerstitial nephritis (Rabadi *et al*., 2012). (iv) Hemodynamic abnormalities: Angiotensin II (Ang II) promotes TLR4 expression by increasing AP-1-dependent TLR4 gene transcription in mouse mesangial cells and podocytes (Bondeva *et al*., 2007). Blockade of RAS by candesartan (Lv *et al*., 2009) or aldosterone receptor antagonist (Liu *et al*., 2012) could attenuate TLR4 expression and the associated downstream proinflammatory and apoptotic events. (v) Oxidative stress: TLR4 mediates oxidative stress induced hemorrhagic shock. In vitro, H_2O_2 induced TLR4/myD88 signaling in macrophages (Powers *et al*., 2006). In addition, oxidative stress induces the formation of an end product of lipid oxidation, ω-(2-carboxyethyl) pyrrole (CEP), which may promote angiogenesis and endothelial migration through TLR2 signaling in a MyD88-dependent manner (West *et al*., 2010). Therefore, the metabolic substrates associated with diabetes may directly interact with TLRs or indirectly promote the production of TLR endogenous ligands which then trigger the downstream events and the development of diabetes and diabetic complications. Further investigation into the mechanisms of TLR signaling activation in the diabetic state will facilitate the identification of more endogenous ligands for TLRs, which could have implications for targeting TLR signaling.

2.14 Therapeutic Modulation of TLRs

TLRs fulfill many of the criteria that are required to be considered potential therapeutic targets (Elizabeth *et al*., 2010). These include over expression in disease, knockout mice being resistant to disease in disease models, ligands exacerbating inflammation in disease models and genetic differences in TLRs (or their signaling proteins) correlating with risk of disease (Elizabeth *et al*., 2010). The studies described above suggest the possibility that TLR-mediated inflammation is key in the pathological mechanisms

underlying diabetes and the consequent development of complications, and may be suppressed by anti-inflammatory treatments (Devaraj *et al*., 2010). Besides, the ability of TLRs to initiate and propagate inflammation makes them attractive therapeutic targets (Dunne *et al*., 2011). However, as with many targets for anti-inflammatory agents, the approach is empirical. Given the available literature on TLRs in diabetes and its complications, we can be optimistic that targeting them will prove useful. Some of the strategies currently used for decreasing inflammation in diabetes include using statins, PPAR-γ (peroxisome-proliferatoractivated receptor-γ) agonists [for example TZD (thiazolidinedione)], ARBs (angiotensin receptor blockers), phytochemicals and $n-3$ fatty acids (Giacco and Brownlee, 2010). All of these treatments exert their pleiotropic effects by inhibiting TLR mediated inflammation, besides controlling diabetes co-morbidities, such as hyperglycaemia, dyslipidaemia and hypertension.

2.14.1 Statins

Statins are chemically defined as a HMG-CoA (3- hydroxy-3-methylglutaryl CoA) reductase inhibitors and have been shown to effectively lower LDL (low-density lipoprotein)-cholesterol levels and reduce cardiovascular events in diabetic and non-diabetic patients (Dasu *et al*., 2012). The pleiotropic beneficial effects of statins appear to be via anti-inflammatory actions and exceed their cholesterol-lowering effects (Dasu *et al*., 2012). Statin treatment results in a reduction in NF-κB activity and a subsequent decrease in pro-inflammatory cytokines such TNF-α and IL-6. Furthermore, statins inhibit LPS mediated activation of human peripheral mononuclear cells and endothelial cells. Findings indicate that the anti-inflammatory effects of statins may involve TLRs, and it has been shown that statins inhibit TLR4 and TLR2 expression, with a concomitant decrease in TLR signalling and effector cytokine/chemokine release. Therefore, statins, which target TLR mediated signalling pathways in human peripheral

mononuclear cells and endothelial cells, may be a good strategy in preventing the chronic inflammation seen in diabetes and associated CVD (cardiovascular disease). This hypothesis was supported further by results showing the inhibitory effects of simvastatin and atorvatstatin on monocyte TLR4 expression in normolipaemic patients and patients with high cholesterol levels (Dasu *et al*., 2012).

2.14.2 PPAR-γ agonists (TZDs)

The pathophysiology initiating the development of inflammation remains poorly understood, in part due to the complexity of the interaction of multiple cells (monocytes, macrophages, T cells, adipocytes and endothelial cells) and organ systems plus the diversity of intracellular perturbations within these systems that mediate the development of diabetes (Gerber 1998; EDPG, 1999). Increasing our understanding of this biology will require the combination of studying cross-talking signalling networks and the pleiotropic effects of known therapeutic drugs. PPAR-γ is a member of the nuclear hormone receptor superfamily that plays an important role in the regulation of inflammatory and immune reactions (Daynes and Jones, 2002). Their protective effects in inflammatory diseases may be correlated with the suppression of a key pro-inflammatory transcription factor, NF-κB (Dehmer *et al*., 2004), up-regulation of antioxidase (Tao *et al*., 2003), inhibition of pro-inflammatory mediators (Dasu *et al*., 2009) and prevention of monocyte migration, adhesion and infiltration (Ramirez *et al*., 2008). Whether these effects are operational in the immune system is not clear and, if present, whether these changes are linked to inflammation is also unclear. TLR activation by glucose is linked with the inflammation seen in diabetes (Dasu *et al*., 2008). Therefore, to understand the mechanisms of the anti-inflammatory effects of pioglitazone, Dasu *et al*. (2009) investigated whether selective PPAR-γ agonists are effective inhibitors of TLR activation both *in vitro* and *in vivo*. Thus it may be

reasonable to postulate that PPAR-γ agonists could negatively regulate inflammatory responses through TLR pathways and play protective effects in diabetic complications.

2.14.3 ARBs

Ang II (angiotensin II), in addition to stimulating vasoconstriction, also induces an increase in ROS and a pro-inflammatory phenotype via AT1Rs (Ang II type 1 receptors). ARBs are widely used as antihypertensive drugs and have been reported to possess anti-inflammatory effects. Ang II, following engagement of the AT1R, promotes vasoconstriction, oxidative stress, inflammation and atherosclerosis (Dzau, 2001; Nickenig and Harrison, 2002; Suzuki *et al*., 2003). ARBs, such as candesartan, prevent cerebrovascular events and also help reduce the progression of coronary heart disease (Demers *et al*., 2005). Candesartan is widely used for the treatment of high blood pressure (Heeneman *et al*., 2007), the management of chronic heart failure (Tsutamoto *et al*., 2000) and diabetic nephropathy (Donaire and Reulope, 2007), to reverse endothelial dysfunction (Ghiadoni *et al*., 2000) and to attenuate oxidative stress (Koh *et al*., 2003). Candesartan has been reported to have anti-atherosclerotic effects, such as reducing neointimal formation in rats (Igarashi *et al*., 2001) and diminishing vascular inflammation (Koh *et al*., 2006). Given the anti-inflammatory effects of ARBs and the expression of TLRs in inflammatory conditions, such as diabetes, we have shown that candesartan decreases TLR2 and TLR4 protein level and mRNA expression and reduces NF-κB p65-dependent activation, with a concomitant reduction in key inflammatory mediator production *in vitro*. Furthermore, administration of candesartan to mice resulted in significant reduction in TLR2 and TLR4 expression compared with vehicle control C57BLJ/6 mice (Dasu *et al*., 2009). These findings may have pathophysiological and clinical implications for patients with chronic inflammatory diseases, such as diabetes (Creely *et al*., 2007) and atherosclerosis (Shinohara *et al*.,

2007), because these patients have enhanced TLR2 and TLR4 expression, leading to increased inflammation via the expression of inflammatory mediators such as IL-1β, IL-6, TNF-α and MCP-1.

2.14.4 Phytochemicals

Phytochemicals are chemical compounds that occur naturally in plants and may affect human health. Several lines of evidence suggest that diabetes-related pathologies can be prevented or improved by the intake of phytochemicals that can control inflammation. Inflammatory responses in activated cells are regulated by master regulators of inflammation such as NF- κB (Giacco and Brownlee, 2010). Moreover, TLR2 and TLR4 are reported to mediate inflammation in activated cells via NF-κB signaling (Medzhitov and Janeway, 2002). Therefore, targeting these inflammatory receptors using phytochemicals may be a useful strategy to prevent or ameliorate the development of diabetes and its related diseases. Several plant-derived components can modulate inflammatory responses via various mechanisms, some of which are dependent on TLRs, whereas others are TLR-independent by attenuating downstream NF-κB signalling. Recent studies have shown that certain phytochemicals inhibit TLR-mediated pro-inflammation. Capsaicin, the spicy component in chili peppers, has anti-inflammatory properties in addition to its metabolic properties. It exerts its anti-inflammatory effects in macrophages by inhibiting MCP-1 and IL-6 secretion, and NF-κB inactivation (Dasu *et al*., 2012).

