

CHAPTER ONE

1.0. INTRODUCTION

1.1 BACKGROUND OF THE STUDY

Diabetes mellitus (DM) is defined as a metabolic disorder of multiple aetiology characterized by chronic hyperglycaemia with impairment of carbohydrate, fat and protein metabolism resulting from defects in insulin secretion, insulin action, or both (World Health Organisation (WHO) 1999, 2019). The effects of untreated DM may results in long-term dysfunction, damage and eventual failure of various organs in the body. DM usually presents with characteristic symptoms such as excessive thirst, polyuria (excessive urination), polyphagia (excessive food intake), blurring of vision and weight loss. In its most severe forms, acute complications such as ketoacidosis or hyperosmolar hyperglycaemic state (HHS) may arise with grave consequences if not treated promptly. Sometimes, DM (especially type 2 DM) may be asymptomatic, with detection on routine screening. The asymptomatic presentation often delays diagnosis, and consequently, hyperglycaemia of sufficient degree to cause functional and pathological alterations are present for a long period before the diagnosis is confirmed. Hence, some individuals with delayed diagnosis usually present with one form of chronic DM complication or another (Olamoyegun *et al.*, 2015).

The long-term effects of DM include the progressive development of specific complications like retinopathy, nephropathy and neuropathy, with associated risk of foot ulcers, limb amputation, and features of autonomic dysfunction (WHO, 2019). People with DM are also at higher risk of cardiovascular, peripheral vascular disease (PVD) and cerebrovascular diseases. The development of DM complications is a significant cause of morbidity and mortality and

constitutes an ever-increasing burden to healthcare authorities in developing and developed nations.

Several pathogenetic processes have been postulated to be involved in the development of DM. These include processes that destroy the pancreas' beta cells, with resultant deficiency of insulin secretion or resistance to its actions. The abnormalities of carbohydrate, protein and fat metabolism are due to deficient insulin action on target tissues, resulting from insensitivity to or lack of insulin.

Cardiovascular diseases have been proposed as the primary cause of hospitalization and death among patients with DM. Therefore, it is regarded as a cardiovascular event equivalent to patients with previous myocardial infarction (Pop-Busui, 2010). Studies have earlier shown that endothelial dysfunction, endomyocardial fibrosis, the direct toxic effect of hyperglycaemia on cardiomyocytes and autonomic neuropathy play an important role in the pathogenetic mechanism of diabetic cardiomyopathy (Lin *et al.*, 2008; Vinik *et al.*, 2018).

Diabetic cardiac autonomic neuropathy (DCAN) is defined as the impairment of the autonomic control of the cardiovascular system in the setting of DM after exclusion of other etiologies (Agashe and Petak, 2018). It is common in many patients with DM, with a prevalence of 35% and 65% in type 1 DM (T1DM) and type 2 DM (T2DM), respectively. DCAN is a risk marker for all-cause mortality and cardiac related deaths, stroke, coronary events, silent myocardial ischaemia (SMI), heart failure, arrhythmia, progression of nephropathy and sudden death (Yang, 2011). An effective way to detect DCAN at an early stage has not been discovered, but researchers and patients highly desire it. Despite intensive medical research on the mechanism of DCAN and new medical treatment modalities for patients who suffer from this complication, it

remains a significant cause of morbidity and mortality in DM patients and warrants further testing of novel therapeutic approaches.

Carvedilol is a non-selective β -adrenoceptor blocker and a selective α_1 -adrenoceptor blocker. So far, it has been widely used as a class 1 recommended drug in the treatment of heart failure and some cases of hypertension with or without DM. Unlike the first generation of β -blockers that have been shown to worsen glycaemic control, reduce insulin sensitivity and cause deranged lipid metabolism, several studies have shown that these effects are not observed with carvedilol (Bakris *et al.*, 2004; Huang *et al.*, 2007; Ahmad, 2017). Although carvedilol has been consistently shown to improve mortality in heart failure that is associated with cardiac autonomic neuropathy (CAN), its mechanistic role in the management of DCAN is yet to be fully established (Bloom *et al.*, 2014; Li *et al.*, 2014; Potočník *et al.*, 2017; Vinik *et al.*, 2018).

1.2 STATEMENT OF THE PROBLEM AND JUSTIFICATION

DM is a common disease with increasing prevalence worldwide. According to the 2017 International Diabetes Federation (IDF) Atlas, about 425 million people worldwide, which approximates to 8.8% of adults aged 20–79 years, are estimated to have DM, of which 79% live in low and middle income countries (Cho *et al.*, 2018). However, according to the 2019 IDF Atlas, about 463 million people worldwide, or 9.3% of adults, are now estimated to have DM. The IDF further projects that by 2045; 700 million people will have DM (Saeedi *et al.*, 2019). Over 70% of mortality in DM is due to cardiovascular complications. The annual health costs incurred by DM and its complications account for 6–12% of all healthcare expenditure (Cho *et al.*, 2018). The diabetic population of people aged over 20 years is increasing at 1.9 million per

year. There is no cure or prevention for T1DM, which causes approximately 10% of DM cases in Europe and North America (Chan, 2016; Cho *et al.*, 2018).

Patients with DM often suffer from blurred vision, fatigue, and polyuria at the initial stage and gradually progress into severe complications such as kidney failure, cardiovascular disease and DM neuropathy, to name a few. Among all DM complications, neuropathy is, unfortunately, the most severe and pressing problem as early nerve degeneration is hard to diagnose (Dobretsov *et al.*, 2007; Deli *et al.*, 2013). Nerve cells and their supporting blood vessel cells are most vulnerable to high blood glucose. None is insulin-independent for glucose transportation, with excessive intracellular glucose results in oxidative stress and cell death. The death of supporting blood vessel cells also accelerates the death of nerve cells. Consequently, over 60% of DM patients are affected by DM neuropathy (Vinik *et al.*, 2000).

The heart is an essential organ highly innervated by autonomic nerves, which are responsible for maintaining cardiovascular homeostasis (Gordan *et al.*, 2015; Dampney, 2016). Neuropathy in the heart is called DCAN. It is one of the most overlooked of all serious complications of DM. It can cause abnormalities in the control of heart rate, with disruption of central and peripheral vascular dynamics, increased risk of mortality and morbidity, and reduced quality of life for people with DM (Yang, 2011). This complication is currently not curable and not reversible after significant neuronal loss; thus, early diagnosis of DCAN is critical.

An early diagnostic technique needs to be based on non-invasive measurements, sensitive, accurate, and easy to use for human applications. One proper non-invasive method for assessing autonomic function in various physiological and pathophysiological conditions, including evaluating autonomic dysfunction in DM patients, is the use of heart rate variability (HRV) time series. It has been shown that abnormalities of power spectral density of HRV autonomic

function are present early in DM patients with subsequent progressive decline in autonomic function (Li *et al.*, 2015). Since, currently, there is no cure for DCAN and, for most cases, damage to nerves in DCAN is not reversible, there is a need to investigate other therapeutic modalities, especially such that have been used in the management of autonomic neuropathy from other conditions.

Why use animals? Animal models are used to study the genetics and pathogenesis of diseases with their accompanying complications, develop new treatment modalities, and discover preventative strategies (Rees and Alcolado, 2005; Rodrigues, 2016). Animal models, mainly rodents like rats and mice, play an essential role in investigating human diseases. Due to their small size, easy availability, the possibility of breeding under standardised conditions, as well as economic considerations, rodents are mostly preferred to other animal models like pigs or dogs (Islam and Loots, 2009; King, 2012; Dhuria *et al.*, 2015). Moreover, large syntenic regions between the rodent and human genomes exist; thus, linkages can be easily translated between the two genomes (Waterston *et al.*, 2002).

1.3 AIM AND OBJECTIVES

1.3.1. Aim

This study investigated the effects of carvedilol on streptozotocin-induced DCAN rats.

1.3.2. Objectives

The objectives of this study are to

1. induce DM in experimental rats;
2. investigate the outcome of DM and DCAN induction;
3. investigate the effect of carvedilol on biochemical markers of autonomic nervous system in streptozotocin-induced DCAN rats;
4. investigate the effect of carvedilol on HRV indices in streptozotocin-induced DCAN rats; and
5. investigate the effect of carvedilol on cardiac histology and nerve densities in streptozotocin-induced DCAN rats.

1.4 RESEARCH HYPOTHESES

Hypothesis 1

DCAN will be induced following streptozotocin (STZ) induction in Wistar rats.

Hypothesis 2

Carvedilol will ameliorate features of DCAN in STZ-induced rat models.

1.5 RESEARCH QUESTIONS

1. What will be the outcome of a single high dose of STZ and multiple low doses of STZ combined with a high-fat diet on induction of DM in experimental rats?
2. What will be the Holter HRV pattern of DCAN following STZ-induced rat models?
3. What will be the effect of carvedilol in ameliorating DCAN features in STZ-induced rats?
4. What will be the effect of carvedilol on nerve densities in the hearts of STZ-induced rats?

1.6 SCOPE OF THE STUDY

This study only investigated the role of carvedilol on DCAN with respect to HRV indices, laboratory biomarkers and histological cardiac nerve density.

1.7 SIGNIFICANCE OF THE STUDY

There has been increasing recognition of the significant relationship between the autonomic nervous system and cardiovascular morbidity and mortality, especially in DM patients. Moreover, despite intensive medical research on the mechanism of DCAN and new medical treatment options for patients living with this pathology, it remains a significant cause of morbidity and mortality in DM patients and warrants further testing of novel therapeutic approaches. Carvedilol has been widely used in treating heart failure and hypertension with or without DM due to its effect on the autonomic system. Its role in the management of DCAN, however, has not been established. This study, therefore, hoped to investigate the intervention of carvedilol in STZ-induced DCAN in a rat model. The study's findings will help propose a more extensive multicentre human study to further evaluate this premise and advocate its therapeutic application for an increasingly large segment of the DM population.

CHAPTER TWO

2.0. LITERATURE REVIEW

2.1. DIABETES MELLITUS

DM is a chronic metabolic syndrome characterized by chronic hyperglycaemia resulting from the combination of resistance to insulin action, inadequate insulin secretion, and excessive or inappropriate glucagon secretion (Ahmad, 2017; Vinik *et al.*, 2018). It is now broadly categorized into six subtypes, although two (T1DM and T2DM) are more common than others (WHO, 2019). T1DM is characterized by destruction of the insulin-producing beta cells of the islets of Langerhans in the pancreas, resulting in relative or absolute insulin deficiency. This type of DM can be further classified as idiopathic or immune-mediated. The majority of T1DM cases are of the immune-mediated nature, in which beta cell loss is due to a T cell-mediated autoimmune attack (Echeverri and Tobon, 2013; Katsarou *et al.*, 2017; Roep *et al.*, 2021). T2DM is characterized by insulin resistance, which is the defective responsiveness of body tissues to insulin. The typical outcome of either T1DM or T2DM is abnormally high blood glucose levels. It is the complications caused by this abnormal condition that lead to serious health problems.

2.2. EPIDEMIOLOGY

Worldwide, DM has reached alarming epidemic proportions. In 2019, about 500 million people (9.3% of adults aged 20–79 years) lived with DM worldwide. The estimated number of those living with DM has increased by 62% in the last decade—from 285 million in 2009 to 463 million at present. Currently, half (50.1%) of the people with DM do not know that they have the disease (Saeedi *et al.*, 2019).

Since 2000, the IDF has reported the national, regional and global prevalence and occurrence of DM. In 2009, 285 million people were estimated to have DM, increasing to 366 million in 2011 and 425 million in 2017. A total of 463 million were estimated to be living with DM in 2019, representing 9.3% of the global adult population aged between 20 and 79 years. This number is projected to increase to 700 million (10.9%) in 2045 (Saeedi *et al.*, 2019). The prevalence of DM in females in 2019 was estimated to be 9.0%, while it was 9.6% in males. The increase in DM prevalence with age gives an estimate of 19.9% (111.2 million) in people aged 65 and 79.

DM prevalence differs by World Bank income group, with a higher prevalence among high-income countries (10.4%) and middle-income countries (9.5%) compared to low-income countries (4.0%). The prevalence is projected to reach 11.9%, 11.8% and 4.7% in high-, middle- and low-income countries, respectively, by 2045. Of all people living with DM, 67.0% are living in urban areas. Although the prevalence of DM is higher in urban than in rural areas (10.8% vs. 7.2%), there appears to be a rising trend among rural dwellers which may reflect, a degree of urbanisation or "westernisation" of rural areas. For example, a recent report from Pakistan stated the prevalence of DM to be only slightly higher in urban (28.3%) than in rural (25.3%) areas (Saeedi *et al.*, 2019).

Table 2.1: Global prevalence of diabetes mellitus, and comparative prevalence estimates (%) of diabetes (among people aged 20–79 years) in 2017 and projection for 2045 (IDF, 2017).

At a glance	2017	2045
Total world population	7.5 billion	9.5 billion
Adult population (20–79 years)	4.84 billion	6.37 billion
Diabetes global estimates		
Prevalence (20–79 years)	8.8% (7.2–11.3%)	9.9% (7.5–12.7%)
Number of people with diabetes (20–79 years)	424.9 million (346.4–545.4 million)	628.6 million (477.0–808.7 million)
Number of deaths due to diabetes (20–79 years)	4.0 (3.2–5.0) million	-
Total healthcare expenditures for diabetes (20–79 years), R=2* 2017 USD	USD 727 billion	USD 776 billion

Key: USD, United States dollars

Table 2.2: Top ten countries/territories with highest prevalence of diabetes (among people aged 20–79 years) in 2017 and projection for 2045 (IDF, 2017).

2017			2045		
Rank	Country/territory	Number of people with diabetes (range)	Rank	Country/territory	Number of people with diabetes (range)
1	China	114.4 million (104.1–146.3)	1	India	134.3 million (103.4–165.2)
2	India	72.9 million (55.5–90.2)	2	China	119.8 million (86.3–149.7)
3	United States	30.2 million (28.8–31.8)	3	United States	35.6 million (33.9–37.9)
4	Brazil	12.5 million (11.4–13.5)	4	Mexico	21.8 million (18.6–22.1)
5	Mexico	12.0 million (6.0–14.3)	5	Brazil	20.3 million (18.6–22.1)
6	Indonesia	10.3 million (8.9–11.1)	6	Egypt	16.7 million (9.0–19.1)
7	Russian Federation	8.5 million (6.7–11.0)	7	Indonesia	16.7 million (14.6–18.2)
8	Egypt	8.2 million (4.4–9.4)	8	Pakistan	16.1 million (11.5–23.2)
9	Germany	7.5 million (6.1–8.3)	9	Bangladesh	13.7 million (11.3–18.6)
10	Pakistan	7.5 million (5.3–10.9)	10	Turkey	11.2 million (10.1–13.3)

2.3. MORTALITY IN DIABETES

Over 4.0 (3.2–5.0) million people died from DM in 2017, equivalent to one death every eight seconds (IDF, 2017). DM accounted for 10.7% of global all-cause mortality among people in this age group. This mortality was higher than the combined number of deaths from infectious diseases (1.1 million deaths from HIV/AIDS, 1.8 million from tuberculosis, and 0.4 million from malaria) in 2015. About 46.1% of deaths due to DM among the 20–79 years age group occurred in people under the age of 60 years (IDF, 2017).

In Nigeria, an estimated 1.6 million deaths were directly caused by DM, while high glucose accounted for another 2.2 million deaths in 2012 (IDF, 2015). The main contributors to DM mortality were reported to be hyperglycaemic emergencies, diabetic foot and cardiovascular diseases. In Nigeria, the majority of DM patients present when complications have already set in (Ogbera, 2014). Acute complications of DM (DKA, HHS, and hypoglycaemia) are frequent Hospital emergencies in Nigeria, with high mortalities recorded (Oguejiofor *et al.*, 2014).

Table 2.3: Proportion of deaths from diabetes in 2017 among people younger than 60 years in IDF regions (IDF, 2017).