2.14.5 Omega-3 fatty acids

SFAs directly induce inflammatory responses in macrophages via TLR2/TLR4, whereas long-chain *n*−3 PUFAs, such as DHA and EPA, are known to inhibit TLR2/TLR4 expression, activity and downstream signalling, and are considered as

potential anti-inflammatory agents (Zhau *et al*., 2011). Fish oil contains high concentrations of DHA and EPA, and is considered to be a good source of *n*−3 PUFAs (Todoric *et al*., 2006). TLR2/TLR4 activity and PPAR-*γ* activation is involved in the prevention of high-fat or high-energy-diet-induced tissue inflammation and remodeling by long-chain *n*−3 PUFAs, suggesting that the anti-inflammatory mechanisms of *n*−3 PUFA action are diverse and require detailed investigation. Furthermore, *n*−3 PUFAs need many cofactors, such as folic acid, vitamins, tetrahydrobiopterin, minerals and l-arginine, for their physiological functions. Hence these cofactors should also be provided in adequate amounts to bring about the anti-inflammatory actions of *n*−3 PUFA in obesity and diabetes (Dasu *et al*., 2012).

2.15 The Future of Modulators of TLRs

Increasing evidence using well-characterized approaches suggests the involvement of TLRs in metabolic disturbances and bridging immune responses to metabolic homoeostasis. However, efforts to identify modulators of TLR-dependent signaling and inflammation in diabetes and complications are significantly understudied. To date, there are no approved therapeutic agents targeting TLR2/TLR4 that have been shown to play a pivotal role in initiating and propagating persistent inflammation in diabetes. On the other hand, there are a variety of small-molecule inhibitors, compounds or antibodies under different developmental stages for allergy (TLR4, Pollinex Quatro; Allergy Therapeutics), pain management (TLR4, AV411; Avigen), autoimmunity, chronic inflammation and ischemia/reperfusion injury (TLR2, OPN305; Opsona Therapeutics), and inflammatory bowel disease and rheumatoid arthritis (TLR2, OPN401; Opsona Therapeutics) targeting the TLRs. The availability of TLR structures may now aid medicinal chemistry in the rational design of small-molecule agonists or antogonists. Non-traditional approaches to drug discovery, such as miRNAs

(microRNAs), which regulate genes involved in immune responses, have been identified (Dasu *et al*., 2012).

2.16 Streptozotocin Induced Diabetes Rat Model

2.16.1 Streptozotocin

Streptozotocin (STZ) is a glucosamine-nitrosourea which is commonly used to induce experimental diabetes in animals (Szkudelski, 2001; Leiter, 1982). It specifically targets beta cells, entering via the glucose transporter GLUT2 and causing alkylation of DNA (Melmed *et al*., 1973). DNA damage induces activation of poly ADP-ribosylation, depletion of cellular NAD^+ and ATP, and formation of superoxide radicals, leading to the destruction of beta cells (Szkudelski, 2001). The effectiveness of STZ depends on the level of GLUT2 expression, which in turn may be influenced by age, sex, strain, or species (Leiter, 1982; Kramer *et al*., 2009).

2.16.2 Metformin

Metformin is a biguanide that lowers blood glucose levels primarily by improving insulin sensitivity in the liver, where it effectively inhibits gluconeogenesis (Hundal *et al*., 2000), whereas it does not have marked hypoglycaemic effects (Arguand *et al*., 1993).

The discovery of metformin began with the synthesis of galegine-like compounds derived from *Gallega officinalis*, a plant traditionally employed in Europe as a drug for diabetes treatment for centuries (Godarzi and Brier-Ash, 2005). The clinical usefulness of metformin was discovered by Stern in 1950. They observed that the dose–response of metformin was related to its glucose lowering capacity and that metformin toxicity also displayed a wide security margin (Godarzi and Brier-Ash, 2005). Metformin acts primarily at the liver by reducing glucose output and, secondarily, by augmenting glucose uptake in the peripheral tissues,

chiefly muscle. These effects are mediated by the activation of an upstream kinase, liver kinase B1 (LKB-1), which in turn regulates the downstream kinase adenosine monophosphatase co-activator, transducer of regulated CREB protein 2 (TORC2), resulting in its inactivation which consequently down regulates transcriptional events that promote synthesis of gluconeogenic enzymes. Inhibition of mitochondrial respiration has also been proposed to contribute to the reduction of gluconeogenesis since it reduces the energy supply required for this process. Metformin's efficacy, beneficial cardiovascular and metabolic effects, and its capacity to be associated with other antidiabetic agents makes this drug the first glucose lowering agent of choice when treating patients with type 2 diabetes mellitus (TDM2).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 EXPERIMENTAL ANIMALS

Thirty Albino Wistar rats weighing 180-200g and comprising of 15 males and 15 females were purchased from the Animal House, Faculty of Pharmaceutical Sciences, Usmanu Danfodiyo University, Sokoto. The animals were housed under similar conditions in standard cages at room temperature for a period of 28 days (19/10/2017 to 17/11/2017) in the Department of Pharmacology, Faculty of Pharmaceutical Sciences, Usmanu Danfodiyo University, Sokoto.

3.2 EXPERIMENTAL DESIGN

The rats were randomly divided into three equal groups of 10 rats each as follows:

GROUP 1: Non-diabetic rats as controls

GROUP 2: Diabetic untreated rats

GROUP 3: Diabetic rats treated with metformin.

3.3 INDUCTION OF DIABETES MELLITUS

Experimental DM was induced by a single intraperitoneal injection of freshly dissolved streptozotocin (60mg/kg) (Akbarzadeh *et al*., 2007) in normal saline maintained at 37°C to Groups 2 and 3 rats. A similar volume of normal saline was given to control rats (Group 1). Glucose (Munro limited, Lagos, Nigeria) solution at 10%, was used as rat drinking water for 24 hours to prevent increased glucose level in the blood. Streptozotocin causes degeneration of beta-cells of the islets of Langerhans leading to outburst of insulin (Akbarzadeh *et al*., 2007).

3.4 INCLUSION AND EXCLUSION CRITERIA

Seventy two hours following intraperitonial streptozotocin (60mg/kg) injection, DM was confirmed by estimating fasting blood glucose through the tail artery after the rats fasted overnight and only rats from Groups 2 and 3 that have fasting blood glucose level of ≥7.0mmol/L which was considered as diabetic (Akbarzadeh *et al*., 2007) were used in the study. Similarly, rats from group 1 were also tested and only those with FBG of < 7.0mmol/L were included in the study as controls.

3.5 TREATMENT

The following treatment was administered after the induction of DM:

Metformin was given orally (250mg/kg) to Group 3 rats which was adjusted weekly based on fasting blood glucose estimation. Metformin treatment ran daily for a period of 28 days with weekly measurement of body weight, fasting blood glucose estimation and adjustment of metformin administration.

3.6 SAMPLE COLLECTION

On the last day of the metformin treatment (Day 28), the rats were fasted overnight and anaesthetized by dropping each in a transparent plastic jar saturated with chloroform vapor (Dallatu *et al.*, 2009). Blood sample was obtained through cardiac puncture and immediately aliquoted into Fluoride Ethylenediamine tetra acetic acid (EDTA) (Agary, China) and plain containers (Agary, China). The blood sample containers were appropriately labeled with the corresponding animal identification number. Serum and plasma were obtained by centrifuging the samples at 3000rpm for 5 min and separated into pre-labelled plain containers and refrigerated at 4ºC. Humane procedure was adopted throughout the experiment (Dallatu *et al.*, 2009).

3.7 Analytical Methods

3.7.1 Estimation of Plasma Glucose

Plasma glucose concentrations were estimated using Oxidase-Peroxidase Method as reported by Trinder *et al.* (1969).

3.7.1.1 Principle

Glucose oxidase (GOD) catalyses the oxidation of glucose to give hydrogen peroxide and gluconic acid. In the presence of enzyme peroxidase (POD), the hydrogen peroxide is broken down and the oxygen released reacts with 4-aminophenazone (4-aminoantipyrine) and phenol to give a pink color. The absorbance of pink color is directly proportional to the concentration of glucose in the sample.

3.7.1.2 Procedure

Glucose reagent, 0.5ml was dispensed into a two clean test tubes labelled as test and standard and 10µl of the plasma was added for test and 10µl of glucose standard was added for the standard. The mixtures were incubated for 15 minutes at 37^{o}C after which they were each diluted with 1.5ml of distilled water. The absorbance was then read at 490nm. The plasma glucose concentrations were calculated as follows:

Plasma glucose concentration = $\frac{absorbance\ of\ Test}{absorbance\ of\ standard}$ x concentration of standard (5.5mmol/L)

3.7.2 Estimation of Serum Toll-Like Receptor 2

Estimation of serum toll-like receptor 2 was performed according to manufacturer's instruction as follows:

3.7.2.1 Principle

This uses enzyme linked immunosorbent assay-double antibody sandwich principle to assay Toll-like receptor 2 (TLR2) level in the sample. The microelisa stripplate coated by purified Toll-like receptor 2 antibody to make solid phase antibody, then add TLR2

to wells, combine with TLR2 antibody labelled by HRP, become antibody-antigen-enzyme-antibody complex. After washing completely to remove the uncombined enzyme, add chromogen solution A and chromogen solution B, the color of the liquid changes into blue. And at the effect of acid, the color finally becomes yellow. The color change is measured spectrophotometrically at a wavelength of 450nm. The concentration of TLR2 in the sample is then determined by comparing the OD of the samples to the standard curve.

3.7.2.2 Procedure

Standard dilution: One original standard reagent was provided in the kit. Serial dilutions of the standard were made in the steps below.

Ten (10) standard wells were marked on the microtiter plate coated with purified toll-like receptor 2 (TLR2) antibody to make solid phase antibody. Standard solution, 100µl was added to wells 1 and 2 separately and standard solution, 50µl was added to the wells separately and mixed gently and then 100µl was taken out from the wells separately and added it to wells 3 and 4 separately. Standard solution, 50µl was added to wells 3 and 4 separately. The contents were mixed gently and 50µl was taken out of wells 3 and 4 separately and discarded. Then 50µl from wells 3 and 4 were taken out separately and added them to wells 5 and 6 separately. Standard solution of 50µl was added to wells 5 and 6 and mixed gently. Then 50µl was taken out from wells 5 and 6 separately and added to wells 7 and 8 separately. Standard solution of 50µl was added to wells 7 and 8 separately and mixed gently. Then 50µl were taken out from wells 7 and 8 added it to wells 9 and 10 separately. Standard solution was added to wells 9 and 10 separately and mixed gently. Then 50µl were taken out from wells 9 and 10 and discarded it. Finally, 50µl remained in each well after diluting (density: 24ng/mL, 16ng/mL, 8ng/mL, 4ng/mL, 2ng/mL respectively).

Addition of Sample: blank and sample wells were set. (To blank well, sample and HRP conjugate was not added. Only chromogen solutions A and B and stop solution were allowed; the other steps were the same). Sample dilution, 40µl was added to sample wells followed by addition of 10µl of serum (final sample diluted was 5-fold). Care was taken to avoid touching the bottom of the wells during addition samples and it was then mixed gently.

Addition of HRP Conjugate reagent: HRP conjugate reagent, 50µl was taken into each well except blank well.

Incubation: after closing plate with closure plate membrane, it was incubated for 30 minutes at 37°C.

Preparation of solution: diluted wash solution concentration (30 x) into deionized water to prepare 600ml of wash solution (1x).

Washing: operated according to washing methods.

Colour development: Chromogen solutions A and B, 50µl each were added to each well, covered and incubated for 10 minutes at 37°C.

Stopping the reaction: stop solution of 50µl was added into each well to stop the reaction (the blue colour changed into yellow immediately).

Final measurement: blank well was set as zero, measured the optical density (OD) under 450nm wavelength which was carried out within 15 minutes after adding the stop solution.

Assay Range

1 ng/mL – 30 ng/mL

3.7.3 Estimation of Serum Toll Like Receptor 4

Estimation of serum toll-like receptor 4 was performed according to manufacturer's instructions as follows:

3.7.3.1 Principle

This uses enzyme linked immunosorbent assay-double antibody sandwich principle to assay Toll-like receptor 4 (TLR4) level in the sample. The microelisa stripplate coated by purified Toll-like receptor 4 antibody to make solid phase antibody, then add TLR4 to wells, combine with TLR4 antibody labelled by HRP, to become antibody-antigen-enzyme-antibody complex. After washing completely to remove the uncombined enzyme, add chromogen solution A and chromogen solution B, the color of the liquid changes into blue. And at the effect of acid, the color finally becomes yellow. The color change is measured spectrophotometrically at a wavelength of 450nm. The concentration of TLR4 in the sample is then determined by comparing the OD of the samples to the standard curve.

3.7.3.2 Procedure

Standard dilution: One original standard reagent was provided in the kit. Serial dilutions of the standard were made in the steps below.

Ten (10) standard wells were marked on the microtiter plate coated with purified toll-like receptor 4 (TLR4) antibody to make solid-phase antibody. Standard solution, (100µ) was added to wells 1 and 2 separately, and standard solution, 50µl was added to the wells 1 and 2 separately and mixed gently, and then 100µl was taken out from the wells separately, and added to wells 3 and 4 separately. Standard solution (50µl) was added to wells 3 and 4 separately. The contents were mixed gently and, 50µl was taken out from wells 3 and 4 separately and discarded. Then 50µl from wells 3 and 4 were taken out from wells 3 and 4 separately and added to wells 5 and 6 separately. Standard solution of (50µl) was added to wells 5 and 6 and mixed gently. Then, 50µl were taken

out from wells 5 and 6 separately, and added to wells 7 and 8 separately. Standard solution of 50µl was added to wells 7 and 8 separately and mixed gently.

Then, 50µl was taken out from wells 7 and 8, and added to wells 9 and 10 separately. Standard solution (50µl) was added to wells 9 and 10 separately and mixed gently. Then, 50µl was taken out from wells 9 and well 10 and discarded it. Finally, 50µl remained in each well after diluting. (density:18ng/mL, 12ng/mL, 6ng/mL, 3ng/mL, 1.5ng/mL respectively).

Addition of Sample: blank and sample wells were set. (To blank well, sample and HRP conjugate was not added. Only chromogen solutions A and B, and stop solution were allowed; the other steps were the same). Sample dilution 40µl was added to sample wells, followed by addition of 10µl of serum (final sample diluted is 5-fold). Care was taken to avoid touching the bottom of the wells during addition of samples, and it was then mixed gently.

Addition of HRP Conjugate reagent: HRP conjugate reagent 50µl was taken into each well, except blank well.

Incubation: after closing plate with closure plate membrane, it was incubated for 30 minutes at 37°C.

Preparation of solution: diluted wash solution concentration (30 x) into deionized water to prepare 600ml of wash solution (1x).

Washing: operated according to washing methods.

Colour development: Chromogen solutions A and B, 50µl each were added to each well, covered and incubated for 10 minutes at 37°C.

Stopping the reaction: stop solution of 50µl was added into each well to stop the reaction (the blue colour changed into yellow immediately).

Final measurement: blank well was set as zero, measured the optical density (OD) under 450nm wavelength which was carried out within 15 minutes after adding the stop solution.

ASSAY RANGE

0.4 ng/mL – 20 ng/mL

3.7.4 GLYCOSYLATED HEMOGLOBIN GHb (Ion Exchange Resin Method) (Trivelli *et al*., 1971)

3.7.4.1 Principle

Glycosylated haemoglobin (GHb) has been defined operationally as the fast fraction haemoglobins (Hb A1a, A1b A1c) which elute first during column chromatography. The non-glycosylated haemoglobin which consists of the bulk of haemoglobin, has been designated HbA0. A haemolysed preparation of whole blood is mixed continuously for 5 mins with a weakly binding cation exchange resin. The labile fraction is eliminated during the hemolysate preparation. Hemolysate preparation and the mixing period, a filter separator is used to remove the resin from the supernatant. The percent glycosylated hemoglobin is determined by measuring absorbance of the ratio of the absorbances. The ratio of the absorbances of GHb and THb of the control and test is used to calculate the percent GHb of the glycosylated haemoglobin (Ghb) and the total haemoglobin fraction (THb).

3.7.4.2 Hemolysate Preparation

0.5ml lysing reagent was dispensed into tubes labelled as Control (C) and Test (T). 0.1ml of the reconstituted control and well mixed blood sample was added into the appropriately labelled tubes. It was mixed until complete lysis was evident, then it was allowed to stand for 5 mins.

3.7.4.3 GLYCOSYLATED HEMOGLOBIN (GHb) SEPARATION (Trivelli *et al*., 1971).

Caps were removed from the ion exchange resin tubes and labelled as Control and test. 0.1ml of the hemolysate from step A was added into the appropriately labelled ion exchange resin tubes. A resin separator was inserted into each tube so that the rubber sleeve is appropriately 1cm above the liquid level of the resin suspension. The tubes were mixed on a vortex mixer continuously for 5mins. The resin was allowed to settle, resin separator was pushed into the tubes until the resin was firmly packed. The supernatant was poured directly into a cuvette and each absorbance was measured against distilled water.