IDF region	Number of deaths due to diabetes before age 60 (range)	Proportion of all deaths due to diabetes occurring before age 60
Africa	0.23 million (0.16–0.39)	77.0%
Europe	0.16 million (0.13–0.22)	32.9%
Middle East and North Africa	0.16 million (0.12–0.21)	51.8%
North America and Caribbean	0.13 million (0.11–0.14)	45.0%
South America and Central America	0.09 million (0.08–0.11)	44.9%
South East Asia	0.58 million (0.47–0.69)	51.5%
Western Pacific	0.48 million (0.43–0.60)	38.0%

2.4. CLASSIFICATION OF DIABETES MELLITUS

Diabetes had been previously classified into five distinct types (ADA, 2008), namely:

Insulin-dependent diabetes mellitus (IDDM) or "juvenile-onset" diabetes mellitus or T1DM, Non-insulin-dependent diabetes mellitus (NIDDM) or "adult-onset" diabetes mellitus or T2DM, Gestational diabetes mellitus (GDM), malnutrition-related diabetes, and other types.

These types were characterised by fasting hyperglycaemia, elevated random blood glucose, or plasma glucose levels during an oral glucose tolerance test (OGTT). The WHO in 1999 also classified DM into four classes considering both the aetiology and clinical stages of the disease. The two main types were T1DM, which accounts for 5–10% of all cases, and T2DM, which accounts for 90–95%. The third group comprises other less common types of DM caused by or associated with certain specific conditions and syndromes. The last group includes DM diagnosed during pregnancy, GDM (WHO, 1999; ADA, 2008), as shown in table 2.4. However, this classification has been shown to be less than satisfactory for distinguishing these different types of DM, especially the two major types; hence the need for the latest WHO 2019 classification. According to this classification, the distinction between the two main types—T1DM and T2DM—had historically been based on the age of onset, degree of loss of β -cell function, associated insulin resistance, presence of DM associated autoantibodies, and requirement of insulin treatment for survival (IDF, 2017). However, none of these features unequivocally distinguishes one type of DM from the other nor accounts for the entire spectrum of DM phenotypes (WHO, 2019).

Table 2.4: Disorders of glycaemia: aetiological types and clinical stages (WHO, 1999).

Stages Types	Normoglycaemia	Hyperglycaemia			
	Normal glucose tolerance	Impaired glucose regulation IGT and/or IFG	Diabetes Mellitus		
			Not insulin requiring	Insulin requiring for control	Insulin requiring for survival
Type 1 • Autoimmune • Idiopathic	←————→				→
Type 2* • Predominantly insulin resistance • Predominantly insulin secretory defects	←————→			→	
Other specific types*	←————→			→	
Gestational diabetes*	←————→			→	

*In rare instances patients in these categories (e.g. Vacor Toxicity, type 1 presenting in pregnancy, etc.) may require insulin for survival.

Table 2.5: Aetiological classification of diabetes mellitus (WHO, 2019).

Type 1 diabetes
Type 2 diabetes
Hybrid forms of diabetes
Slowly evolving immune-mediated diabetes of adults
Ketosis prone type 2 diabetes
Other specific types (details in Table 2.6)
Monogenic diabetes
- Monogenic defects of β -cell function
- Monogenic defects in insulin action
Diseases of the exocrine pancreas
Endocrine disorders
Drug- or chemical-induced
Infections
Uncommon specific forms of immune-mediated diabetes
Other genetic syndromes sometimes associated with diabetes
Unclassified diabetes
This category should be used temporarily when there is not a clear diagnostic category especially close to the time of diagnosis of diabetes
Hyperglycemia first detected during pregnancy
Diabetes mellitus in pregnancy
Gestational diabetes mellitus

Table 2.6: Other specific types of diabetes (WHO, 2019).

Monogenic diabetes; Monogenic defects of β-cell function (mutated gene followed by clinical syndrome) GCK MODY, HNF1A MODY HNF4A MODY, HNF1B RCAD mtDNA 3243 MIDD, KCNJ11 PNDM KCNJ11 DEND, 6q24 TNDM	Monogenic defects in insulin action (mutated gene followed by clinical syndrome) INSR Type A insulin resistance INSR Leprechaunism, AGPAT2 CGL, INSR Rabson-Mendenhall syndrome LMNA FPLD, PPARG FPLD, BSCL2 CGL
Diseases of the exocrine pancreas Fibrocalculous pancreatopathy, Cystic fibrosis, Pancreatitis, Neoplasia, pancreatectomy, Trauma, Haemochromatosis, Others	Endocrine disorders Cushing's syndrome, Acromegaly Pheochromocytoma, Glucagonoma Somatostatinoma, Hyperthyroidism, others
Other genetic syndromes: Down syndrome, Friedreich's ataxia Huntington's chorea, Porphyria Klinefelter's syndrome, Turner's syndrome, Lawrence-Moon-Biedel syndrome Myotonic dystrophy, Prader-Willi syndrome.	Drug- or chemical-induced diabetes Glucocorticoids, Thyroid hormone Thiazides, Alpha-adrenergic agonists Beta-adrenergic agonists, Dilantin Pentamidine, Nicotinic acid, Pyrinuron Interferon-alpha, STZ, Others
Insulin autoimmune syndrome (autoantibodies to insulin); Stiff man syndrome Anti-insulin receptor antibodies, others	Infections Congenital rubella Cytomegalovirus, Others

Key: MODY = maturity-onset diabetes of the young; RCAD = renal cysts and diabetes; MIDD = maternally inherited diabetes and deafness; PNDM = permanent neonatal diabetes; TNDM = transient neonatal diabetes; DEND = developmental delay epilepsy and neonatal diabetes; FPLD = familial partial lipodystrophy; CGL = congenital generalized lipodystrophy.

2.4.1 Type 1 diabetes mellitus

T1DM (characterised by β -cell destruction, usually leading to absolute insulin deficiency) was previously referred to as IDDM or juvenile-onset DM. It results from a progressive cell-mediated autoimmune destruction of the pancreatic β -cells, which leads to complete insulin deficiency (Knip and Siljander, 2008). According to the WHO 1999 classification, T1DM was divided into two: immune-mediated (Type 1A) and idiopathic (Type 1B).

Although T1DM typically presents in childhood and adolescence, onset can also occur in adults, and 84% of people living with T1DM are adults (Chiang *et al.*, 2014). A variation of T1DM that develops later in life, usually after age 30, is called latent autoimmune DM of adulthood (LADA) and is sometimes referred to as type 1.5 DM.

2.4.1.1 Immune-mediated diabetes

This form of DM, which accounts for only 5–10% of all DM cases, results from cell-mediated autoimmune destruction of the pancreatic β -cells i.e., it is an autoimmune disease in which the immune system mistakenly destroys the insulin-producing pancreatic beta-cells. It typically develops more rapidly than other types of DM. It is often diagnosed in children and adolescents and sometimes occurs in young adults. An individual with this type of DM requires regular insulin administration for survival. Markers of the immune destruction of the β -cells, which include islet cell autoantibodies (ICA), autoantibodies to insulin, glutamic acid decarboxylase (GAD65), and tyrosine phosphatases IA-2 and IA-2 β , are usually present in 85–90% of people diagnosed with this condition (Pihoker *et al.*, 2005). The disease has strong human leukocyte antigen (HLA) associations, with linkage to the *DQA* and *DQB* genes, and the *DRB* genes influence it. These *HLA-DR/DQ* alleles can be either protect against or predispose to the disease.

Immune-mediated DM patients are also prone to other autoimmune diseases which includes; Graves' disease, Addison's disease, Hashimoto's thyroiditis, vitiligo, celiac disease, myasthenia gravis, autoimmune hepatitis, and pernicious anaemia (Lernmark and Latorre, 2015).

2.4.1.2 Idiopathic diabetes

Some forms of T1DM have no known aetiologies; hence the name idiopathic. Some of these patients have permanent insulinopenia and are prone to ketoacidosis, but they have no evidence of autoimmunity. Although only a minority of patients with T1DM fall into this category (<5%), majority in this category are of African or Asian ancestry. Patients with this form of DM suffer from episodic ketoacidosis and exhibit varying degrees of insulin deficiency between episodes. This form of DM is strongly inherited, lacks immunological evidence for β -cell autoimmunity, and is not HLA-associated (Lernmark and Latorre, 2015). The absolute requirement for insulin replacement therapy in affected patients may be intermittent.

Type 1 DM represents around 10% of all cases of DM, affecting approximately 20 million people worldwide (ADA, 2019). Although T1DM affects all age groups, most people are diagnosed around preteen or teen period (Yau *et al.*, 2000). In Europe, the average annual incidence in patients under 15 years of age increases by 3.4%, with the steepest rise in those under five years old. T1DM results from an autoimmune reaction to proteins of the pancreatic islets cells (Maahs *et al.*, 2010). There is a higher incidence of other autoimmune disorders in family members of T1DM patients.

There are three variants of autoantibodies:

- i) Islet cell cytoplasmic antibodies (ICCA): The main antibodies found in over 90% of patients with T1DM are against the islet cell cytoplasmic proteins. The presence of ICCA is a good accurate predictor of the future development of T1DM.
- ii) Islet cell surface antibodies (ICSA): Autoantibodies against islets cell surface antigens have also been described in about 80% of T1DM patients. Fewer patients with T2DM have been shown to be positive for ICSA.
- iii). Specific antigenic targets of islet cells: Over 80% of newly diagnosed T1DM patients have antibodies to glutamic acid decarboxylase (GAD). Anti-GAD antibodies decline over time in T1DM. The presence of anti-GAD antibodies is a strong predictor of the future development of T1DM in high risk populations. Anti-insulin antibodies (IAAs) have been identified in T1DM patients and relatives at risk of developing T1DM. These IAAs are detectable even before the initiation of insulin treatment in T1DM. IAA is seen in around 40% of young children with T1DM (Ozougwu *et al.*, 2013).

2.4.1.3 PATHOGENESIS OF TYPE 1 DM

Type 1 DM is a chronic autoimmune disorder associated with the selective destruction of insulin-producing pancreatic β -cells. The onset of clinical disease usually represents the end stage of β -cell destruction preceding T1DM (Katsarou *et al.*, 2017). Several features typify T1DM as an autoimmune disease:

1. Immune competent cell in pancreatic islets;
2. Association of susceptibility to disease with the class II (immune response) genes of the major histocompatibility complex (MHC; human leukocyte antigens HLA);

3. Presence of islet cell-specific autoantibodies;
4. Alterations of T cell mediated immunoregulation (CD4⁺ T cell);
5. The involvement of TH1 and monokines producing interleukins in the disease process;
6. Response to immunotherapy and;
7. The associated occurrence of other organ specific autoimmune diseases in affected individuals or relations.

Activation of islet antigen-specific CD4⁺ T cells appears to be necessary for development of DM in animal models of T1DM (Roep and Peakman, 2012). CD4⁺ islet-specific T-cell clones derived from DM NOD mice, when injected into prediabetic or non-diabetes prone mice was observed to induce insulitis and DM. It was equally shown that CD4⁺ T cells are required to induce insulitis, while CD8⁺ T cells contributed to severity of damage (Ozougwu *et al.*, 2013).

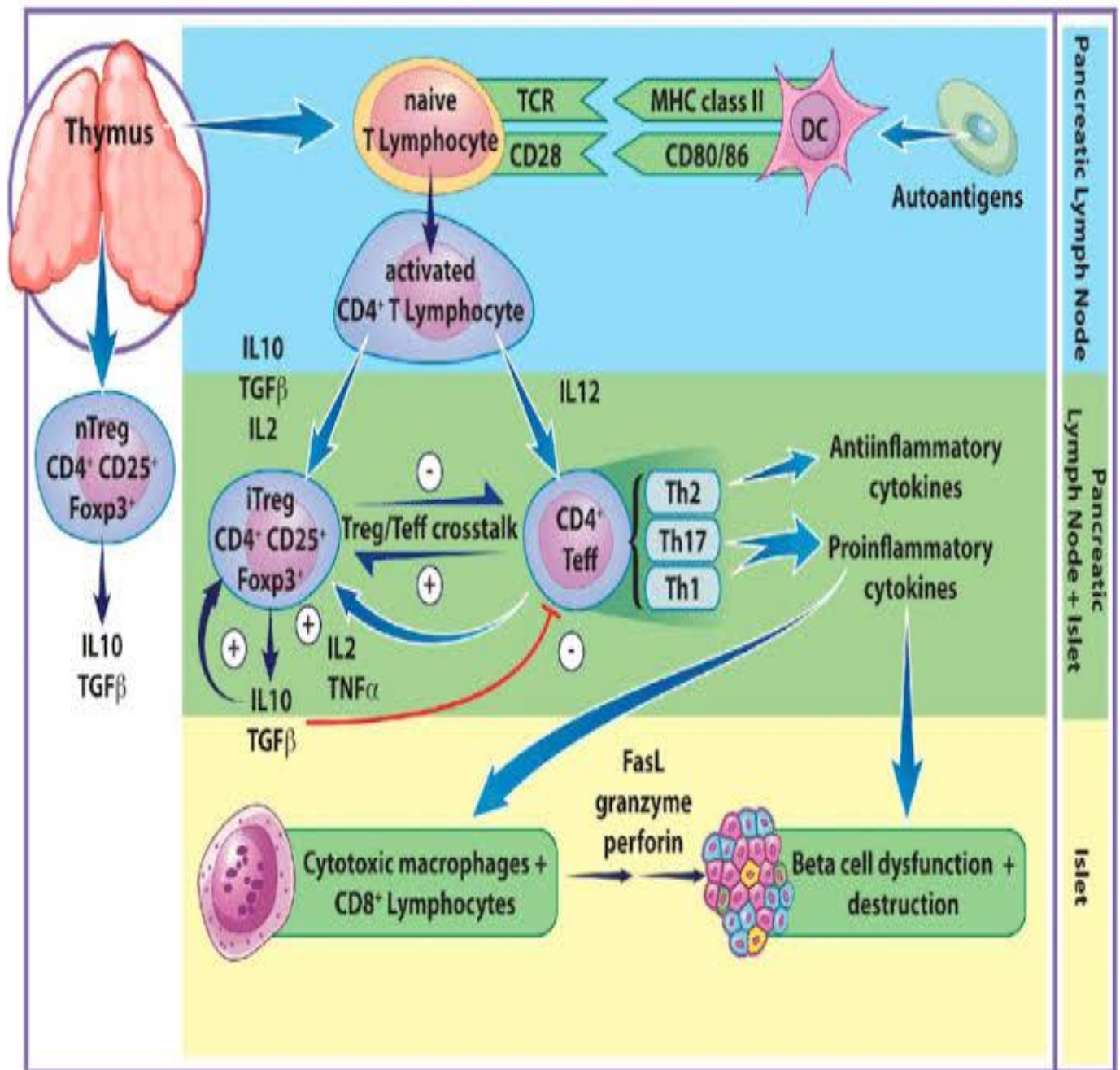


Figure 2.1: Illustration of the pathogenesis of T1DM (Ozougwu *et al.*, 2013).

Key: TGF, Transforming growth factor; MHC, Major histocompatibility complex; IL, Interleukins; TNF, Tumor necrosis factor; TREG, Regulatory T cells

2.4.1.4 PATHOPHYSIOLOGY OF TYPE 1 DIABETES MELLITUS

The autoimmune destruction of pancreatic β -cells leads to a deficiency of insulin secretion, resulting in the associated metabolic derangements in T1DM. In addition to the deficient insulin secretion, the function of pancreatic α -cells is also abnormal, and there is excessive secretion of glucagons in T1DM patients. Typically, hyperglycaemia inhibits glucagon secretion; however, in individuals with T1DM, glucagon secretion is not suppressed by hyperglycaemia (Ozougwu *et al.*, 2013). The resultant inappropriately elevated levels of glucagon further exacerbate the metabolic defects due to insulin deficiency. The most prominent example of this metabolic disruption is that patients with T1DM rapidly develop diabetic ketoacidosis in the absence of insulin administration. Although deficiency of insulin is the primary defect in T1DM, there is also a defect in the administration of insulin. Multiple biochemical mechanisms account for the impairment of tissue's response to insulin. Insulin deficiency leads to uncontrolled lipolysis with elevated plasma free fatty acids, suppressing metabolism of glucose in peripheral tissues (Ozougwu *et al.*, 2013). The impaired glucose utilization and insulin deficiency also decrease the expression of several genes necessary for target tissues to respond typically to insulin, such as glucokinase (in liver) and the GLUT 4 class of glucose transporters (in adipose tissue). The significant metabolic derangements, which result from insulin deficiency in T1DM are impaired glucose, lipid and protein metabolism, as shown in the figure below.