3.7.4.4 TOTAL HEMOGLOBIN (THb) FRACTION

5.0ml of distilled water was dispensed into tubes labelled as control and test. 0.2ml of the hemolysate from step A was added into the appropriately labelled tube. It was mixed well.

Each absorbance was read against distilled water (Trivelli *et al*., 1971).

3.7.4.5 CALCULATIONS

Ratio of control R_C = Absorbance of Control GHb/Absorbance of Control THb

Ratio of test (R_T) =Abs. Test GHb/ Abs. Test THb

GHb in % Ratio of Test(R_T)/ Ratio of Control(R_C) x 10(value of control).

3.7.5 Body Weight- Gravimetric Method

The rats were measured individually in each group by placing them in a container that was at zero reading on a weighing balance. The values were taken in grams.

3.8 DATA ANALYSIS

The data generated were analyzed using statistical package JMP SAS software Cary, USA and Invivostat version 3.7 and presented as mean±standard error of mean (mean±SEM). The mean values of the plasma glucose, TLR2, TLR 4 and glycated haemoglobin obtained from the three groups of the experimental rats were compared using multivariate analysis of variance (MANOVA). Mean difference with p-value of less than 0.05 ($p<0.05$) was considered as significant.

Plasma glucose concentrations and body weight for treatment and sex effects measured overtime (weeks) was analyzed using mixed model analysis. Least significant difference (LSD) was used in the post-hoc test.

CHAPTER FOUR

4.0 RESULTS

4.1 GENERAL OBSERVATIONS

During the 28 days period of the study, all the male and female wistar rats of the control group, diabetic non-treated group and diabetic treated group appeared apparently healthy. The diabetic treated with metformin group showed little signs of weakness and poor feeding at the beginning of treatment. They later appeared apparently healthy.

4.2 BODY WEIGHT ANALYSIS

The results of body weight for the different treatment groups showed that there were no significant difference between the weight of rats in control (137.8 ± 5.50g), diabetic non-treated (147.2 ± 5.50g) and diabetic treated groups (142.9 ± 5.99g), $p = 0.4901$. Based on sex, the results showed that the weight of male wistar rats (151.8 ± 4.75g) used for the study was significantly higher than the weight of female wistar rats (133.4 ± 4.50g), $P = 0.0080$. Also, all the rats used for the study showed a significant growth from the first to the fourth weeks of the study, $p< 0.0001$ as shown in Fig.4.1.

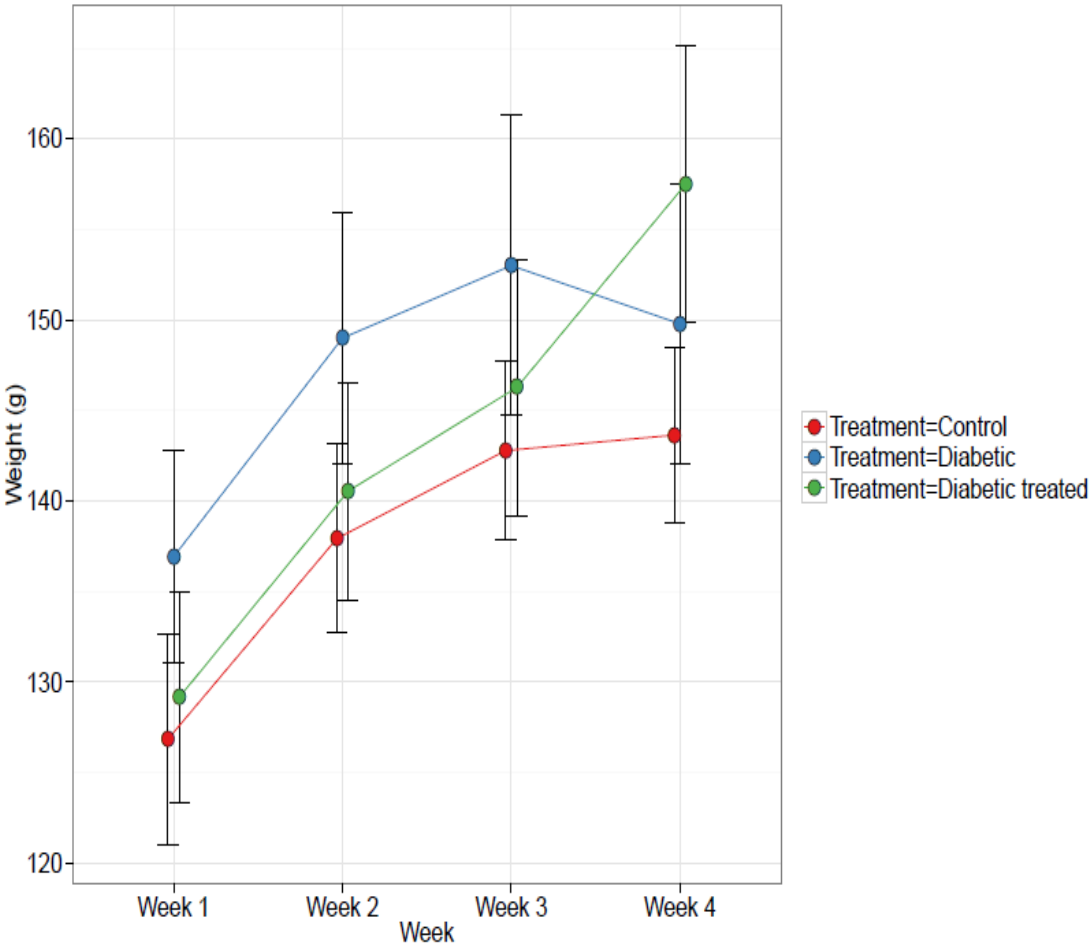

Fig.4.1: Shows the mean weekly body weight of Control, Diabetic and Diabetic treated rats.

4.3 EFFECTS OF DIABETES ON TLR2 SERUM CONCENTRATION

The results of TLR2 for the different treatment groups are represented in Fig. 4.2. The results showed that the presence of DM in wistar rats significantly increased the concentration of TLR2 in both diabetic treated (2.19 ± 0.21ng/ml) and diabetic non-treated (2.18 ± 0.17ng/ml) groups compared to controls (1.72±0.17ng/ml), p = 0.04. However, the results showed no significant difference in TLR2 concentration between diabetic non-treated rats (2.18 ± 0.17) in Group 2 and diabetic treated rats (2.19 ± 0.21) in Group 3 (p = 0.2791).

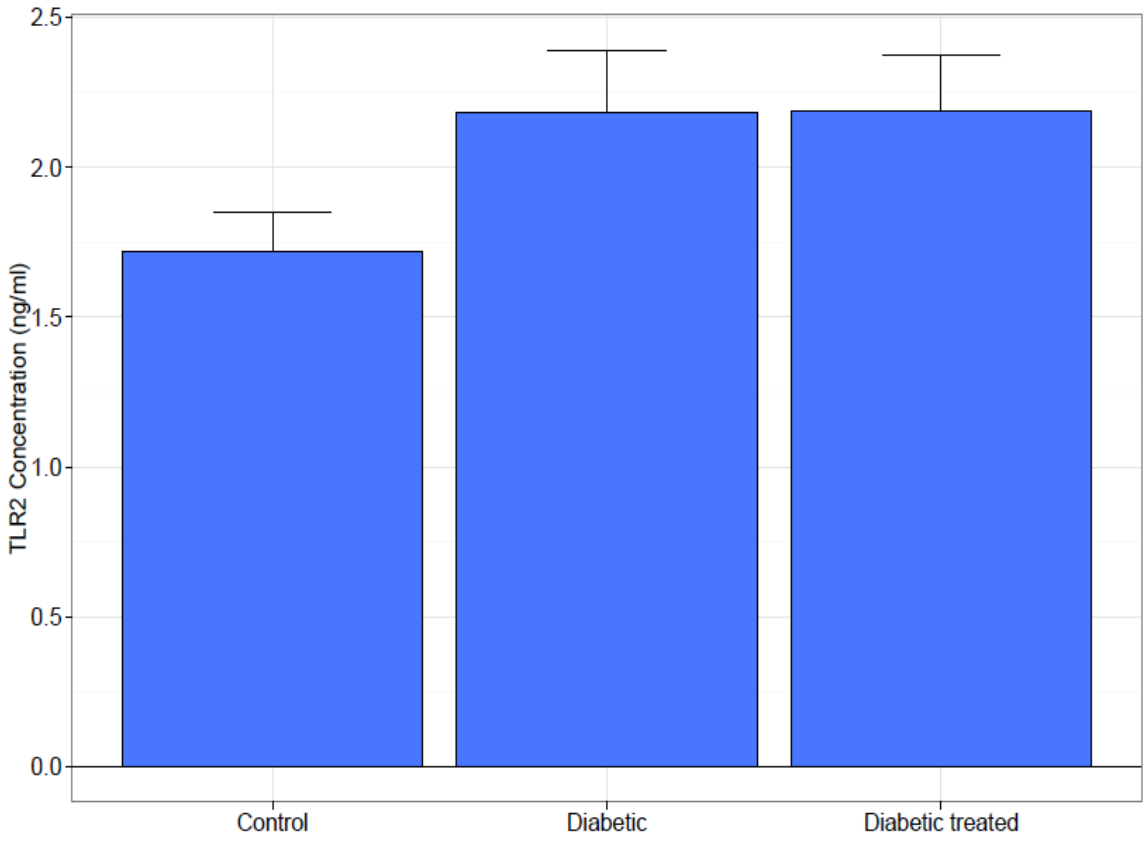

Fig.4.2: Bar graph showing the mean TLR2 concentration comparison between control, Diabetic and Diabetic treated rats.

4.4 EFFECT OF DIABETES ON TLR4 SERUM CONCENTRATION

The pattern of TLR4 in the different animal groups is illustrated in Fig. 4.3. The Figure showed that the presence of DM in wistar rats has no significant effect on the concentration of TLR4 in both diabetic treated (2.20 ± 0.23ng/ml) and diabetic non-treated (2.18 ± 0.19ng/ml) groups compared to controls (2.25 ± 0.19), p =0.9657. The results also showed no significant difference in TLR4 concentration between diabetic non-treated rats (2.18 ± 0.19) in Group 2 and diabetic treated rats (2.20 ± 0.23) in Group 3 (p = 0.8359).

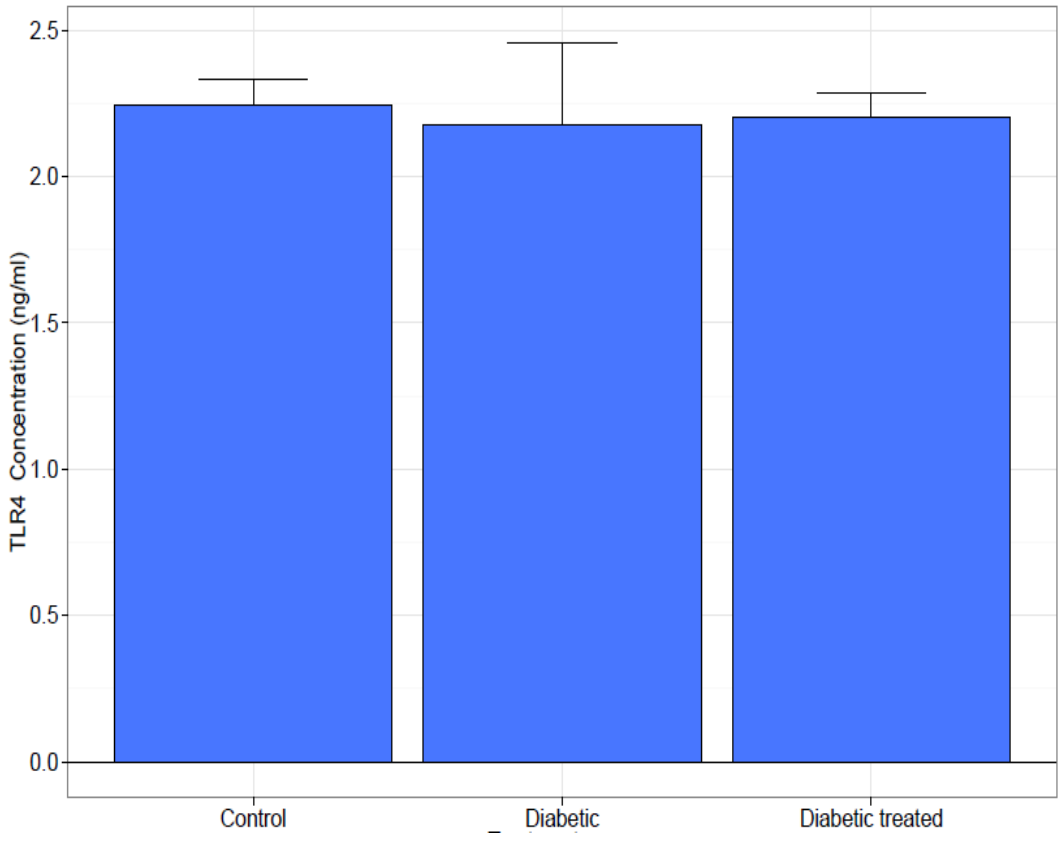

Fig.4.3: A bar graph showing the mean TLR4 concentration comparison between Control, Diabetic and Diabetic treated rats.

4.5 EFFECT OF SEX ON SERUM TLR2 AND TLR4 CONCENTRATIONS IN DIABETIC RATS

Table 4.1 shows the effect of sex on TLR2 and TLR4 concentrations in diabetic rats. The result showed that sex has no significant effect on TLR2 concentration between male (1.86 ± 0.15) and female (2.15 ± 0.15) rats ($p = 0.1790$). There was no significant difference between TLR4 concentrations of male (2.24 ± 0.17) and female (2.18 ± 0.16) rats ($p = 0.7656$).

Table 4.1 also shows no significant difference exists in HBA1C levels between male (6.79 ± 0.40) and female (7.74 ± 0.38) rats ($p = 0.0761$), but the results showed that FBG was significantly higher in female (10.64 ± 0.78) than male (8.06 ± 0.80) rats ($p = 0.0328$).

Table 4.1: Effect of Sex on serum TLR2 and TLR4 concentration

Parameter	Male (n = 18)	Female (n = 19)	P-value
TLR2 (ng/ml)	1.86 ± 0.15	2.15 ± 0.15	0.1790
TLR4 (ng/ml)	2.24 ± 0.17	2.18 ± 0.16	0.7656
FBG (ng/ml)	8.06 ± 0.80	10.64 ± 0.78	0.0328
HBA1C (mmol/mol)	6.79 ± 0.40	7.74 ± 0.38	0.0761

Values are mean ± SEM, n= number of subjects.

4.6 EFFECTS OF METFORMIN TREATMENT ON FASTING BLOOD GLUCOSE LEVEL IN DIABETIC RATS

Results of fasting blood glucose for the different treatment groups are represented in Fig. 4.4. The results showed that diabetic treated rats of Group 3 (11.58 ± 1.12) produced no significant difference (p = 0.1139) compared to diabetic non-treated rats (10.84 ± 0.90) of Group 2. It also showed that the metformin treatment failed to lower the fasting blood glucose levels of diabetic treated rats (11.58 ± 1.12) to a level statistically similar to that of non-diabetic control Group (6.51 ± 0.90), p = 0.0014.