T1DM

Sequence of Metabolic Derangements in Type 1 Diabetes

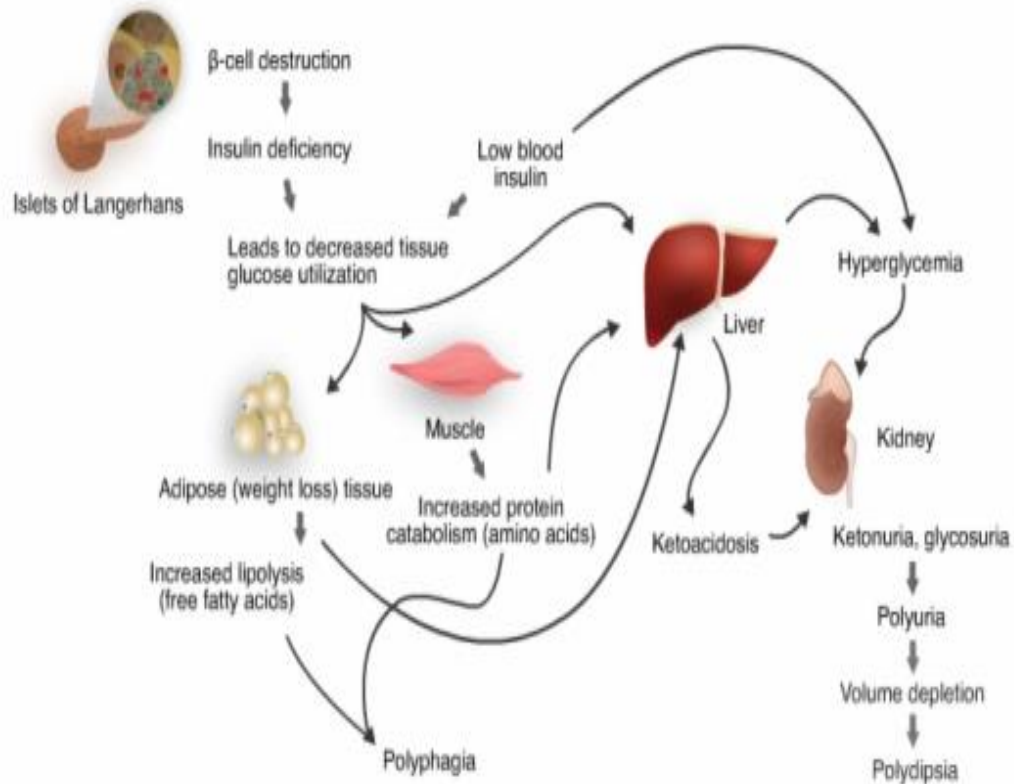


Figure 2.2: Pathophysiology of type 1 diabetes mellitus (Ozougwu *et al.*, 2013)

2.4.2 Type 2 diabetes mellitus

Type 2 DM is sub-classified into (i) predominant insulin resistance with relative insulin deficiency and (ii) predominant insulin secretory defect with insulin resistance. This form of DM accounts for approximately 90–95% of all DM cases and was previously referred to as non-insulin-dependent DM or adult-onset DM. It includes individuals who have insulin resistance with relative (rather than absolute) insulin deficiency (Ferrannini, 1998; Petersen and Shulman, 2018). At least initially and often throughout their lifetimes, these individuals rarely need insulin treatment to survive. Most patients with this form of DM have obesity, especially abdominal obesity, and have a positive family history of DM. Ketoacidosis rarely occurs spontaneously in this type of DM; except in association with the stress of another illness such as infection. T2DM is frequently asymptomatic for many years because the hyperglycaemia develops gradually. At the initial stages, it is often not severe enough for the patient to notice any of the typical symptoms of DM. Nevertheless, such patients are at increased risk of developing both macrovascular and microvascular complications, which may already be present even at the point of diagnosis (Olamoyegun *et al.*, 2015). Insulin secretion is also defective in these patients and insufficient to compensate for insulin resistance. The risk of developing this form of DM increases with age, obesity, prior GDM, hypertension, dyslipidaemia, prior prediabetes, and lack of physical activity. It is often associated with a stronger genetic predisposition compared with T1DM. T2DM is usually preceded by prediabetes, which may be present for many years before developing into overt DM. People with prediabetes may delay or prevent progression to DM by lifestyle modifications, including weight loss and participation in physical exercise, as demonstrated by DM Prevention Program (DPP) and other similar studies (DPP Group, 2002;

Tuso, 2014). Although commonly found among older individuals, T2DM is increasingly being diagnosed among adolescents, especially in developed countries, because of increasing obesity. This type of DM is commonly managed with dietary modifications and oral anti-diabetic agents (OADs) but also may require insulin, especially during acute complications and in secondary failure of OADs (Glechner *et al.*, 2018).

2.4.3 Gestational diabetes mellitus

GDM is defined as any degree of glucose intolerance with onset or first detection during pregnancy (ADA, 2003). GDM may entail situation in which glucose intolerance preceded or diagnosed concomitantly with the pregnancy. Approximately 7% of pregnancies are reported to be complicated with GDM, accounting for more than 200,000 cases annually. The prevalence of GDM may range from 1 to 14% of all pregnancies depending on the diagnostic tests used and the population studied. GDM is often diagnosed during the second or third trimester of pregnancy. It is also a risk factor for the development of T2DM (Zhu and Zhang, 2016; Alejandro *et al.*, 2020).

2.4.4 Other specific types of DM

The frequency of the other specific types of DM is low compared to T1DM, T2DM, and GDM. The aetiologies of these other types include the following: genetic defects in β -cell function, diseases of the exocrine pancreas, genetic defects in insulin action and endocrinopathies (Cushing's syndrome, acromegaly and glucagonoma), and drugs like nicotinic acid, pentamidine, thiazide diuretics, beta-blockers, corticosteroids, and protease inhibitors (Table 2.6).

2.5 Diagnosis of Diabetes

The WHO criteria are commonly used for the diagnosis of DM (Table 2.7). The criteria use fasting plasma glucose (FPG), casual (random) plasma glucose (RPG), or OGTT. Recently, glycated haemoglobin (HbA1C) was approved for the diagnosis of DM.

Criteria for the diagnosis of diabetes (modified)

HbA1C $\geq 6.5\%$. The test is performed in a laboratory using a National Glycohemoglobin Standardization Program (NGSP).*

OR

2. Fasting Plasma Glucose ≥ 126 mg/dl (7.0 mmol/l).

Fasting is defined as no caloric intake for at least eight hours.*

3. Two-hour postprandial plasma glucose ≥ 200 mg/dl (11.1 mmol/l) during an OGTT. The test is performed as recommended by the WHO, using a glucose load containing the equivalent of 75 g anhydrous glucose dissolved in water.*

4. In a patient with classic symptoms of hyperglycaemia or hypoglycaemic crisis, random plasma glucose ≥ 200 mg/dl (11.1 mmol/l)

*In the absence of unequivocal hyperglycaemia symptoms, criteria 1–3 should be confirmed by repeated testing.

Table 2.7: World Health Organization criteria for diagnosis of DM (WHO, 2019).

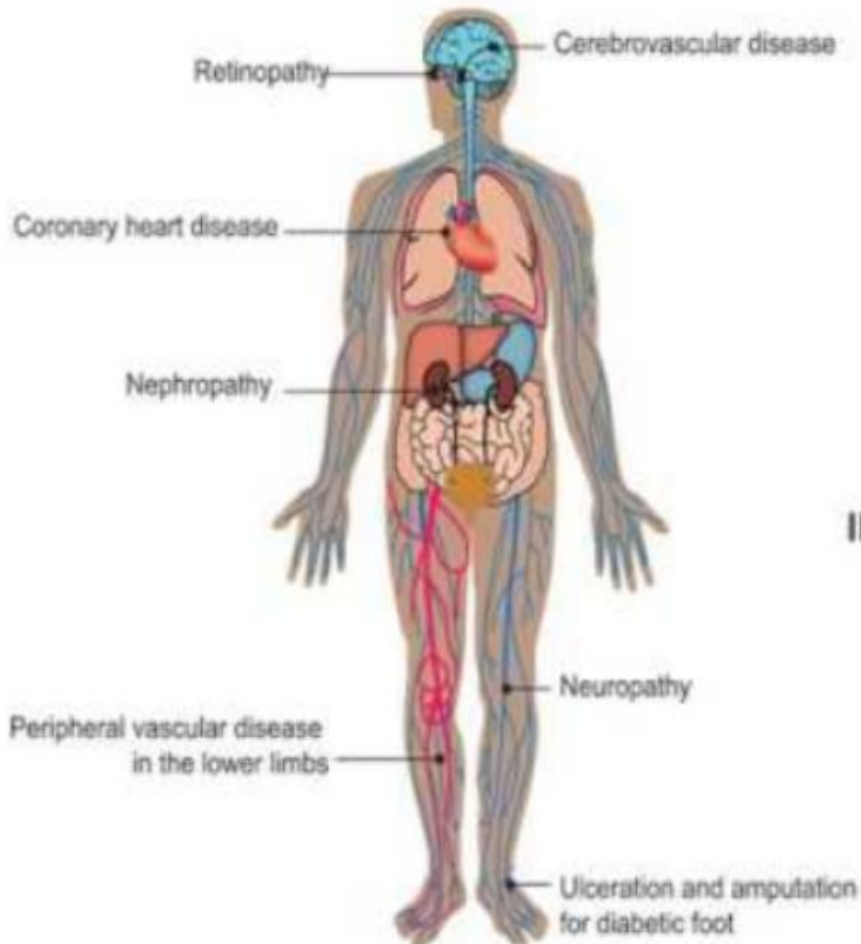
Impaired fasting glucose	• Fasting plasma glucose 6.1 to 6.9 mmol/l (110 mg/dl to 125 mg/dl) • 2-h postprandial plasma glucose <7.8 mmol/l (140 mg/dl)
Impaired glucose tolerance (fulfils both criteria)	• Fasting plasma glucose <7.0 mmol/l (126 mg/dl) • 2-h postprandial plasma glucose $\geq$7.8 (140 mg/dl) and <11.1 mmol/l (200mg/dl)
Diabetes mellitus (fulfils any criterion)	• Fasting plasma glucose $\geq$7.0 mmol/l (126 mg/dl) • 2-h postprandial plasma glucose $\geq$ 11.1 mmol/l (200 mg/dl) • HbA1c $\geq$ 48 mmol/mol
Diagnostic criteria for gestational DM:	• fasting plasma glucose 5.1–6.9 mmol/L • or 1-hour post-load plasma glucose $\geq$ 10.0 mmol/L • or 2-hour post-load plasma glucose 8.5–11.0 mmol/L

2.6 Complications of Diabetes

If not controlled, hyperglycaemia can lead to complications, divided into two broad categories: acute and chronic complications.

Acute complications include diabetic ketoacidosis (DKA), Hyperosmolar hyperglycaemic state (HHS), hypoglycaemia and lactic acidosis. In contrast, chronic complications may be microvascular (such as DM eye disease [retinopathy], DM kidney disease [nephropathy], and DM neuropathy) or macrovascular (stroke, myocardial infarction, peripheral arterial disease and gangrene). The macrovascular complications are accelerated atherosclerosis, secondary to dyslipidaemia, primarily increased plasma low-density lipoprotein cholesterol. The result is an increased incidence of stroke and myocardial infarction (WHO, 2019).

Major diabetes complications



I. Diabetic angiopathy:

1. Microangiopathy:

- 1) nephropathy;
- 2) retinopathy;
- 3) angiopathy of lower extremities.

2. Macroangiopathy:

- 1) heart (ischemic heart disease);
- 2) brain
- 3) angiopathy of lower extremities.

II. Diabetic neuropathy:

- 1) central (encephalopathy);
- 2) peripheral;
- 3) visceral (dysfunction of inner organs).

Figure 2.3: Complications of diabetes mellitus in various organ systems (Pop-Busui, 2010).

2.6.1 Microvascular Complications of DM

2.6.1.1 Diabetic Retinopathy

Patients with DM often develop eye complications, such as corneal abnormalities, cataracts, glaucoma, iris neovascularization, and neuropathies. The most common and potentially most debilitating of these complications is DM retinopathy, which is regarded as the leading aetiology of new onset blindness in individuals aged 25–74 years in the United States (Cheung *et al.*, 2010).

Diabetic retinopathy is about the most common microvascular complication of DM. The risk of developing DM retinopathy or other microvascular complications of DM is influenced by the duration and severity of hyperglycaemia, cigarette smoking, pregnancy, dyslipidaemia, and hypertension. Most patients with T1DM develop evidence of retinopathy within 20 years of diagnosis, compared to patients with T2DM in whom retinopathy is found at diagnosis or shortly after diagnosis (Yau *et al.*, 2012; Lee *et al.*, 2015; Nentwich, 2015).

As evidenced by elevated blood urea /creatinine levels and proteinuria, renal disease is an excellent predictor of retinopathy; DM-related micro-angiopathies cause both conditions, and the presence and severity of one reflect that of the other. Aggressive treatment of nephropathy may slow the progression of DM retinopathy and neovascular glaucoma. A study by Ito *et al.* indicated that in patients with T2DM, reduced peripheral nerve conduction velocity is associated with early DM retinopathy. The report included 42 patients with T2DM (42 eyes) who had no DM retinopathy or mild non-proliferative diabetic retinopathy (NPDR). The study observed that the NPDR group had significantly lower tibial motor conduction velocity and sural sensory conduction velocity than did patients with no DM retinopathy. Furthermore, logistic regression

analysis showed that the conduction velocities were independent risk factors for mild non-proliferative eye disease (Ito *et al.*, 2018).

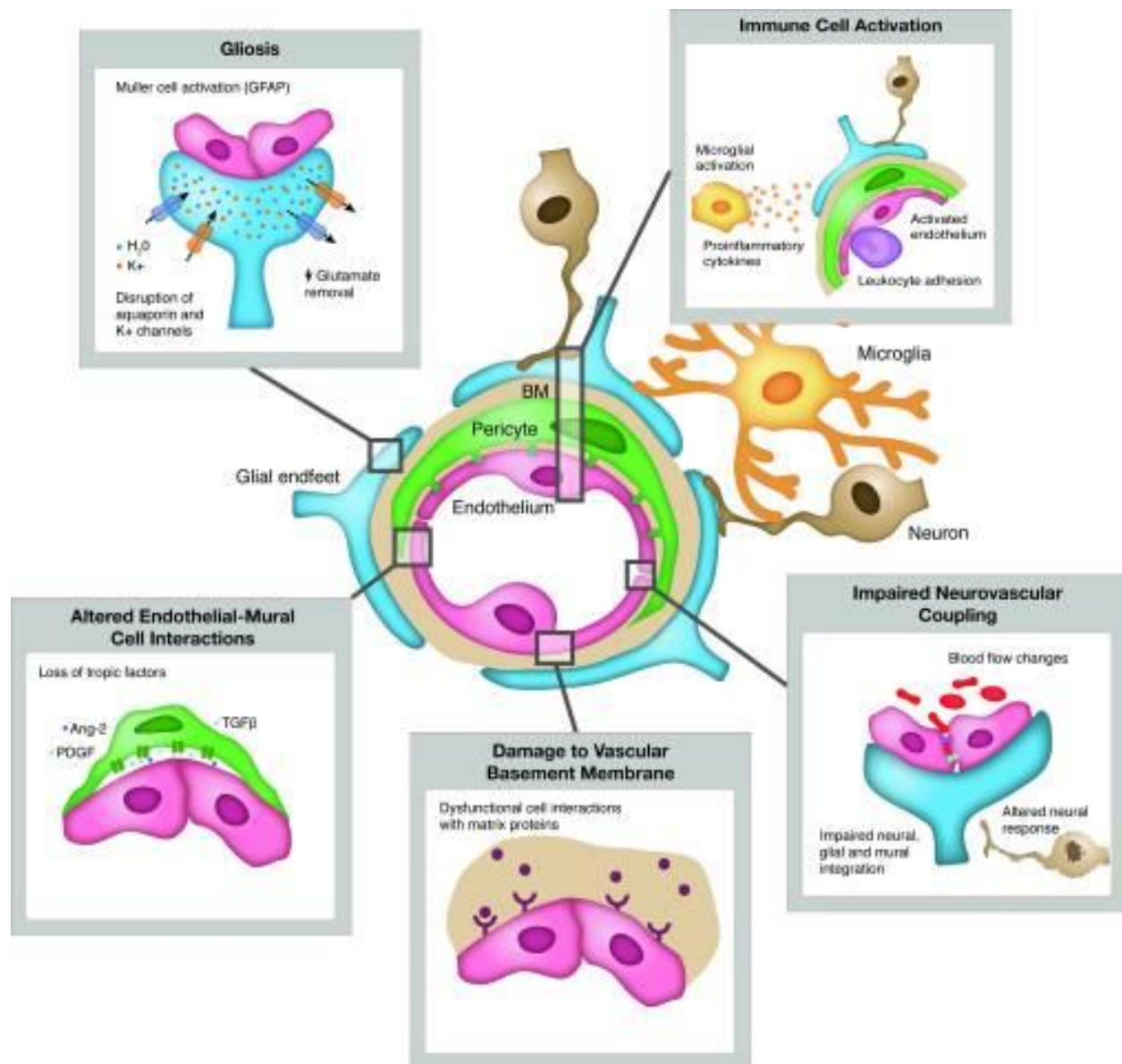


Figure 2.4: The neurovascular unit and its disruption by diabetes (Duh *et al.*, 2017).