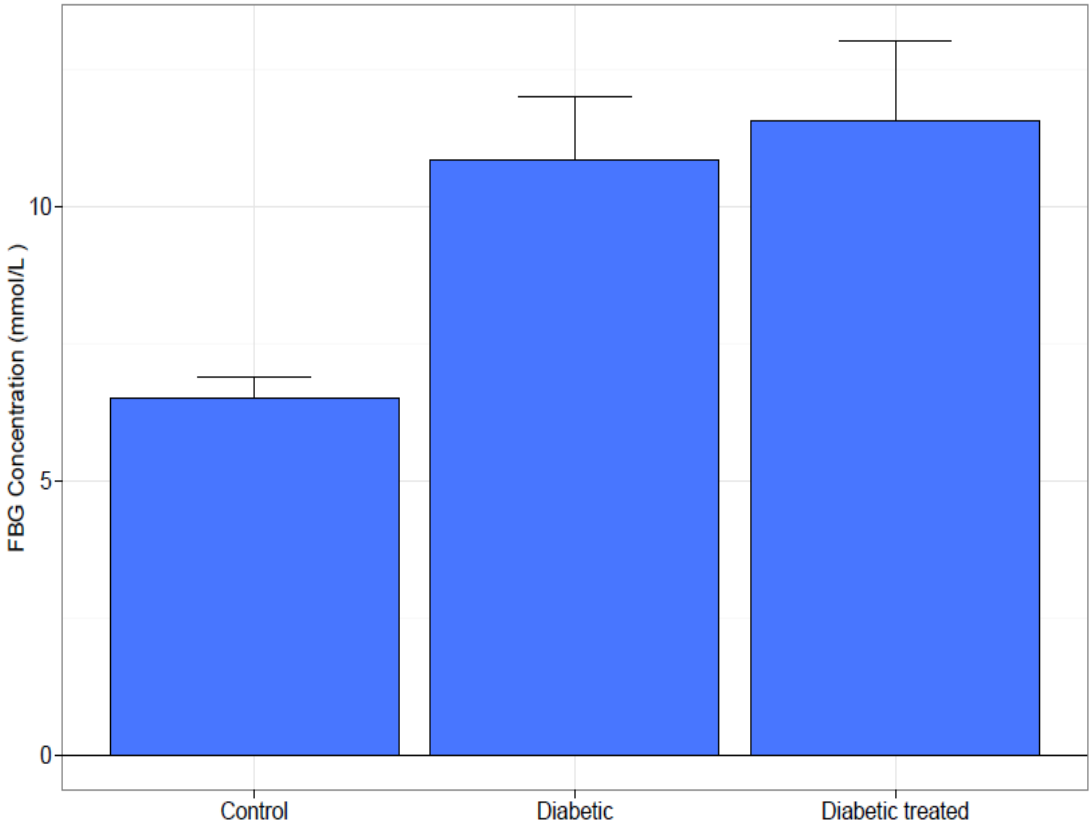

Figure 4.4: A bar graph showing the mean fasting blood glucose concentrations comparison between Control, Diabetic and Diabetic treated rats.

4.7 EFFECTS OF METFORMIN TREATMENT ON BLOOD GLYCATED HEMOGLOBIN IN DIABETIC RATS

Fig. 4.5 showed the results of blood glycated hemoglobin concentration for the different treatment groups. The results showed that metformin treatment on diabetic rats of Group 3 (6.45 ± 0.55) produced a significant difference (p = 0.0007) in HBA1C levels compared to non-treated diabetic rats (8.77 ± 0.44) of Group 2. It also showed that the metformin treatment lowered the blood glycated hemoglobin levels of diabetic treated rats (6.45 ± 0. 0.55) to a level statistically similar to that of control Group (6.32 ± 0.44), when compared with diabetic non-treated group.

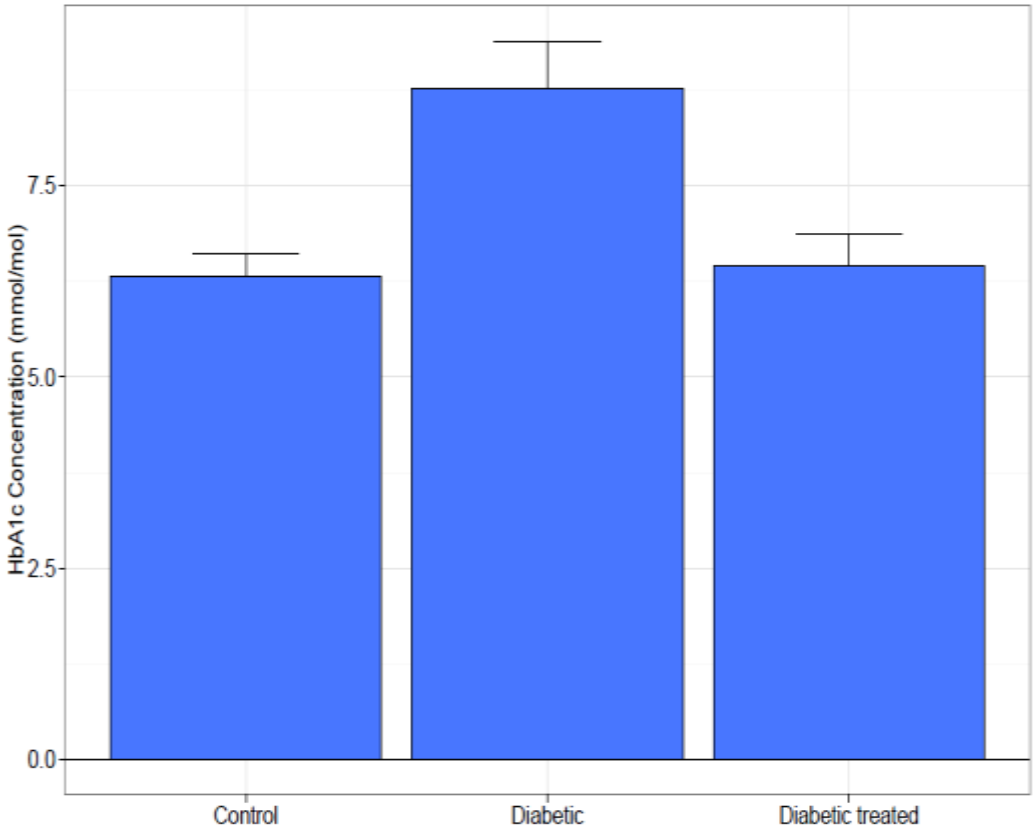

Figure 4.5: Bar graph showing the mean glycated hemoglobin concentration comparison between Control, Diabetic and Diabetic treated rats.

CHAPTER FIVE

5.0 DISCUSSION

Diabetes mellitus is a common metabolic endocrine disease characterized by the disturbance of glucose and fat metabolism and rise of glucose level in the plasma (Orchard, 1996). Its pathologic and physiological characteristics are the absolute or relative insulin hyposecretion and increased activity of pancreatic glucagon which result in the metabolic disturbance. In this study, TLR2, TLR4, FBG, HBA1C were assayed in control, diabetic treated and non-treated rats.

From the current study, it was observed that there was no significant difference between the weight of rats in control, diabetic non-treated and diabetic treated rats used for the study. The study also showed that the weight of male rats is significantly higher than the female rats, and overall, the wistar rats used showed a significant growth from the first week of study to the fourth. Although polyphagia has many causes, there is a strong link between polyphagia and diabetes (Metzger *et al*., 2010). Diabetes affects the body's ability to utilize and produce glucose which is necessary for normal body function (Orchard, 1996). When blood glucose is low, hunger sets in and risk of overeating, thus gaining weight (Bel Marra health report, 2016).

At present, there is no physiological explanation for major sex differences with regards to weight loss (Lovejoy and Sainsbury 2009), but some trials indicate that women lose less weight than males including the more harmful visceral fat mass (Lovejoy and Sainsbury 2009).

Hyperglycaemia induces inflammation, increases apoptosis and induces oxidative stress (Dasu *et al*., 2010). In the current study, levels of TLR2 in experimental diabetic rats showed a significant increase in both diabetic treated and diabetic non-treated group as

compared to control. However, the results showed no significant difference in TLR2 levels between diabetic non-treated and diabetic rats treated with metformin. Several studies carried out are in conformity with our findings. Ahmad *et al.* (2012) demonstrated that the levels of TLR2 were upregulated in obese individuals. In addition, they showed that obese type 2 diabetic patients had higher levels of TLR2 in comparison to obese patients without type 2 DM. Creely *et al.* (2007) showed increased TLR2 levels in the adipose tissue of type 2 DM with strong correlates to endotoxin levels. Duarte *et al.* (2012) revealed higher mRNA levels of TLR2 in gingival biopsies from type 2 diabetic patients with chronic periodontitis in comparison to healthy patients. Another study identified that free fatty acids and high glucose levels upregulate the levels of TLR2 and TLR6 which resulted in increased activity of monocytes and increased production of superoxides, which are released in an NF-kB-dependent manner (Dasu and Jialal, 2011). Interestingly, another study showed that not only TLR2 has been more highly expressed on the immune cells of type 2 diabetic patients than on those of healthy subjects, but also the levels of TLR2 ligands such as hyaluronan, Heat Shock Protein 60, Heat Shock Protein 70, High Mobility Group Box 1 and endotoxin were elevated (Dasu *et al*., 2010).

Also in this study, the induction of DM in rats had no significant effect on the concentration of TLR4 in both diabetic treated and diabetic non-treated as compared to control, and the difference between the expression of TLR4 in diabetic non-treated and diabetic treated was not significant. Few reports agree with our findings such as Creely *et al.* (2007) who reported non increase in TLR4 activation in adipose tissue. Furthermore, they demonstrated that circulating endotoxin levels are associated with atherosclerosis. A study by Dandona *et al.* (2013) reported a decrease in TLR4 expression in mononuclear cells as a result of short term insulin infusions administered

to diabetic patients. Melody *et al.* (2014) showed decreased TLR4 protein expression in a cardiac circle membrane extract during conditions of prolonged (46h) insulin infusions in healthy equine subjects. Contrary to our findings, an overwhelming number of research demonstrated a clear increase in levels of both TLR2 and TLR4 under high glucose concentrations in both clinical and experimental conditions (Shi *et al*., 2006; Devaraj *et al*., 2008; Wong and Weng, 2008; Curtiss and Tobias, 2009; Lu *et al*., 2015). The expression and function of TLR4 are elevated in monocyes of diabetic patients (Dasu *et al*., 2008). Abnormalities in glucose concentration have been reported to elevate and activate TLR4 to promote the secretion of inflammatory cytokines in mouse messengial cells and contribute to diabetic nephropathy (Lu *et al*., 2015). Miao *et al.* (2012) demonstrated an increased expression of TLR4 which correlated with CD68+ cell infiltration suggesting a possible role for TLR4 in mediating monocyte/macrophage recruitment and tubule interstitial inflammation in diabetic nephropathy. Kim *et al.* (2007) using TLR2-/-/TLR4-/- knockout mice and NOD mice have demonstrated that TLR2 senses beta-cell death and contributes to the instigation of autoimmune DM. Again, Dasu *et al.* (2010) reported an increased TLR2 and TLR4 expression, intracellular signaling and TLR- mediated inflammation in monocytes with a significant relationship with HBA1C levels in diabetic patients.

In agreement to our findings of TLR2 increase in DM, levels of ligands of TLRs such as HSP60, HMGB1 also increases which has clear implications in DM as evidenced from researches leading to disease progression, however, this study did not analyse ligands of TLR2 and TLR4. Diabetic patients have high level of circulating HMGB1 and HSP60 which could trigger TLR2 and TLR4 activation, leading to a proinflammatory state (Dasu *et al*., 2010). Study by Melody *et al.* (2014) demonstrated that acute and prolonged insulin exposure has a potent protective effect on the

expression of TLR4, a key regulator of innate immune and inflammatory responses in the mammalian heart and this could be a promising pharmacological agent in the treatment of cardiometabolic diseases (Melody *et al*., 2014).

The variation in our findings as opposed to most of the findings of researches carried out could be as a result of the method of analysis which employed real time polymerase chain reaction (RT-PCR), immunofluorescence and flow cytometry techniques (Dasu *et al*., 2008; Lu *et al*., 2015). Also, the measurement of TLR2 and TLR4 was done in a dose- and time-dependent manner which induced a marked increase in TLR2, TLR4, mRNA and protein expression (Mohan *et al*., 2008). Duration of experiment may also influence the findings. The study by Lu *et al*. (2015) who found that hyperglycaemia induced over expression and activation of TLR4in endothelial cells ranged between 6-8 weeks period and to induce diabetes, 6 weeks old C57BL/6 mice were given five consecutive intraperitoneal injections of streptozotocin (60mg/kg body wt/day) (Lu *et al*., 2015). Dasu *et al*. (2008) determined that high-glucose-induced TLR2 and TLR4 expression persists for 48-72 hours before returning to normal glucose levels. The physiological consequences of this prolonged expression remain to be elucidated. This may possibly indicate that increased expression of TLR2/TLR4 is time monitored. Lu *et al.* (2015) demonstrated that high glucose challenge enhances the expression of TLR4 and the secretion of inflammatory factors by human endothelial cells. The results suggest an important role of hyperglycaemia-induced expression and activation of TLR4 in diabetic retinopathy. Again, it shows possibly why we have a variation from the majority of work done in this field.

Although the findings of this study did not completely agree with those of other studies and possible reasons listed as to why there is a variation, majority of work cited showed a positive relationship between hyperglycaemia and increased expression of both TLR2

and TLR4. The fundamental question of how high glucose activates TLR2 and TL4 and how this leads to increased inflammation needs to be determined. Dimerization is a critical event in the functional activation of TLRs and results in cytokine production (Dasu *et al*., 2008). TLR2 activity requires heterodimerization with TLR1 or TLR6 to recognize ligands. Using luciferase reporter assays and real-time RT-PCR, Dasu *et al.* (2008) showed that high glucose induces TLR2 and TLR6 heterodimerization, resulting in NF-κB activation and cytokine production. MD-2 is required for TLR4 ligation with endotoxin (Akashi *et al*., 2000).

In this study, the FBG and HBA1C levels showed a significant positive relationship between the controls, diabetic and diabetic treated groups. However, there was an inverse relationship between the FBG and HBA1C levels in the male and female wistar rats. The association between the FBG and HBA1C level as found in our study depends on the extent of glycaemic control. Glycaemic control monitoring can be achieved by HBA1C and FBG among other tests (Emmanuel *et al*., 2016). Haddadinezhad and Ghazaleh (2010) and Saiedullah *et al.* (2011) reported a higher than normal HBA1C and FBG in diabetic patients. Rohlfing *et al*. (2002), Saiedullah *et al*. (2011) and Kaur *et al*. (2014) reported a significant correlation between HBA1C and FBG. These studies reported a relationship between HBA1C and age but an insignificant difference between males and females as opposed to our findings. Again, several studies have revealed a strong positive correlation between HBA1C and FBG (Raja *et al*., 2013, Lipska *et al*., 2013; Kaur *et al*., 2014). This is because the level of HBA1C is proportional to the level of glucose in the blood and normal levels of glucose produce a normal amount of HBA1C. Thus, the fraction of HBA1C increases in a predictable way as the average amount of plasma glucose increases and this serves as a marker for average blood glucose levels over the previous 8-12 weeks prior to measurement (Roszyk *et al*.,

2007). Therefore, circulating red blood cells from the oldest (120 days old) to the youngest contribute to by all the level of HBA1C at any point in time (NGSP, 2010).

Studies reported a significant relationship between HbA1c and FPG and the relationship was strongest in the age group below 30years but the difference was insignificant between males and females (Haddadinezhad and Ghazaheh, 2010; Kaur *et al*., 2014). It is important to note that FBG test ascertains the glucose levels for the past few days but since blood glucose levels fluctuate throughout the day, glucose records are imperfect indicators of changes in the body due to hyperglycaemia (Emmanuel *et al*., 2016). However, HBA1C is a "weighted" average of blood glucose levels during the preceding 120 days, meaning that glucose levels in the preceding 0 days contribute substantially more to the level of HbA1c than do glucose levels 90-120 days earlier. This is supported by data from actual practice showing that HbA1c level improved significantly already after 20 days since glucose-lowering treatment intensification (Sidorenkov *et al*., 2011). This explains why the level of HbA1c can increase or decrease relatively quickly with large changes in plasma glucose; it does not take 120 days to detect a clinically meaningful change in HbA1c after a change in mean plasma glucose (Rohlfing *et al*., 2002; NGSP, 2010).

A benefit of metformin is the anti-inflammatory effect manifested by a decrease in the production of IL-1B, IL-6 and TNF alpha (Arai *et al*., 2010; Hyun *et al*., 2013). In the present study, metformin treatment had no effect on the level of TLR2 and TLR4 in both male and female. A very limited number of studies reported its ability to influence TLRs concentration. To the best of our knowledge, only a few reports link attenuated TLR2 and TLR4 activity with protection of the infracted heart in rats treated with metformin and these include a study by Soraya *et al*. (2012). A study carried out by Peixoto *et al.* (2017) to determine if metformin treatment attenuates the TLR signaling

pathways triggered by inflammation in skeletal muscle of hyperinsulinaemic/hyperglycaemic streptozotocin induced rats, they found a relationship between muscular TLR4, P-AMPK and NF-KB content and insulin sensitivity. Metformin treatment may prevent attenuation of activation of the inflammatory pathway leading to disease progression.

TLR4 contributes to the development of non-alcoholic fatty liver disease (NAFLD) and metabolic syndrome. It is unclear whether anti-diabetic metformin affects TLR4 expression of blood monocytes, thereby protecting or improving inflammatory parameters. But in a study carried out by Agnieskzia *et al.* (2016) demonstrated that metformin significantly decreased the TLR4 level on monocytes originating from all NAFLD patients in a dose dependent manner. However, its efficacy depended largely on the presence of obesity.

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATIONS

6.1 CONCLUSION

It is concluded in my findings of the present study that:

TLR2 is significantly increased in diabetic non-treated and diabetic treated group compared with control, but the difference between the diabetic non-treated and diabetic treated group is not significant.

This study also revealed that the TLR2/TLR4 levels in both males and females is not different among the diabetic non-treated, diabetic treated and the control groups.

Metformin treatment produced no significant difference in TLR2 and TLR4 levels in diabetic non-treated and diabetic treated groups compared with control groups. Also, FBG and HBA1C levels were significantly higher in diabetic treated and diabetic non-treated compared with controls.