Clinical Features

In the initial stages of DM retinopathy, patients are generally asymptomatic; in the more advanced stages of the disease, patients may experience floaters, blurred vision, vision distortion, and progressive visual acuity loss (Klein *et al.*, 2009). Signs of DM retinopathy include the following:

- Microaneurysms: These are the earliest clinical sign of DM retinopathy, and they occur secondary to an outpouching of capillary walls due to pericyte loss. The microaneurysm appears as small, red dots in the superficial layers of the retina.
- Dot and blot haemorrhages: They appear similar to microaneurysms if they are small. They occur as rupture of the microaneurysms in the retina's deeper layers, such as the inner nuclear and outer plexiform layers.
- Flame-shaped haemorrhages: These are splinter haemorrhages that occur in the more superficial nerve fibre layers.
- Retinal oedema and hard exudates: They are caused by the breakdown of the blood-retina barrier thereby leading to leakage of serum proteins, lipids, and protein from the vessels.
- Cotton-wool spots: These are caused by nerve fibre layer infarctions from occlusion of precapillary arterioles. Microaneurysms and areas of vascular hyperpermeability frequently border them.
- Venous loops and venous beading: They frequently occur adjacent to areas of non-perfusion. They reflect increasing retinal ischaemia, and their occurrence is the most significant predictor of progression to proliferative DM retinopathy (PDR).
- Intra-retinal microvascular abnormalities: These are due to remodelled capillary beds without proliferative changes and can usually be seen on the borders of the non-perfused retina.

- Macular oedema: This is the leading cause of visual impairment in patients with DM.

Nonproliferative DM retinopathy

- Mild: There is the presence of at least one microaneurysm
- Moderate: includes the presence of haemorrhages, microaneurysms, and hard exudates
- Severe: characterized by haemorrhages and microaneurysms in 4 quadrants, with venous beading in at least two quadrants, and intra-retinal microvascular disruptions in at least one quadrant.

Proliferative diabetic retinopathy

- Neovascularization: the hallmark of PDR
- Pre-retinal haemorrhages: appear as blood pockets within the potential space between the posterior hyaloid and the retina; as blood pools within this space, the haemorrhages may appear boat-shaped.
- Haemorrhage into the vitreous: may appear as a diffuse haze or as clumps of blood clots within the vitreous humour.
- Fibrovascular tissue proliferation: It is associated with neovascular complex although may appear avascular when the vessels have already regressed.
- Traction retinal detachments: This may appear tented up, immobile, and concave
- Macular oedema.

Diagnosis

Laboratory studies of HbA1c levels are essential in the long-term follow-up care of patients with DM and retinopathy.

Imaging studies used in the diagnosis of DM retinopathy include the following:

- Fluorescein angiography: Microaneurysms is seen as pinpoint, hyperfluorescent lesions in the early phases of the angiogram and typically leak in the latter phases.
- Optical coherence tomography scanning: This is administered to determine the thickness of the retina, the presence of swelling within the retina and vitreomacular traction.
- B-scan ultrasonography



Figure 2.5: Fundus photograph of early background diabetic retinopathy showing multiple microaneurysms (Crawford *et al.*, 2009).

Pathophysiology

The exact mechanism by which DM causes retinopathy remains unclear, but several theories have been postulated to explain the disease's typical course and history (Crawford *et al.*, 2009; Ruta *et al.*, 2013).

Growth hormone

Growth hormone appears to play a vital role in the development and progression of DM retinopathy. Diabetic retinopathy is reversible in women with postpartum haemorrhagic necrosis of the pituitary gland (Sheehan syndrome). This resulted in the controversial practice of pituitary ablation to treat or prevent DM retinopathy in the 1950s. This technique however, is no longer been practiced due to systemic complications and the discovery of laser therapy. It should also be noted that DM retinopathy has been reported in patients with hypopituitarism as well.

Platelets and blood viscosity

The variety of haematologic abnormalities seen in DM, such as increased erythrocyte aggregation, decreased red blood cell deformability, and increased platelet aggregation and adhesion, predispose the patient to sluggish circulation, endothelial damage, and focal capillary occlusion. This leads to retinal ischaemia, which may contribute to the development of DM retinopathy.

Aldose reductase and vasoproliferative factors

DM causes abnormal glucose metabolism as a result of decreased levels or activity of insulin. Increased blood glucose levels may have a structural and physiologic effect on retinal capillaries, leading to both functional and structural incompetence.

In specific tissues, a persistent increase in blood glucose levels shunts excess glucose into the aldose reductase pathway, converting sugars into alcohol (e.g., glucose into sorbitol and

galactose to dulcitol). Intramural pericytes of retinal capillaries is affected by this increased sorbitol level, which eventually leads to the loss of their primary function (i.e., autoregulation of retinal capillaries). This results in weakness and saccular outpouching of capillary walls. The microaneurysms thus formed are the earliest detectable signs of DM retinopathy. Ruptured microaneurysms result in retinal haemorrhages either superficially (flame-shaped haemorrhages) or in deeper retina layers (blot and dot haemorrhages) (figure 2.6).

Using nail fold video capillaroscopy, a high prevalence of capillary changes has been detected in patients with diabetes, particularly those with retinal damage. This reflects generalized microvessel involvement in both T1DM and T2DM. Increased permeability of the new vessels results in leakage of proteinaceous material and fluids, which clinically appear as exudates and retinal thickening. If the exudation and swelling involve the macula, a reduction in central vision may be experienced.

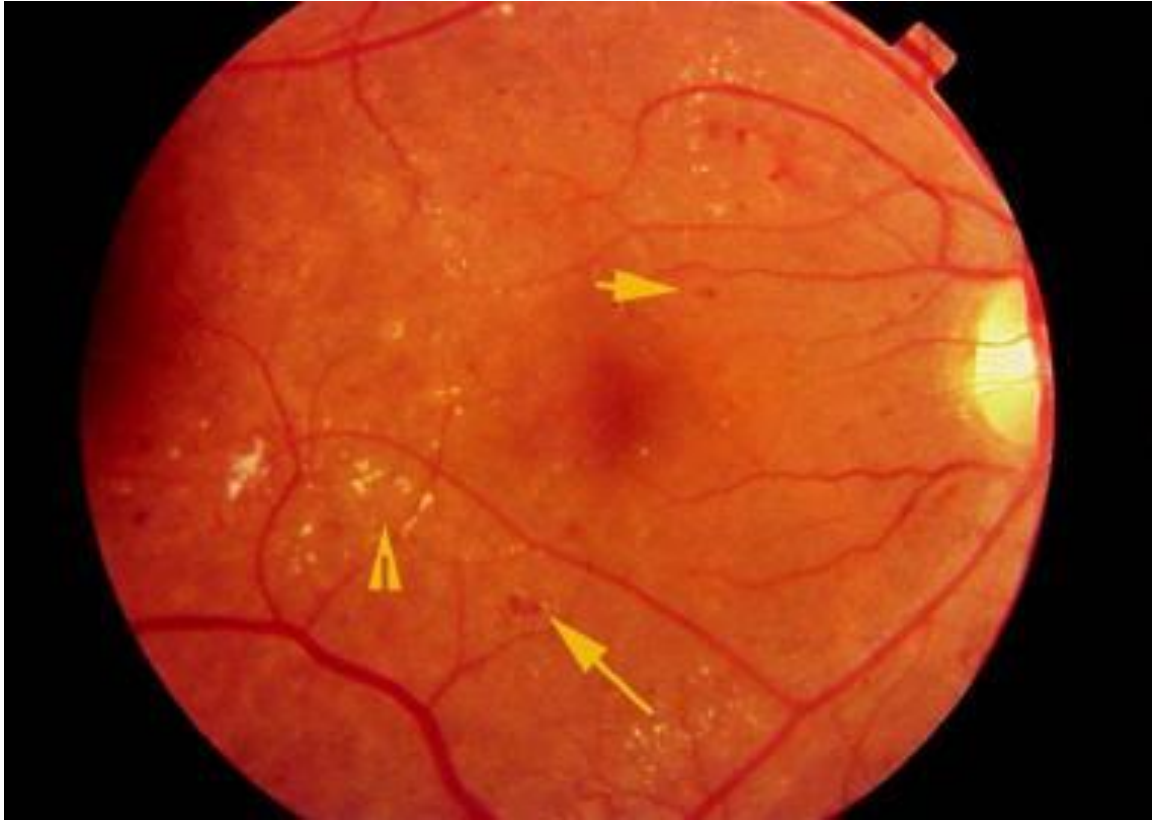


Figure 2.6: Retinal findings in background diabetic retinopathy, including blot haemorrhages (long arrow), microaneurysms (short arrow), and hard exudates (arrowhead) (Crawford *et al.*, 2009).

Macular oedema

Macular oedema is the most common cause of blindness in patients with NPDR although it may also complicate PDR cases (Figure 2.7A and B).

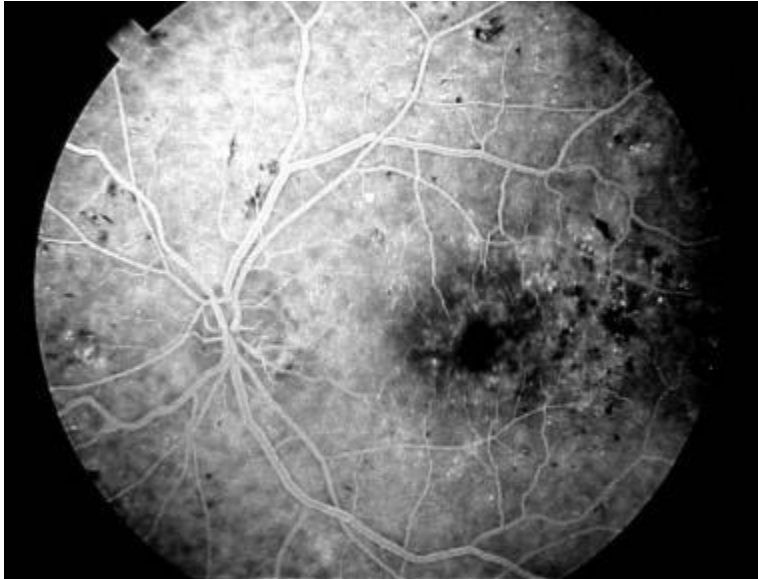


Figure 2.7A: Fluorescein angiogram demonstrating foveal dye leakage caused by macular oedema (Crawford *et al.*, 2009).



Figure 2.7B: Fundus photograph of clinically significant macular oedema demonstrating retinal exudates within the fovea (Crawford *et al.*, 2009).

Another theory to explain macular edema development focuses on increased diacylglycerol levels from the shunting of excess glucose. This is due to activation of protein kinase C, which, in turn, affects retinal blood dynamics, especially permeability and flow, leading to leakage of fluid and retinal thickening.

Hypoxia

As DM retinopathy progresses, eventual closure of the retinal capillaries occur, leading to hypoxia. Infarction of the nerve fibre layer result in the formation of cotton wool spots, with concomitant stasis in axoplasmic flow.

More extensive retinal hypoxia stimulates compensatory mechanisms which provide oxygen in the eye tissues. Venous calibre abnormalities, such as venous beading, loops, and dilation, signify increasing hypoxia and are commonly seen bordering the areas of capillary non-perfusion. Intraretinal microvascular distortion represent either new vessel growth or remodelling of pre-existing vessels through endothelial cell proliferation within the retinal tissues to act as shunts through areas of non-perfusion.

Neovascularization

Further increases in retinal ischaemia trigger the production of vasoproliferative factors that stimulate new vessel formation. New vessels arise from the retinal venules and penetrate the internal limiting membrane thereby forming capillary networks between the inner surface of the posterior hyaloid face and the retina (Hartnett *et al.*, 2017).

Neovascularization is commonly observed at the borders of perfused and non-perfused parts of the retina. It most commonly occurs along the vascular arcades and at the optic nerve head. The new vessels breakthrough and grow along the surface of the retina into the scaffold of the posterior hyaloid face. The new vessels rarely cause visual compromise naturally although they

are fragile and highly permeable. The delicate vessels are disrupted easily by vitreous traction, which leads to haemorrhage into the vitreous cavity or the pre-retinal space.

These new blood vessels are initially associated with a small amount of fibroglial tissue formation. However, the degree of fibrous tissue formation increases as the density of the neovascular frond increases. In later stages, the blood vessels may regress, leaving only avascular fibrous tissue networks adherent to both the posterior hyaloid face and retina. Tractional forces are exerted on the retina as the vitreous contracts through these fibroglial connections. Traction may cause retinal oedema, retinal heterotopia, and both tractional retinal detachments and retinal tear formation with subsequent detachment (Flaxel *et al.*, 2020).

Risk factors for retinopathy

Duration of diabetes

In patients with T1DM, no clinically significant retinopathy can be seen in the first five years after the initial diagnosis of DM. After 10–15 years, 25–50% of patients may show some signs of retinopathy. The prevalence increases to 75–95% after 15 years and approaches 100% after 30 years of being diagnosed with diabetes. PDR is rare in the first decade of diagnosis of T1DM, but its prevalence increases to 14–17% by 15 years, and then rises steadily after that (Nentwich, 2015). In patients with T2DM, the incidence of diabetic retinopathy increases with the disease duration. Among these patients, 23% have NPDR after 11–13 years, 41% after 14–16 years, and 60% after 16 years (Song and Wong, 2014).

Hypertension and hyperlipidaemia

Systemic hypertension, in the setting of DM nephropathy, correlates well with the presence of retinopathy. Independently, hypertension may also complicate DM in that it may result in

hypertensive retinal vascular changes, which can then be superimposed on the pre-existing diabetic retinopathy, further compromising retinal blood flow. Proper management of hyperlipidaemia may result in less retinal vessel leakage and hard exudate formation, although the reason behind this is unclear (Do *et al.*, 2015).

Pregnancy

Pregnant women with PDR fare poorly without treatment, but those who have had prior pan-retinal photocoagulation remain stable throughout pregnancy. Pregnant women without DM retinopathy run a 10% risk of developing NPDR during their pregnancies; of those with pre-existing NPDR, 4% progress to the proliferative type. A study by Morrison *et al.* found that among pregnant women with DM retinopathy, progression of the eye disease was associated with a longer duration of DM, to have had diabetic retinopathy before pregnancy, and to have higher blood pressure in the second trimester (Morrison *et al.*, 2016).

Prognosis

Prognostic factors that reduce the risk of visual loss include the following:

- Circinate exudates of recent onset
- Well-defined leakage
- Good perifoveal perfusion

Prognostic factors that increase the risk of visual loss include the following:

- Diffuse oedema/multiple leaks
- Lipid deposition in the fovea
- Macular ischaemia
- Cystoid macular oedema
- Preoperative visual acuity less than 20/200.

Hypertension

Approximately 8,000 eyes become blind yearly because of DM. The treatment of DM retinopathy entails tremendous costs. However, it has been estimated that this represents only one-eighth of the costs of Social Security payments for vision loss in the USA. This cost does not compare to the non-monetary cost in terms of loss of productivity and quality of life. The Early Treatment for DM Retinopathy Study found that laser surgery for macular oedema reduces the incidence of moderate visual loss from 30% to 15% over three years. The Diabetic Retinopathy Study also found that adequate scatter laser pan-retinal photocoagulation reduces the risk of severe visual loss ($< 5/200$) by more than 50% (Amoaku *et al.*, 2020).

Patient Education

One of the essential aspects in the management of diabetic retinopathy is patient education (Duh *et al.*, 2017). Patients must be informed that they play an integral role in their eye care.