TLR2 and TLR4 levels are not different between male and female rats. FBG is significantly different between male and female rats but difference between male and female rats on HBA1C levels is not significant

6.2 RECOMMENDATION

From this study, it is recommended that:

4.0 Estimation of TLR 2 and TLR4 in DM patients investigation be included as seen from the current study, Toll like receptor 2 is increased in hyperglycaemic conditions, targeting them may prove to be therapeutic. More so, clinical and experimental results support the belief that the ability to target key receptors or signaling in innate immunity might prevent uncontrolled inflammation and limit the progression of many diseases including diabetes.

5.0 Further research needs to be done in this aspect to have a clearer picture and role of TLR2/TLR4 in diabetes mellitus. It is evident from literature reviews that TLR2 and TLR4 have a significant role to play in diabetes mellitus and implicated in disease progression,

6.0 Such studies have never been done in this area and the prevalence of DM is increasing, hence the need for it. The menace of diabetes mellitus cannot be over emphasized, and TLR seems to be a promising avenue for research. Besides, the ability of TLR to initiate and propagate inflammation makes them attractive therapeutic targets

7.0 Similar study needs to be conducted on human subjects to further validate the findings in this study.

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APPENDIX I

TLR2 Reagents

8.0	**Closure plate membrane**	**2**
9.0	**Sealed bags**	**1**
10.0	**Micro elisa strip plate**	**1**
11.0	**Standard, 36 ng/mL**	**0.5 ml x 1 bottle**
12.0	**Standard Diluent**	**1.5 ml x 1 bottle**
13.0	**HRP-Conjugate Reagent**	**6 ml x 1 bottle**
14.0	**Sample Diluent**	**6 ml x 1 bottle**
15.0	**Chromogen Solution A**	**6 ml x 1 bottle**
16.0	**Chromogen Solution B**	**6 ml x 1 bottle**
17.0	**Stop Solution**	**6 ml x 1 bottle**
18.0	**Wash Solution**	**(20 ml x 30 fold) x 1 bottle**
19.0	**Distilled water**	**1 litre**
20.0	**Stored at 2 – 8^{0}C, returned to 18 – 30^{0}C before use**	

TLR4 Reagents

21.0	**Closure plate membrane**	**2**
22.0	**Sealed bags**	**1**
23.0	**Micro elisa strip plate**	**1**
24.0	**Standard, 27 ng/mL**	**0.5 ml x 1 bottle**
25.0	**Standard Diluent**	**1.5 ml x 1 bottle**
26.0	**HRP-Conjugate Reagent**	**6 ml x 1 bottle**
27.0	**Sample Diluent**	**6 ml x 1 bottle**
28.0	**Chromogen Solution A**	**6 ml x 1 bottle**
29.0	**Chromogen Solution B**	**6 ml x 1 bottle**
30.0	**Stop Solution**	**6 ml x 1 bottle**
31.0	**Wash Solution**	**(20 ml x 30 fold) x 1 bottle**
32.0	**Distilled water**	**1 litre**
33.0	**Stored at 2 – 8^{0}C, returned to 18 – 30^{0}C before use**	
34.0		

Glycosylated haemoglobin (GHb) reagents

35.0	**R1 ion exchange resin**	**50 x 3 ml**
36.0	**R2 lysing reagent**	**12 x 12.5 ml**
37.0	**Control (10% GHb)**	**1 x 1 ml**
38.0	**A resin separators**	**50 Nos**
39.0	**Stored at 2-8^{0}C**	

Reagents preparation

40.0 The ion exchange resin tubes and the lysing reagent are ready to use.
41.0 Reconstitute the control with 1ml of distilled water
42.0 Allow to stand for 10mins with occasional mixing
43.0 Reconstituted control is stable for at least 7 days when stored at 2-8^{0}C tightly sealed and at least 4 weeks when stored at -20^{0}C.
44.0 Haemolysate preparation
45.0 Dispense 0.5 ml lysing reagent into tubes labelled as Control and Test
46.0 Add 0.1 ml of the reconstituted control and well-mixed blood sample into the appropriately labelled ion exchange resin tubes. Mix until complete lysis is evident.
47.0 Allow to stand for 5 mins

APPENDIX II

Formula for Reagents Preparation

48.0 Volume of original solution required = R x V/O

49.0 Where; R: Required concentration, V: Required volume, O: Original concentration

Statistical Notations

MANOVA Analysis

First, the total sum-of-squares is partitioned into the sum-of-squares between groups (SSbg) and the sum-of-squares within groups (SSwg). This can be expressed as:

ANOVA

$$\sum_i \sum_j (Y_{ij} - GM_{(y)})^2 = n \sum_j \left(\bar{Y}_j - GM_{(y)} \right)^2 + \sum_i \sum_j (Y_{ij} - \bar{Y}_j)^2$$

$$SS_{Total(y)} = SS_{bg(y)} + SS_{wg(y)}$$

For MANOVA, it becomes:

$$n_{km} \sum_k \sum_m (DT_{km} - GM_{(DT)})^2 = n_k \sum_k \left(D_k - GM_{(D)} \right)^2 + n_m \sum_m \left(T_m - GM_{(T)} \right)^2 +$$

$$\left[n_{km} \sum_k \sum_m \left(DT_{km} - GM_{(DT)} \right)^2 - n_k \sum_k \left(D_k - GM_{(D)} \right)^2 - n_m \sum_m \left(T_m - GM_{(T)} \right)^2 \right]$$

$$SS_{bg} = SS_D + SS_T + SS_{DT}$$

The full factorial design is:

$$\sum_i \sum_k \sum_m (Y_{ikm} - GM_{(ikm)})^2 = n_k \sum_k \left(D_k - GM_{(D)}\right)^2 + n_m \sum_m \left(T_m - GM_{(T)}\right)^2 +$$
$$\left[n_{km} \sum_k \sum_m \left(DT_{km} - GM_{(DT)}\right)^2 - n_k \sum_k \left(D_k - GM_{(D)}\right)^2 - n_m \sum_m \left(T_m - GM_{(T)}\right)^2 \right]$$
$$+ \sum_i \sum_k \sum_m (Y_{ikm} - DT_{km})^2$$

Matrix equations:

$$\sum_i \sum_k \sum_m (Y_{ikm} - GM)(Y_{ikm} - GM)' = n_k \sum_k (D_k - GM)(D_k - GM)'$$
$$+ n_m \sum_m (T_m - GM)(T_m - GM)' + [n_{km} \sum_{km} (DT_{km} - GM)(DT_{km} - GM)'$$
$$- n_k \sum_k (D_k - GM)(D_k - GM)' - n_m \sum_m (T_m - GM)(T_m - GM)']$$
$$+ \sum_i \sum_k \sum_m (Y_{ikm} - DT_{km})(Y_{ikm} - DT_{km})'$$

WILK'S LAMBDA

$$\Lambda = \frac{\left|S_{error}\right|}{\left|S_{effect} + S_{error}\right|}$$

Approximate Multivariate F For Wilks Lambda Is:

$$F(df_1, df_2) = \left(\frac{1-y}{y}\right)\left(\frac{df_2}{df_1}\right)$$

$$where\ y = \Lambda^{1/s},\ s = \sqrt{\frac{p^2(df_{effect})^2 - 4}{p^2 + (df_{effect})^2 - 5}},$$

$$p = number\ of\ DVs,\ df_1 = p(df_{effect})$$

$$df_2 = s\left[(df_{error}) - \frac{p - df_{effect} + 1}{2}\right] - \left[\frac{p(df_{effect}) - 2}{2}\right]$$

Eta Squared

$$\eta^2 = 1 - \Lambda$$

Partial Eta Squared

$$\eta^2 = 1 - \Lambda^{1/s}$$

LIST OF REAGENTS

50.0 Streptozotocin
51.0 Distilled water
52.0 Glucose reagent
53.0 TLR 2 reagent
54.0 TLR 4 reagent
55.0 Glycated haemoglobin reagent
56.0 Metformin
57.0 Normal saline
58.0 Glucose solution 10%
59.0 Ion exchange resin R1
60.0 Lysing reagent
61.0 Control
62.0 Resin separators

LIST OF MATERIALS

63.0 Cotton wool
64.0 Syringes
65.0 Hand gloves
66.0 Laboratory coat
67.0 Iron cages
68.0 Plastic bottles
69.0 Rubber plates
70.0 Rubber tubes
71.0 Superglue
72.0 Razor blade
73.0 Weighing Balance
74.0 Rodent chaw and clean water
75.0 Washing soap
76.0 Timer
77.0 Automatic micropipettes
78.0 Precision pipettes and disposable pipette tips
79.0 Distilled water
80.0 Absorbent paper
81.0 Incubator (37^{o}C)
82.0 Standard enzyme reader
83.0 Disposable tubes for sample dilution
84.0 Writing notebook and pen
85.0 Printing paper
86.0 Refrigerator and deep freezer