Excellent glucose control is beneficial in any stage of DM retinopathy: it delays the onset and slows down the progression of DM complications in the eye.

The following symptoms and health concerns must be addressed in any patient education program for those with diabetic retinopathy:

- Systemic diseases (e.g. hypertension, renal disease, and hyperlipidaemia) may contribute to progression of the disease.
- Smoking, although not directly proven to affect the course of DM retinopathy, may further compromise oxygen delivery to the retina. Therefore, all efforts should be made toward the reduction, if not outright cessation, of smoking.

- Visual symptoms (e.g. vision changes, floaters, distortion, redness, and pain) could be manifestations of disease progression and should be reported immediately.

DM, in general, and diabetic retinopathy, in particular, are progressive conditions, and regular follow-up care with a physician is crucial for detection of any changes that may benefit from treatment (Klein *et al.*, 2009; Nentwich, 2015).

2.6.1.2 Diabetic Nephropathy

Diabetic nephropathy is regarded as the leading cause of renal failure in the United States and one of the leading causes of renal failure in developing countries, including Nigeria. It is defined by 24 hour proteinuria >500 mg in the setting of DM or reduction in estimated glomerular filtration rate (eGFR) with or without elevated creatinine and urea levels. However, this proteinuric level is usually preceded by lower degrees of proteinuria, described as “microalbuminuria.” Microalbuminuria is defined as albuminuria of 30–299 mg per 24 hours (or 20–200 mcg/min or 30–300 mcg/mg creatinine) in 2 of 3 urine collections (Mogensen *et al.*, 1983; Prakash and Yadav, 2017). Without intervention, DM patients with microalbuminuria typically progress to proteinuria and overt DM nephropathy. This progression occurs in both T1DM and T2DM (Gross *et al.*, 2005). Over 7% of patients with T2DM may already have microalbuminuria at the point of diagnosis. The overall incidence of microalbuminuria in patients with T1DM was ~12% during seven years and 2% per year in patients with T2DM and the 10-year prevalence after diagnosis was 25% (Prakash and Yadav, 2017). The pathological changes in the kidney include increased glomerular basement membrane thickness, microaneurysm formation, mesangial nodules (Kimmelstiel-Wilson bodies) formation, and other changes (Tecilazich, 2020).

Screening for DM nephropathy or microalbuminuria may be accomplished by either 24-hour urine collection or spot urine measurement of microalbumin. It is important to note that false positive proteinuria may be produced by conditions such as urinary tract infections, exercise, and haematuria (Gross *et al.*, 2005).

2.6.1.3 Diabetic neuropathy

Diabetic neuropathy is recognized by the American Diabetes Association (ADA) as “the presence of signs and symptoms of peripheral nerve dysfunction in people with DM after the exclusion of other causes” (Fowler, 2008). As with other microvascular complications, the risk of developing diabetic neuropathy is related to both the severity and duration of hyperglycaemia and some individuals may possess genetic attributes that affect their predisposition to developing such complications (Pop-Busui, 2010; Bissinger, 2017; Vinik *et al.*, 2018) (Figure 2.8 and 2.9).

DM neuropathy is broadly divided into diffuse neuropathy (distal symmetrical polyneuropathy and autonomic neuropathy), mononeuropathy and polyradiculopathy as shown in table 2.8.

Diabetic autonomic neuropathy is one of the most common forms of neuropathy affecting many organ systems in the body as shown in table 2.9 below.

Table 2.8: Classification of DM neuropathy (Pop-Busui *et al.*, 2017).

Diabetic neuropathies
A. Diffuse neuropathy
DSPN
• Primarily small-fiber neuropathy • Primarily large-fiber neuropathy • Mixed small- and large-fiber neuropathy (most common)
Autonomic
Cardiovascular
• Reduced HRV • Resting tachycardia • Orthostatic hypotension • Sudden death (malignant arrhythmia)
Gastrointestinal
• Diabetic gastroparesis (gastropathy) • Diabetic enteropathy (diarrhea) • Colonic hypomotility (constipation)
Urogenital
• Diabetic cystopathy (neurogenic bladder) • Erectile dysfunction • Female sexual dysfunction
Sudomotor dysfunction
• Distal hypohydrosis/anhidrosis, • Gustatory sweating
Hypoglycemia unawareness
Abnormal pupillary function
B. Mononeuropathy (mononeuritis multiplex) (atypical forms)
Isolated cranial or peripheral nerve (e.g., CN III, ulnar, median, femoral, peroneal)
Mononeuritis multiplex (if confluent may resemble polyneuropathy)
C. Radiculopathy or polyradiculopathy (atypical forms)
Radiculoplexus neuropathy (a.k.a. lumbosacral polyradiculopathy, proximal motor amyotrophy)
Thoracic radiculopathy
Nondiabetic neuropathies common in diabetes
Pressure palsies
Chronic inflammatory demyelinating polyneuropathy
Radiculoplexus neuropathy
Acute painful small-fiber neuropathies (treatment-induced)

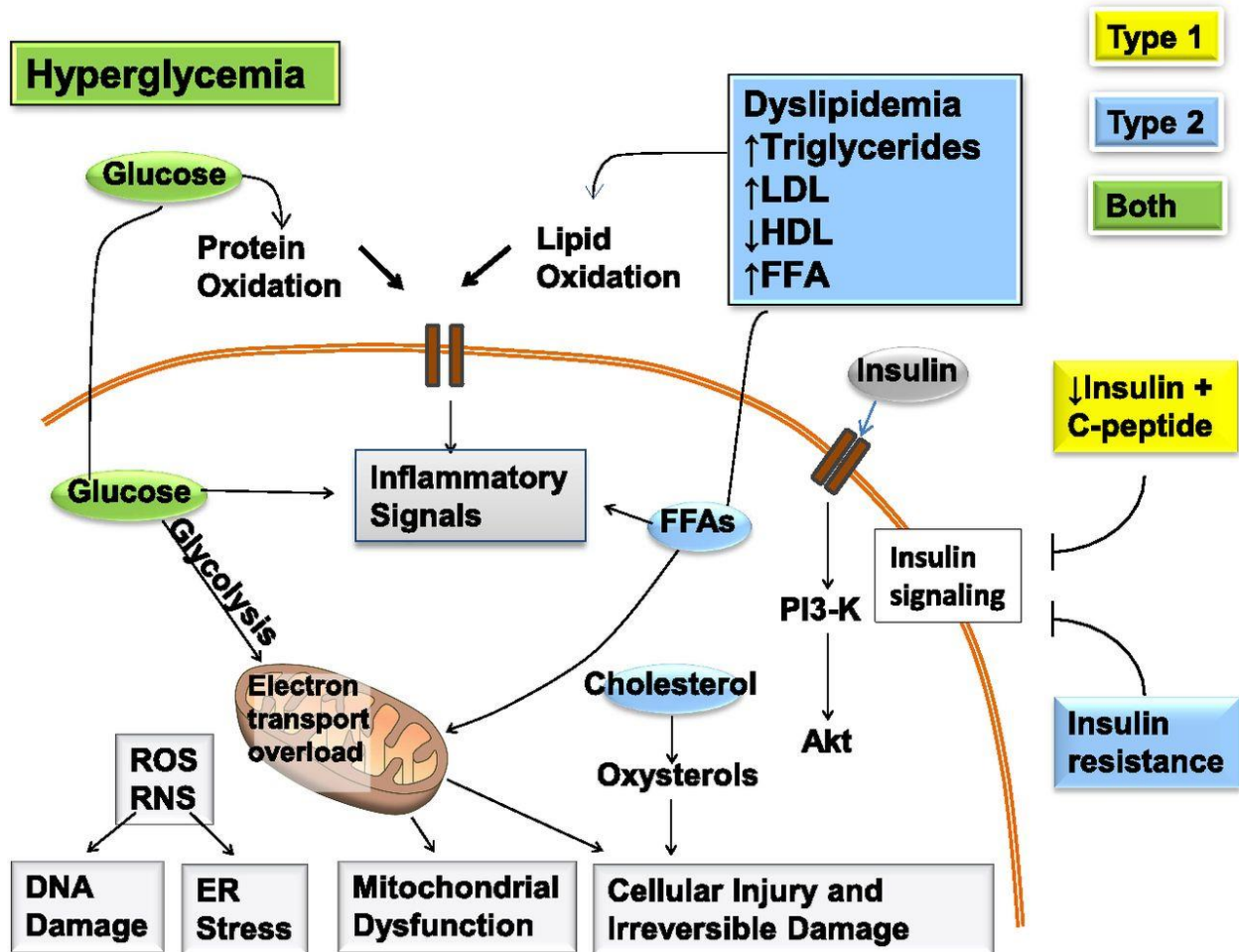


Figure 2.8: Mechanisms of diabetic neuropathy: Factors linked to T1DM (yellow), T2DM (blue), and both (green) cause DNA damage, endoplasmic reticulum stress, mitochondrial dysfunction, cellular injury, and irreversible damage. The relative importance of the pathways in this network will vary with cell type, disease profile, and time (Callaghan *et al.*, 2012).

Key: ER, endoplasmic reticulum; FFA, free fatty acids; HDL, high-density lipoprotein; LDL, low-density lipoprotein; PI3-K, phosphatidylinositol-3 kinase; RNS, reactive nitrogen species; ROS, reactive oxygen species.

2.6.2 EFFECT OF DIABETES ON NERVES

Diabetes cause progressive destruction of the nerve blood vessels leading to the various clinical manifestation of DM neuropathy as shown in figure 2.9.

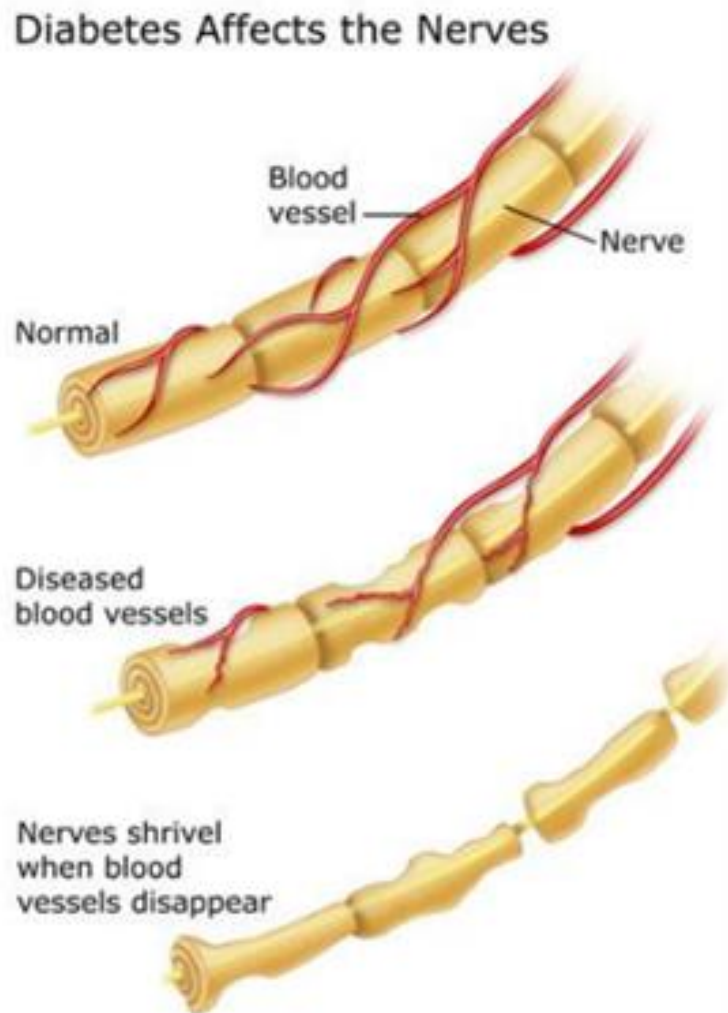


Figure 2.9: Illustration of nerve and blood vessel cells damaged due to DM (Pop-Busui, 2010).

Table 2.9: Clinical manifestation of diabetic autonomic neuropathy (Pop-Busui *et al.*, 2017).

CAN	Gastrointestinal	Urogenital	Sudomotor
Resting tachycardia Abnormal blood pressure regulation • Nondipping • Reverse dipping	Gastroparesis (Gastropathy) • Nausea • Bloating • Loss of appetite • Early satiety • Postprandial vomiting • Brittle diabetes	Bladder dysfunction • Frequency • Urgency • Nocturia • Hesitancy • Weak stream • Dribbling • Urinary incontinence • Urinary retention	Dry skin • Anhidrosis • Gustatory sweating
Orthostatic hypotension (all with standing) • Light-headedness • Weakness • Faintness • Visual impairment • Syncope	Esophageal dysfunction • Heartburn • Dysphagia for solids	Male sexual dysfunction • Erectile dysfunction • Decreased libido • Abnormal ejaculation	
Orthostatic tachycardia or bradycardia and chronotropic incompetence (all with standing) • Light-headedness • Weakness • Faintness • Dizziness • Visual impairment • Syncope	Diabetic diarrhea • Profuse and watery diarrhea • Fecal incontinence • May alternate with constipation	Female sexual dysfunction • Decreased sexual desire • Increased pain during intercourse • Decreased sexual arousal • Inadequate lubrication	
Exercise intolerance	Constipation • May alternate with explosive diarrhea		

2.7. DIABETIC CARDIOVASCULAR AUTONOMIC NEUROPATHY

DCAN is defined as the impairment of autonomic control of the cardiovascular system in the setting of DM after exclusion of other causes. It may affect patients with T1DM or T2DM and may be detected at a subclinical stage through cardiovascular autonomic reflex tests. DCAN has been associated with higher morbidity and mortality with significant cardiovascular changes in DM patients (Agashe and Petak, 2018).

2.7.1. REVIEW OF AUTONOMIC ACTIVITY OF THE HEART

Both the sympathetic and parasympathetic nerves supply and control cardiac activity. They regulate the intrinsic cardiac conduction system through independent receptors. The autonomic nerves are notably denser in the atrium around the sino-atrial region. Sympathetic nerve stimulation increases heart rate, the force of contraction, and the conduction velocity of impulses in the atrioventricular (AV) node. On the other hand, parasympathetic stimulation reduces heart rate, conduction speed, and force of contraction. Both parasympathetic and sympathetic systems interact effectively to achieve optimal control of cardiac functions (Malpas, 2010).

ANS monitoring

ANS: Sympathetic vs. Parasympathetic Responses

			Heart
Receptor	Effector	Response	
Sympathetic	β_1 SA node	$\uparrow$ HR	Parasympathetic
	M_2 SA node	$\downarrow$ HR	
	β_1 AV node	$\uparrow$ conduction velocity and automaticity	
	M_2 AV node	$\downarrow$ conduction velocity	
	β_1 Contractility	$\uparrow$ force of contraction (atria & ventricles)	
	M_2 Contractility	$\downarrow$ contractility (atria)	

Figure 2.10: Effect of autonomic activation on heart functions (Vinik *et al.*, 2013).

Key: ANS, autonomic nervous system; AV, atrioventricular; HR; heart rate; SA, sino-atrial

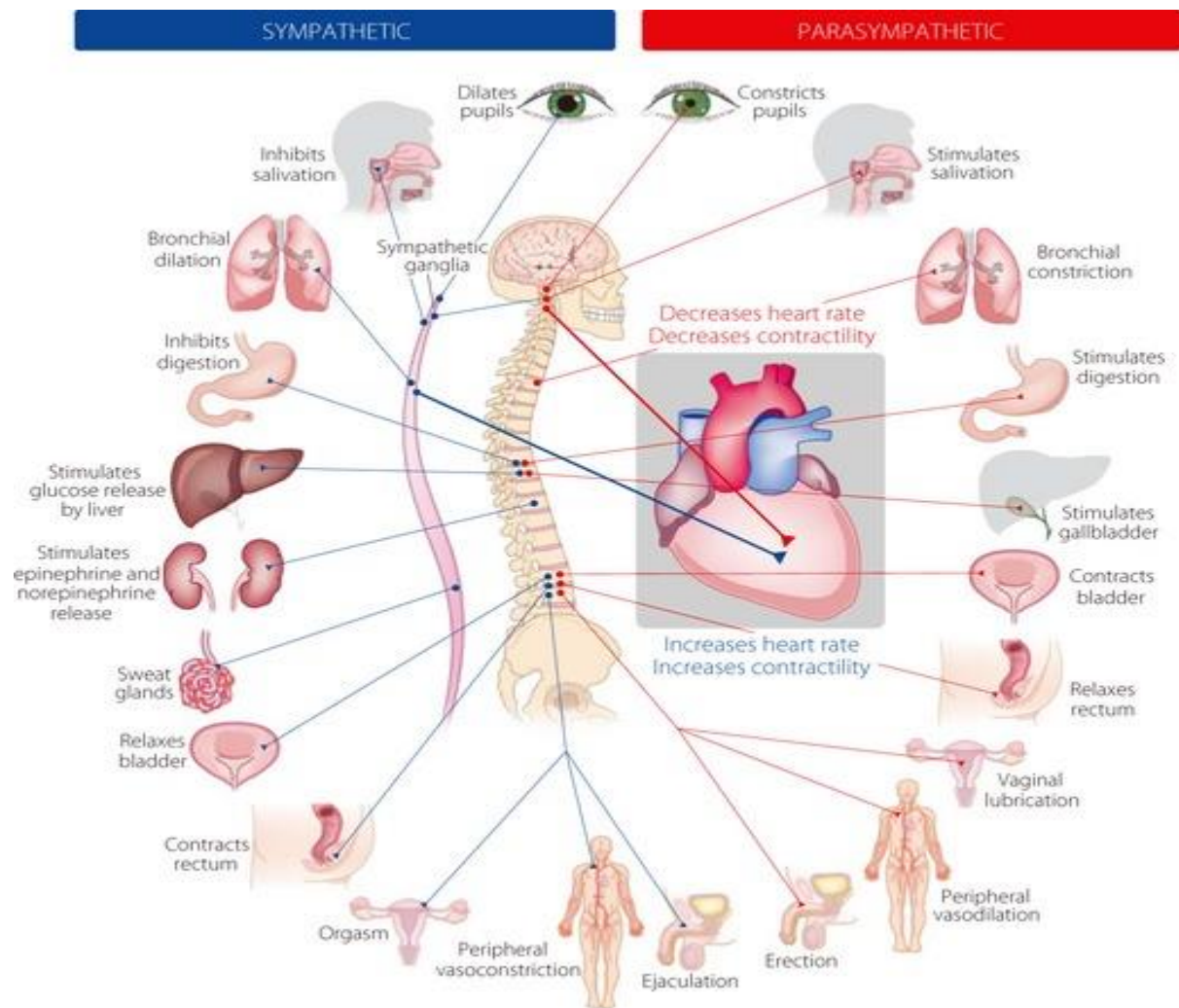


Figure 2.11: Physiological functions of both the sympathetic and parasympathetic nervous system (Vinik *et al.*, 2013).

2.7.2. AUTONOMIC CONTROL OF HEART FUNCTION

Efferent pre-ganglionic vagal nerve (parasympathetic) fibres course from the brainstem towards the heart and connect with post-ganglionic cells in the circumscript epicardial ganglionic plexus. Sympathetic pre-ganglionic fibres synapse with post-ganglionic fibres inside the stellate ganglion. The primary afferent inputs to the central autonomic centres are the carotid sinus nerves and spinal cord afferents (Ardell *et al.*, 2016; Waxenbaum and Varacallo, 2019).

ANS monitoring

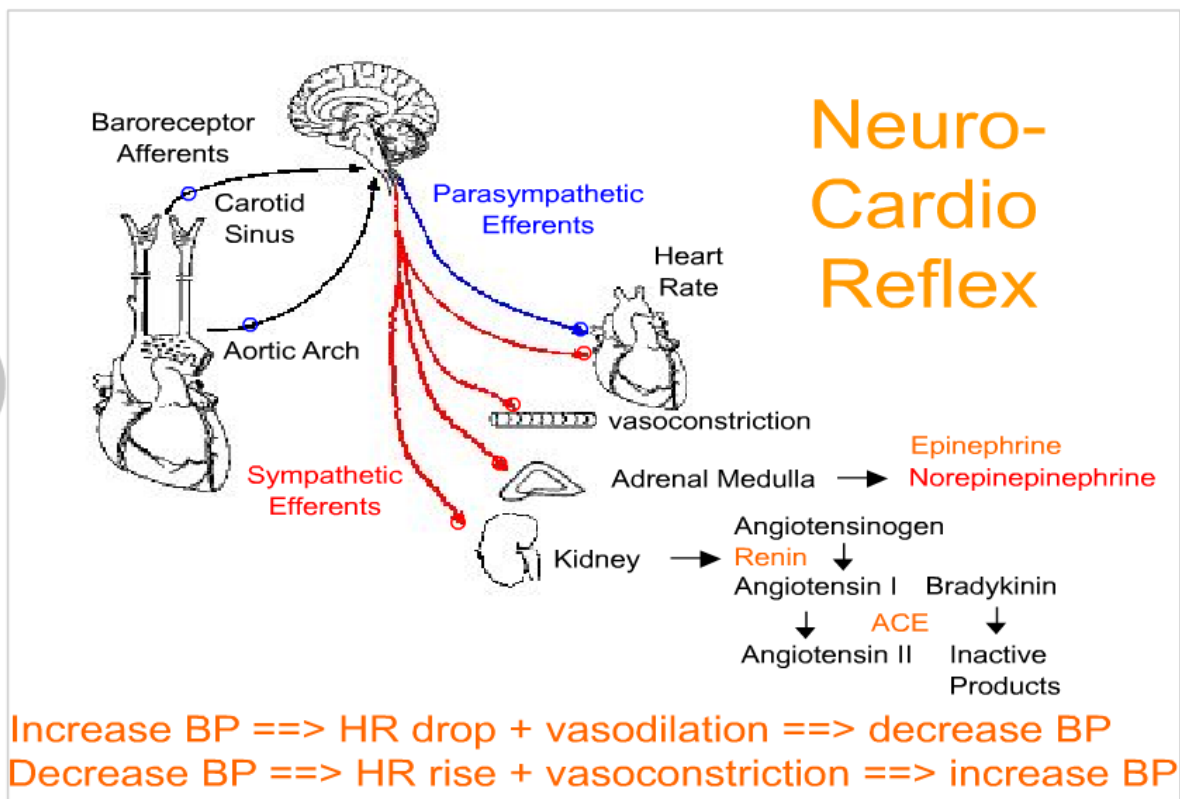


Figure 2.12: Neural control of cardiac functions (Pop-Busui, 2010).

Key: ACE, angiotensin-converting enzyme; ANS, autonomic nervous system; BP, blood pressure; HR; heart rate.

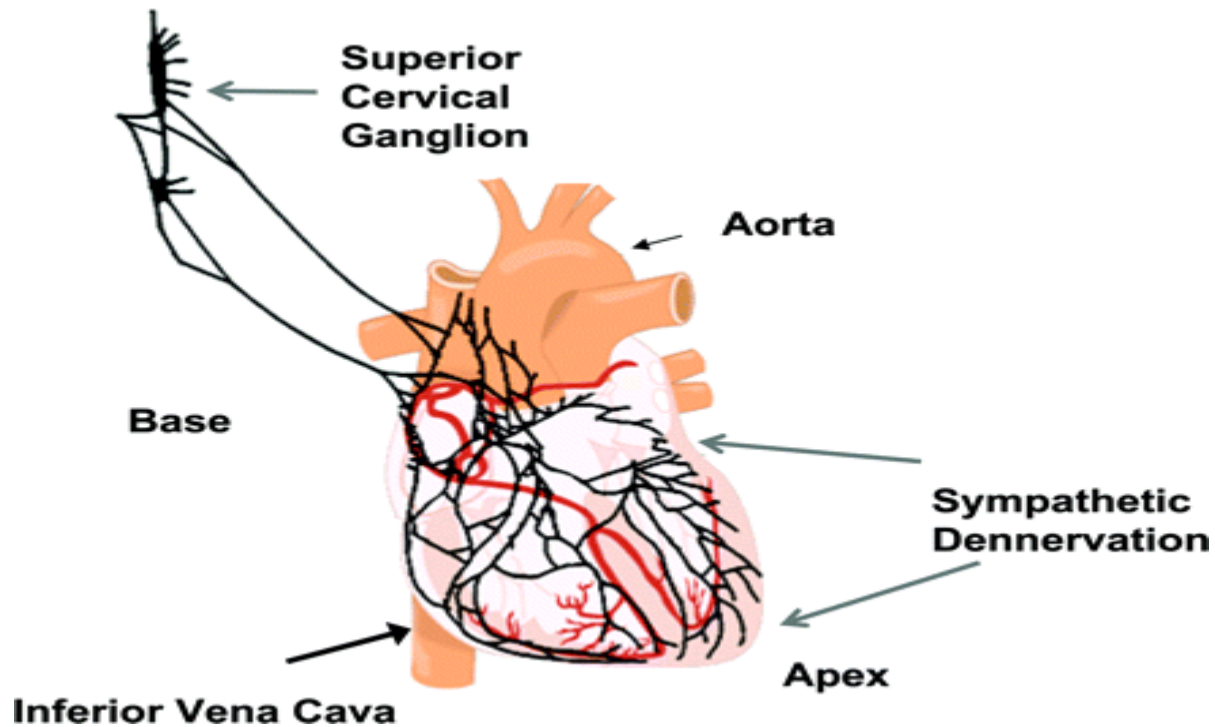


Figure 2.13: The autonomic innervation of the heart and the effects of DM. It has been shown that in DM, in a fashion that parallels the development of peripheral neuropathy, which begins at the tip of the toes and can progress proximally, neuropathy affecting the heart begins at the apex of the ventricles and progresses toward the base modified from (Pop-Busui, 2010).

2.7.3. Pathophysiology of vagosympathetic imbalance

The relationships between cardiac autonomic dysfunction and insulin resistance are complex and interwoven. Some researchers have suggested that DCAN may occur in obese individuals even before the onset of diabetes. In obese individuals, cardiac vagal tests have shown impairment in those with metabolic syndrome, with worsening severity as the component of the syndrome increases (Williams *et al.*, 2019).

Cardiac autonomic dysfunction may occur in individuals with one or more metabolic abnormalities, even in the absence of insulin resistance; therefore, it may precede insulin resistance in the metabolic syndrome. The above hypothesis is supported by a recent observation that progression to diabetes is associated with increased central sympathetic drive, blunted sympathetic responsiveness, and altered norepinephrine disposition. Indeed, vagal depression and sympathetic predominance may contribute to insulin resistance and depression of insulin secretion.

In obese normotensive patients, central fat distribution is associated with higher sympathetic activity. Glucose usage has been found to correlate negatively with low frequency to high frequency ratio (LF/HF) on spectral analysis of heart rate (HR) variations, which means that glucose usage is reduced when sympathetic activity is relatively higher. Hyperinsulinaemia subsequent to insulin resistance may also modulate autonomic activity and induce changes in haemodynamic parameters. Insulin may increase HR slightly, as shown during hyperinsulinaemic euglycaemic clamps in healthy individuals. HR elevation results from both vagal depression and cardiac sympathetic activation as indicated by increased muscle

sympathetic nerve activity (MSNA), plasma catecholamines, and, in some studies, LF/HF ratio (an index of relative sympathetic predominance) (Depace *et al.*, 2018).

The sympathoexcitatory effects of insulin result from a central nervous action and possibly from baroreflex activation secondary to insulin-induced peripheral vasodilation. However, during insulin clamp, the shift in cardiac autonomic activity toward sympathetic predominance was reported to be lower in obese than in lean individuals and in insulin-resistant patients, suggesting that chronic hyperinsulinaemia may prevent further enhancement of cardiac sympathetic tone during an acute rise in insulin level.

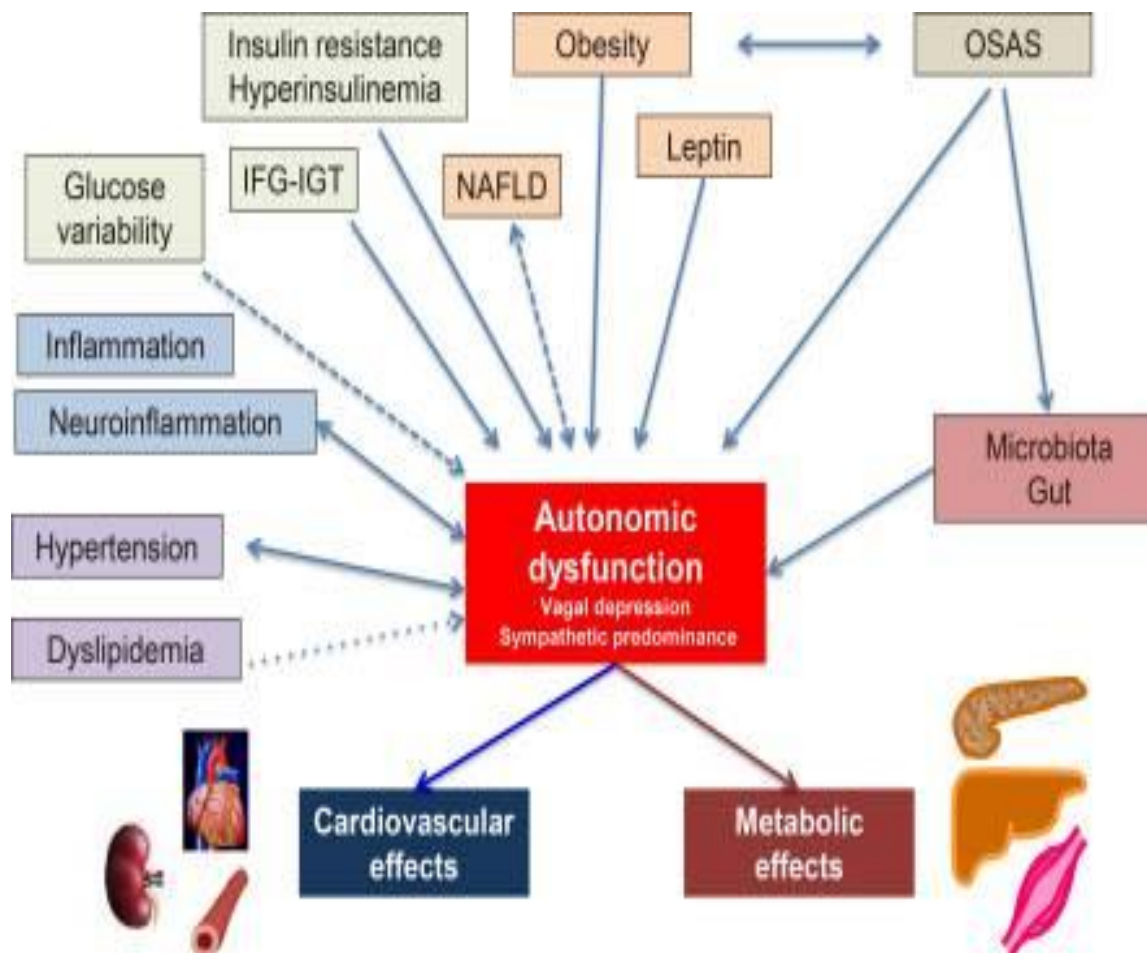


Figure 2.14: Multiple factors in the relationship between metabolic syndrome and autonomic dysfunction (Spallone, 2019).

KEY: IFG, impaired fasting glucose; IGT, impaired glucose tolerance; NAFLD, non-alcoholic fatty liver disease; OSAS, obstructive sleep apnoea syndrome

2.7.4. Vagosympathetic Imbalance and Haemodynamic Changes

Sympathetic activity has been found to be greater and baroreflex sensitivity more severely impaired in individuals with obesity and hypertension than in those with either obesity or hypertension alone, and similarly for individuals with metabolic syndrome and hypertension, suggesting that sympathetic overactivity may contribute to hypertension. Vagal impairment and sympathetic overactivity may also contribute to resting sinus tachycardia. At advanced stages of DCAN, sympathetic activity is depressed, which may induce orthostatic hypotension (OH). Postprandial hypotension may also occur as a result of meal-induced splanchnic vasodilation while the sympathetic response is blunted (Pal *et al.*, 2011; Depace *et al.*, 2018).

2.7.5. Prevalence and Correlates of Cardiovascular Autonomic Neuropathy

In clinical studies including T1DM and T2DM patients, the prevalence of confirmed cardiovascular autonomic neuropathy (CAN; defined by at least two abnormal cardiovascular autonomic reflex test [CART] results) varies with diagnostic methods and from study to study. The prevalence rates increase with age and duration of DM (up to 35% in T1DM and 65% in T2DM patients with long-standing DM) as reported by Vinik *et al* (Vinik *et al.*, 2013, 2018). Fabiyi *et al.*, in a recent study, estimated a prevalence of 82% using HRV indices (Fabiyyi-Edebor and Fasanmade, 2020). Glycaemic control and the presence of microvascular complications (polyneuropathy, retinopathy, and nephropathy) are other correlates of CAN. A contributing role of several cardiovascular risk factors (high blood pressure [BP] or hypertension, smoking, dyslipidaemia, overweight or obesity in T2DM, large waist circumference, high insulin levels in T2DM, and cardiovascular disease) has also been reported. The influence of overweight and obesity is supported by the high prevalence of impaired cardiac vagal activity in non-diabetic

obese patients and the finding of an inverse correlation between HRV and body weight in the general population. This suggests that cardiac autonomic dysfunction precedes the onset of T2DM and may play a role in metabolic disorders.

2.7.6. Cardiovascular Autonomic Neuropathy as a Predictor of Cardiovascular Morbidity and Mortality

CAN is a risk marker for all-cause and cardiac mortality, stroke, coronary events, silent myocardial ischaemia (SMI), heart failure, arrhythmia, sudden death, and progression of nephropathy. A meta-analysis of 15 longitudinal studies, which included 2,900 patients followed up for 1 to 16 years, showed that the diagnosis of CAN based on at least two abnormal CART results determined a relative risk of mortality of 3.45 (95% confidence interval 2.66–4.47; $P < 0.001$) (Maser, Mitchell, Vinik, & Freeman, 2003). Subsequent studies, including the Action to Control Cardiovascular Risk in Diabetes (ACCORD) trial, confirmed the independent predictive value of CAN for all-cause and cardiovascular mortality even after adjusting cardiovascular risk factors. The presence of orthostatic hypotension (OH), a clinical manifestation of severe CAN, impaired the prognosis and was associated with higher mortality risk than the increase in risk associated with vagal cardiac test abnormalities. Prolongation of the QT interval corrected for HR (QTc), which may result from CAN, is also an independent predictor of all-cause and cardiovascular mortality (Buse *et al.*, 2007).

2.7.7. Cardiovascular Disorders Associated with Cardiovascular Autonomic Neuropathy

Several disorders associated with subclinical CAN and subsequent to sympathetic predominance may increase cardiovascular events.

2.7.7.1. Silent Myocardial Ischaemia (SMI)

SMI may be detected by stress ECG or by stress myocardial scintigraphy or echocardiography. In a meta-analysis of 12 studies, including 1,468 DM patients, SMI was present in 20% of those with CAN compared with 10% of those without CAN, with a prevalence rate ratio of 1.96 (Spallone *et al.*, 2011). The Detection of Ischaemia in Asymptomatic Diabetics (DIAD) trial, which assessed the role of routine screening for ischaemia using nuclear stress testing compared with usual care, showed that 1,123 T2DM patients, abnormal Valsalva manoeuvre was the most vital determinant of SMI. The lowest quartile of HR response in the lying-to-standing test was associated with an adjusted hazard ratio of 4.33 for cardiac death or nonfatal myocardial infarction over a mean follow-up period of 4.8 years. In addition, it was reported that CAN enhances the cardiac risk associated with SMI. Thus, CAN testing may be considered the main component of a DM-specific risk pattern for identifying high-risk patients in whom screening for SMI is more effective. However, the efficacy of such a strategy awaits confirmation in a controlled clinical trial (Wackers *et al.*, 2004).

2.7.7.2. Hypertension

A defect in vagal activity may contribute to hypertension through a relative sympathetic override. This is supported by a study in rats with ventromedial hypothalamic obesity that exhibited marked bradycardia and only a mild increase in BP. In these rats, heart vagal tone was increased, and adrenal medulla secretion was enhanced, probably due to hyper-insulinaemia

(Valensi *et al.*, 2003). In addition, increased vagal activity was observed, and cardiac responsiveness to beta-agonist stimulation was also increased. This suggests that high vagal activity may be protective against hypertension associated with obesity (Valensi *et al.*, 2003). In patients with T1DM or T2DM, the prevalence of hypertension has been shown to increase with severity of CAN, which supports the role of CAN in contributing to hypertension in DM patients. Furthermore, a large majority of patients with macrovascular complications, retinopathy, or nephropathy exhibited the CAN-plus-hypertension profile, which is consistent with a deleterious effect of CAN on vascular haemodynamics and structure, in addition to the effects of hypertension (De-Boer *et al.*, 2017; Serhiyenko and Serhiyenko, 2018).

2.7.7.3. Left Ventricular Dysfunction

CAN have been reported to be associated with left ventricular systolic and particularly diastolic dysfunction. However, it is difficult to evaluate the independent role of CAN in these disorders and chronic heart failure because interstitial myocardial fibrosis and microangiopathic or metabolic changes may also contribute to diabetic myocardial disease and left ventricular dysfunction (Abboud ,1982; Valensi *et al.*, 2003).

2.7.7.4. QT Interval Prolongation

QTc prolongation may result from an imbalance in cardiac sympathetic innervation but also metabolic and electrolytic myocardial changes, left ventricular hypertrophy, coronary artery disease, and genetic factors. In a meta-analysis of 17 studies, including 4,584 DM patients, QTc prolongation (corrected QT for HR >441 milliseconds) was a specific (86%) but insensitive (28%) index of CAN. Using 24-hour electrocardiographic recordings, it was shown that the day-night modulation of the QT/RR-interval relationship was altered in CAN patients who were free

of coronary artery disease or left ventricular dysfunction or hypertrophy, with reversed day-night pattern and an increased nocturnal QT-HR dependence (Whitsel *et al.*, 2000).

In T1DM patients, prolonged QTc has been reported frequently during overnight hypoglycaemia and associated with cardiac rate and rhythm disturbances. Reversible QTc prolongation may be induced by hyperglycaemia and acute hypoglycaemia in both healthy and DM patients. These findings support an arrhythmic basis for the “dead in bed” syndrome and possibly a provocative role of hypoglycaemia-induced sympathetic activation in cardiovascular events (Gill *et al.*, 2009). Thus, CAN identify DM patients who are more susceptible to the deleterious effects of hypoglycaemia, particularly among those with coronary artery disease. According to the patient’s risk profile, screening for CAN may also serve to inform the choice of glycaemic control targets and balance the advantage of aggressive glucose-lowering treatment versus its risk. Moreover, QTc measurement may be used for cardiovascular risk stratification (Kolo *et al.*, 2008; Maule *et al.*, 2008; Akintunde *et al.*, 2012; Kolo *et al.*, 2012).

2.7.7.5. Abnormal Circadian Blood Pressure Pattern

An abnormal circadian BP pattern on ambulatory blood pressure monitoring (ABPM) is associated with CAN. Normal circadian BP changes are characterized by lower levels during the night than during the day. Non-dipping and reverse dipping, defined as attenuation or loss of nocturnal BP fall, respectively, may result from CAN. Several studies linked non-dipping to changes in the circadian variation of sympathovagal activity, consisting of a diminished increase in vagal activity and sympathetic predominance during the night. Non-dipping or reverse dipping was associated with left ventricle hypertrophy and was found to predict the progression of nephropathy independently of 24-hour BP level. Thus ABPM may be helpful in patients with

CAN in order to detect disturbance of normal circadian BP variability, to determine risk stratification for cardiovascular mortality and nephropathy progression, and to adjust antihypertensive treatment (Spallone, 2018; Kempler *et al.*, 2020).

2.7.7.6. Exercise Intolerance

In DM patients with CAN, exercise capacity and the responses of HR, BP, and cardiac stroke volume to exercise are diminished, with further decrease in exercise capacity and BP response in patients with both vagal neuropathy and orthostatic hypotension (OH). The severity of CAN correlates inversely with maximal HR increase during exercise, suggesting that CAN contributes to altered exercise tolerance. After exercise, impairment of HR recovery was found in DM patients who had CAN but were free of SMI. CAN testing offers a valuable tool for identifying patients with potentially poor exercise performance and preventing adverse outcomes when introduced to exercise training programs. Thus, CAN testing may be considered before an exercise stress test and before initiating a vigorous physical activity program (Bissinger, 2017).

2.7.7.7. Arterial Stiffness

Using spectral analysis, it was shown that in obese and DM hypertensive patients, the low-frequency peak of systolic BP variations in the standing position, which reflects sympathetic activity, correlated significantly with pulse pressure measured in the supine position, suggesting that increase in arterial stiffness is associated with higher sympathetic activity. In the Pittsburgh Epidemiology of DM Complications study, cardiac autonomic function was evaluated in a childhood-onset T1DM population and was associated with increased arterial stiffness measured 18 years later (Prince *et al.*, 2010). Some experimental data support the role of a possible

protective effect of vagal activity and an aggravating role of sympathetic predominance in arterial stiffness (Malpas, 2010; Liu *et al.*, 2019).

2.8. Detection of subclinical cardiovascular autonomic neuropathy

Detection in Clinical Practice

Standard Tests

Subclinical CAN is a frequent condition that is usually documented with CARTs, which are the gold standard for clinical autonomic testing. These tests consist of HR response to deep breathing, lying to standing, and Valsalva manoeuvre (all called HR tests), and BP response to standing. HR variations during these tests are mainly indices of parasympathetic function, whereas the presence of OH indicates a sympathetic defect. Knowledge of age-related normal ranges of HR test results is mandatory for accurate analysis of the results. These tests are non-invasive, safe, clinically relevant, easy to perform, sensitive, specific, reproducible, and standardized. However, in the absence of data on the potential risk of retinal complications, avoiding the Valsalva manoeuvre in patients with proliferative retinopathy may be appropriate. CARTs need to be performed with avoidance of confounding factors (Spallone *et al.*, 2011).

Patients should be requested to avoid strenuous physical exercise in the 24 hours preceding the tests, and abstain from caffeinated beverages, smoking, and alcohol at least 2 hours before the tests. Tests should be performed while fasting or at least 2 hours after a light meal. In insulin-treated patients, they should be performed at least 2 hours after short-acting insulin administration and not during hypoglycaemia or marked hyperglycaemia. In addition, test results should be interpreted with caution in patients with chronic obstructive pulmonary disease, respiratory failure, obstructive sleep apnoea syndrome, or cardiac diseases, in particular heart

failure. Appropriate washout of interfering drugs, particularly diuretics, sympatholytic agents, and psychoactive drugs, should be considered, and if this is not feasible, results should be interpreted cautiously (Bernardi, 2011).

CARTs should be performed in a standardized way, with HR variations during the tests being analysed with a simple cardiograph or an electrocardiographic computer system. Briefly, the deep-breathing test consists of six deep respiratory cycles in one minute in the recumbent position; the result is expressed as the mean value for the ratio of maximal RR (interval between two consecutive R waves on the ECG) during breathing out to minimal RR during breathing in for each respiratory cycle. For the lying-to-standing test, the patient is asked to remain recumbent for 1 minute and then to stand up quickly; the result is expressed as the ratio of the longest RR interval (approximately the 30th beat after standing up) to the shortest RR interval (approximately the 15th beat). The Valsalva test is carried out with the patient seated. He or she is asked to exhale deeply to maintain 40 mmHg pressure for 15 seconds; the Valsalva ratio is calculated as the longest RR interval after breathing out divided by the shortest RR interval while breathing out. OH is defined as a reduction of systolic BP of at least 20 mmHg or diastolic BP of at least 10 mmHg within 3 minutes of standing and a fall in systolic BP of 30 mmHg in hypertensive patients (Fisher and Tahrani, 2017; Paneerselvam *et al.*, 2019; Kempler *et al.*, 2020).

2.8.1 Staging of Cardiovascular Autonomic Neuropathy

Definite diagnosis and staging of CAN require more than one HR test and the measurement of BP response to standing. One abnormal cardiovagal test result indicates possible or early CAN to be confirmed over time; at least two abnormal cardiovagal tests results among the three HR tests are required for a definite or confirmed diagnosis of CAN. The presence of OH, in addition to HR test abnormalities, indicates severe or advanced CAN (Serhiyenko and Serhiyenko, 2018; Vinik *et al.*, 2018).

Several scientific societies have advocated the use of HRV for definition and diagnosis of CAN in research for standardization of criteria as shown in table 2.10 below.

Table 2.10: Guidelines of scientific societies on screening and diagnosis of CAN in clinical practice, with regard to the indications for assessment modalities, and the candidates to screen (Spallone, 2019).

	Toronto Consensus (2011)	Position Statement ADA (2017)	Position Statement AACE/ACE (2018)	SID/AMD Standards (2018)
Symptoms	Screening	Screening	Screening	Screening
Signs	Screening	Screening	Screening	Screening
CARTs	Gold standard for diagnosis	Possible utility in asymptomatic patients	Screening	Diagnosis
HRV (time and frequency domain indices)	Prognostic information	Research	Clinical use in addition to CARTs	Research
Candidates	Universal screening of symptoms and signs	Those with microvascular complications and/or hypoglycaemia unawareness	From time of diagnosis in those with T2DM, or 5 years after diagnosis in T1DM	In particular in those with high CV risk and complications

Key: CAN, cardiovascular autonomic neuropathy; ADA, American Diabetes Association; AACE, American Association of Clinical Endocrinologists; ACE, American College of Endocrinology; SID, Italian Society of Diabetology; AMD, Italian Association of Clinical Diabetologists; CART, cardiovascular autonomic reflex test; HRV, heart rate variability; T2DM, type 2 diabetes mellitus; T1DM, type 1 diabetes mellitus; CV, cardiovascular.

2.9. Evaluation of Vagosympathetic Activity in Clinical Research

More sophisticated approaches for evaluating CAN may be used in clinical research, including frequency domain measures of HR and BP variations, baroreflex sensitivity, muscle or skin sympathetic nerve activity, plasma catecholamines, and heart sympathetic imaging.

Frequency domain indices can be obtained by spectral analysis of HR variations applied on short (5- to 7-minute) and longer (24-hour) ECG recordings. High frequency (HF) spectral power measures parasympathetic modulation, whereas low frequency (LF) power evaluates sympathetic and parasympathetic modulation. The LF power of BP variations provides a measure of sympathetic modulation (Reyes *et al.*, 2013). Cardiac vagal baroreflex sensitivity can be assessed by analysis of HR and BP response to pharmacologic or spontaneous BP perturbations. Sympathetic outflow can be measured directly via microelectrodes inserted into a fascicle of a distal sympathetic nerve to the skin or muscle, at rest and in response to various physiologic perturbations. Whole-body sympathetic activity is assessed by measuring plasma concentrations of noradrenaline and adrenaline. Cardiac sympathetic innervation may be analysed through scintigraphic studies performed with radiolabelled noradrenaline analogues (iodine-123 [¹²³I]–metaiodobenzylguanidine [MIBG] or carbon-11 [¹¹C]–hydroxyephedrine [HED]) (Reyes *et al.*, 2013).

In October 2009 in Toronto (Canada), expert panels were convened to provide updates on DM neuropathies. They suggested screening for CAN at the diagnosis of T2DM and five years after the diagnosis of T1DM, particularly in patients at greater risk of CAN because of a history of poor glycaemic control, cardiovascular risk factors, DM peripheral neuropathy, and macroangiopathic and microangiopathic DM complications (Spallone, 2019).

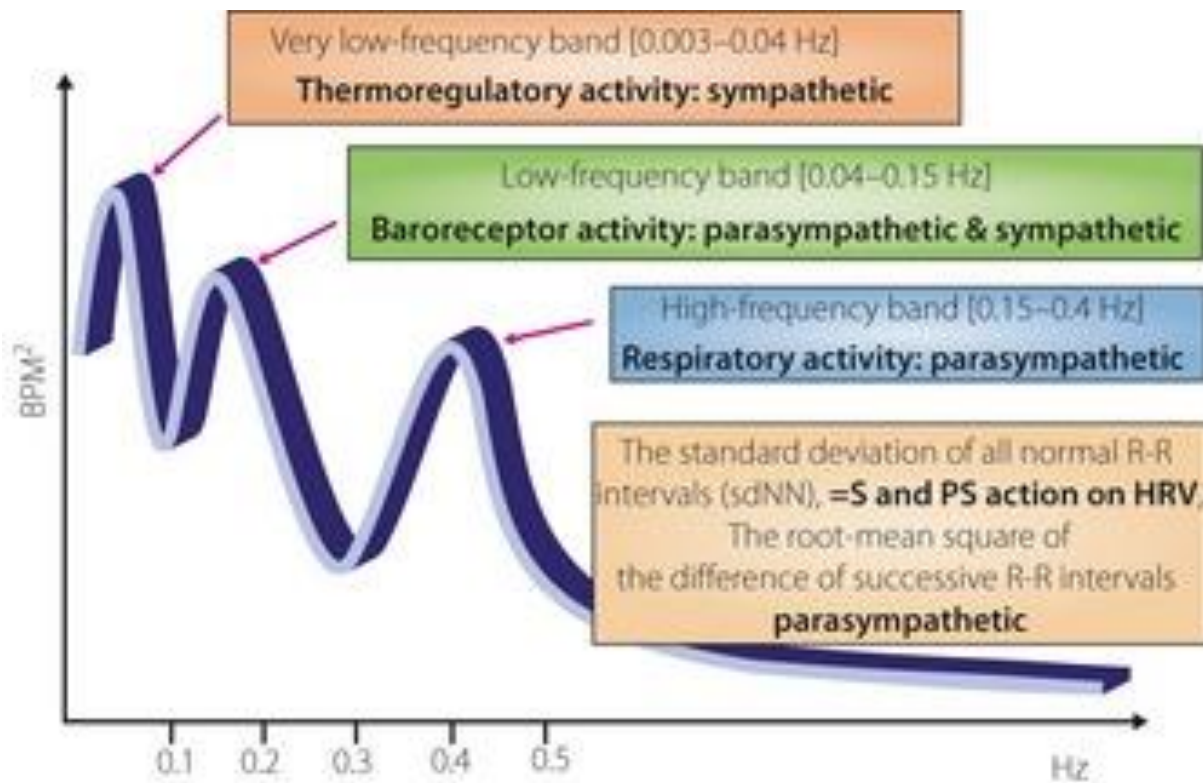


Figure 2.15: Spectral analysis of heart rate variability (Vinik *et al.*, 2013).

Key; HRV, heart rate variability; PS, parasympathetic; S, sympathetic;

sdNN, standard deviations of all normal beat-to-beat intervals.

2.10. Induction of diabetes

Several models have been developed in the past for studying DM, both genetically and through drug induction. One intriguing model of T1DM is the Akita mouse. Ins2C96Y Akita mice, which spontaneously develop insulin-dependent DM at about four weeks of age, express a mutant non-functional insulin isoform (Yang, 2011). They exhibit a significant increase in fasting blood glucose levels, gait disturbances, and sensory neuropathy (nerve conduction slowing) as early as four months of age. Recently, studies that used power spectral analysis found reduced cardiac efficiency, contractibility, HR, and HRV in Akita mice, which all imply possible impairment of cardiac autonomic regulation (Yang, 2011; Li *et al.*, 2019).

Another commonly used method for DM induction is the administration of streptozotocin (STZ). STZ is a toxic chemical that induces DM, but it can also detrimentally affect and alter the functions of many vital organs and can lead to other complications (Lee *et al.*, 2001; Ghasemi *et al.*, 2014). Although STZ-induced animals have been said not to survive more than 5–6 weeks after the onset of DM, thus making the long-term evaluation of the progression of DCAN difficult, Akita mice are known to survive up to 8 months (after DM onset at 3–5 weeks of age) as shown in table 2.11. Alloxan and STZ are the most prominent diabetogenic chemicals in DM research. Both are cytotoxic glucose analogues. Although their cytotoxicity is achieved via different pathways, their mechanisms of beta cell-selective action are identical (Lenzen, 2008; Ighodaro *et al.*, 2017; Sheriff *et al.*, 2020). Table 2.12 shows some of the similarities and differences between the two drugs. This research focuses on STZ since it has been shown to induce both T1DM and T2DM by previous researchers.

Table 2.11: Comparison of popular rodent models

	Onset of Diabetes	Life Span	Severity of Neuropathy	References/sources
Drug-induced (streptozotocin, etc.)	After induction	< 5–6 weeks	strong	(Lo-Giudice <i>et al.</i> , 2002; Srinivasan <i>et al.</i> , 2005).
Akita	3–5 weeks	8 months	strong	(Bufan Yang and Chon, 2011; Chon <i>et al.</i> , 2014).
Non-obese diabetes	11–30 weeks	<5–6 weeks	-	(Srinivasan <i>et al.</i> , 2005; Islam, 2013).

Table 2.12: Comparison of the chemical properties of alloxan and streptozotocin (Ghasemi *et al.*, 2014; Sheriff *et al.*, 2020).

	Alloxan	Streptozotocin
Chemical name	2,4,5,6-tetraoxypyrimidine; 2,4,5,6-pyrimidinetetrone	2-deoxy-2- ([(methylnitrosoamino)carbonyl]amino)- D-glucopyranose
Chemical structure	Oxygenated pyrimidine derivative; barbituric acid derivative (5-ketobarbituric acid)	Cytotoxic methylnitrosourea moiety (N-methyl-N-nitrosourea) attached to the glucose (2-deoxyglucose) molecule; glucosamine derivative
Chemical properties	Very hydrophilic, beta cell-toxic glucose analogue (partition coefficient= 1.8); weak acid	Hydrophilic, beta cell-toxic glucose analogue
Chemical reactivities	Thiol reagent that is reduced to dialuric acid in the presence of GSH and other thiols	DNA-alkylating agent
Mode of toxicity	Generation of ROS	DNA alkylation

Key; DNA, deoxyribonucleic acid; GSH, reduced glutathione; ROS, reactive oxygen species

2.10.1. STZ and diabetes induction

There are several conflicting dose regimens for either T1DM or T2DM induction in the literature. While most researchers hold the fact that T1DM can be induced in rodents by a single high-dose STZ injection, few others make use of multiple doses (Qian *et al.*, 2015; De-Magalhães *et al.*, 2019). T2DM, on the other hand, can be induced by several documented approaches; these include STZ injection alongside the administration of nicotinamide, high fat diet (HFD) administration followed by multiple low-dose STZ injections, and STZ injection during the neonatal period (Davidson *et al.*, 2011; Ghasemi *et al.*, 2014).

T2DM models have been previously induced with a combination of HFD and STZ injection. However, there are differences in the food type, duration of HFD administration, and STZ injection doses among various studies. For instance, Zhang *et al.* fed rats with chow for four weeks, after which a single injection of 45 mg/kg STZ or two STZ injections (30 mg/kg at weekly intervals for two weeks) were administered. Other reports described feeding male Sprague–Dawley rats standard chow or HFD for two weeks and then injecting 50 mg/kg STZ. In another study, a combination of HFD for two weeks and then 35 mg/kg STZ as a single injection was used to develop T2DM rat model. In the present study, Wistar rats were fed a high-fat and high-carbohydrate diet for eight weeks, followed by the administration of low doses of STZ for the development of a diet-induced T2DM animal model (Zhang *et al.*, 2008; Qian *et al.*, 2015; De-Magalhães *et al.*, 2019). This study, therefore, aims to show the effect of high-dose STZ on the one hand compared with multiple low doses after a high-fat diet on cardiac autonomic neuropathy.

2.10.1.1. Mechanisms of STZ action

STZ is a broad-spectrum antibiotic produced by the bacterium *Streptomyces achromogens*. It contains a glucose molecule linked to a highly reactive methyl-nitrosourea moiety that is thought to exert STZ's cytotoxic effects, while the glucose moiety is responsible for the chemical's selective action on pancreatic β cells. STZ has a short half-life due to its rapid metabolism in the liver and elimination by renal excretion. Once STZ is eliminated from the body, further functional impairment of the liver or the kidneys may be attributed to the effects of diabetic hyperglycaemia. This is the basis for studying the mechanisms of STZ-related diabetic complications in most organs of the body. (Vavra *et al.*, 1959; Gajdosík *et al.*, 1999; Mythili *et al.*, 2004).

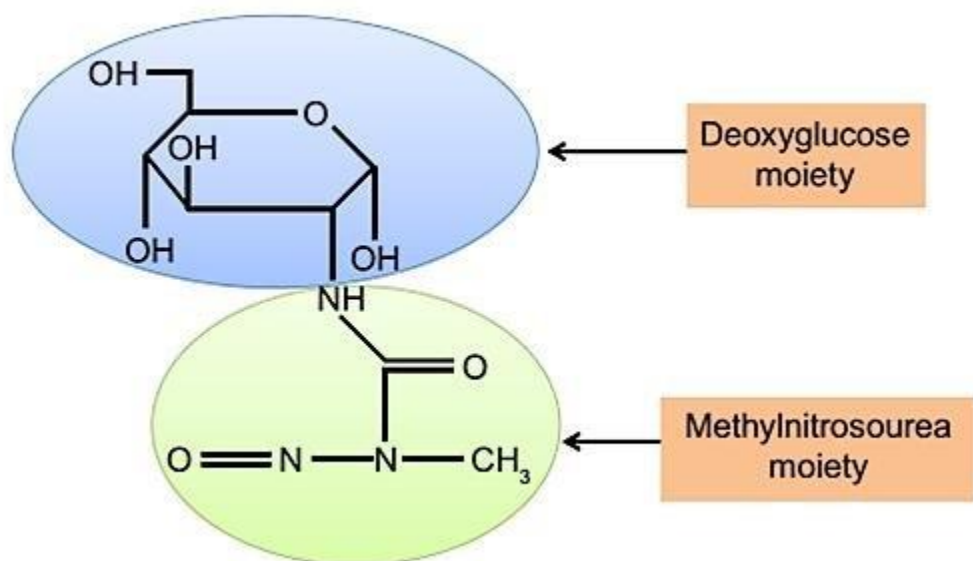


Figure 2.16: Chemical structure of streptozotocin (Wu *et al.*, 2007).

2.11. Management of Diabetes

The goals in caring for patients with DM are to eliminate symptoms and prevent, or at least slow, complications. Reduction in the risk of microvascular complications (eye, nerve, and kidney diseases) is accomplished through control of glycaemia and BP; macrovascular (coronary, cerebrovascular, peripheral vascular) risk reduction is achieved through control of lipidaemia and hypertension, smoking cessation, and aspirin therapy. Management of DM involves lifestyle modification, including weight reduction, regular physical activity, dietary measures, oral anti-diabetic drugs (OAD), use of insulin (especially in T1DM and T2DM during hyperglycaemic emergencies or secondary OAD failure), and non-insulin injectable anti-diabetic agents in individuals with T2DM (Oguejiofor *et al.*, 2014; Leon, 2015). A brief history of the antidiabetic profile is shown in table 2.13 below.

Table 2.13: Brief history of pharmacological agents used for treating T2DM (Quianzon and Cheikh, 2012).

Year	Active Ingredient	Category of medications
before 1920s	no effective drugs	-
1921	first commercially available insulin in USA	Hormones
1946	Insulin	Neutral protamine Hagedorn-
1950	Tolbutamide	Sulfonylureas
1959	Metformin	Biguanides
1996	Troglitazone	Thiazolidinediones
1997	Repaglinide	Meglitinides
1995	Acarbose	α -Glucosidase Inhibitors
2005	Pramlintide	Amylin Agonists
2008	Colesevelam	Bile acid sequestrants
2009	Bromocriptine	Dopamine agonist
2013	Canagliflozin	Sodium GlucoseCo-Transporter 2 Inhibitors
2014	Dapagliflozin	Sodium Glucose Co-Transporter 2 Inhibitors
2016	Lixisenatide	Peptide

2.11.1 Insulin

2.11.1.1 Insulin Regulation

Insulin is the most important regulator of the balance between hepatic glucose production and peripheral glucose uptake and utilization. This metabolic equilibrium is known as glucose homeostasis. However, neural input, metabolic signals, and other hormones (e.g., glucagon) are involved in the integrated control of glucose supply and utilization. Specifically, β -cells sense changes in plasma glucose concentration and respond by releasing corresponding amounts of insulin (Fu *et al.*, 2012). During fasting, low insulin levels stimulate glucose production by activating hepatic gluconeogenesis and glycogenolysis while also reducing glucose uptake in insulin-sensitive tissues such as skeletal muscle and fat. Insulin promotes the mobilization of stored precursors such as amino acids and free fatty acids (lipolysis). Glucagon, secreted by pancreatic alpha cells when blood glucose or insulin levels are low, stimulates glycogenolysis and gluconeogenesis by the liver and renal medulla. Postprandially, the glucose load, elicits a rise in insulin and a fall in glucagon levels, leading to a reversal of the processes that occur in the fasting state. Insulin, an anabolic hormone, promotes carbohydrate storage and fat and protein synthesis. The major portion of postprandial glucose is utilized by skeletal muscles—an effect of insulin-stimulated glucose uptake. Other tissues, most notably the brain, utilize glucose in an insulin-independent fashion (Ojha *et al.*, 2019).

2.11.1.2 Insulin structure

Insulin is a small poly-peptide protein with a molecular weight of 6,000 Daltons. It is composed of two peptide chains referred to as the A chain and B chain. Two disulfide bonds link both chains (between the cystein residues at positions A7 and B7, and A20 and C19), and an additional disulfide bridge is formed within the A chain (connecting the cystein residues at A6 and A11), which is essential for determining the tertiary structure and receptor binding of the molecule. In most species, the A chain consists of 21 amino acids and the B chain, 30 amino acids. The iso-electric point (point of least ionisation/water solubility) of insulin is at a pH of 5.4. Human insulin aggregates into dimers, hexamers, and more complex crystalline structures in the presence of zinc ions and low pH, as found in the secretory granules (Weiss *et al.*, 2000).

The amino acid sequence of insulin differs among species, although specific segments of the insulin biomolecule are highly conserved. This includes the positions of the three disulfide bonds, the two ends of the A chain, and the C-terminal residues of the B chain. These similarities in the amino acid sequence of insulin lead to a three-dimensional conformation of insulin that is very similar among species. Insulin from a specie of animal is therefore very likely to be biologically active in another specie. For example, bovine insulin has been widely used to treat human patients (Fu *et al.*, 2012).

2.11.1.3 Insulin Biosynthesis

Insulin is produced in the pancreatic islet beta cells initially as a single-chain 86-amino-acid precursor polypeptide called preproinsulin. Subsequent proteolytic processing removes the amino-terminal signal peptide, giving rise to proinsulin. Proinsulin is structurally related to insulin-like growth factors 1 and 2, which bind weakly to the insulin receptor. Cleavage of an

internal 31-residue fragment from proinsulin generates the C-peptide and the A and B chains of insulin. The mature insulin molecule and C-peptide are stored together and co-secreted from secretory granules in the beta cells. Since C-peptide is cleared more slowly than insulin, it is a valuable marker of insulin secretion. It allows discrimination of endogenous and exogenous sources of insulin in the evaluation of hypoglycaemia. Pancreatic beta cells also co-secrete islet amyloid polypeptide (IAPP) or amylin, a 37-amino-acid peptide, along with insulin. The role of IAPP in normal physiology is incompletely defined. However, it is the major component of the amyloid fibrils found in the pancreatic islets of patients with T2DM, and an analogue is sometimes used in treating T1DM and T2DM (Weiss *et al.*, 2000; Fu *et al.*, 2012).

Human insulin can be produced by recombinant DNA technology; structural alterations at one or more amino acid residues modify its physical and pharmacologic characteristics (figure 2.17).

BIOSYNTHESIS OF INSULIN:

Insulin is synthesized as **preproinsulin** in pancreatic **β -cells**. It contains a **signal peptide** which directs the nascent polypeptide chain to the rough **endoplasmic reticulum**. Then it is cleaved as the polypeptide is translocated into lumen of the RER, forming **proinsulin**. Proinsulin is transported to the trans-Golgi network (TGN) where immature granules are formed.

Proinsulin undergoes maturation into active insulin through action of cellular endopeptidases known as **prohormone convertases** (**PC1** and **PC2**), as well as the exopeptidase **carboxypeptidase E**. The endopeptidases cleave at 2 positions, releasing a fragment called the **C-peptide**, and leaving 2 peptide chains, the B- and A- chains, linked by 2 disulfide bonds. The cleavage sites are each located after a pair of basic residues and after cleavage these 2 pairs of basic residues are removed by the carboxypeptidase. The **C-peptide** is the central portion of proinsulin, and the primary sequence of proinsulin goes in the order "B-C-A".

The resulting mature insulin is packaged inside mature granules waiting for metabolic signals (such as leucine, arginine, glucose and mannose) and vagal nerve stimulation to be exocytosed from the cell into the circulation.

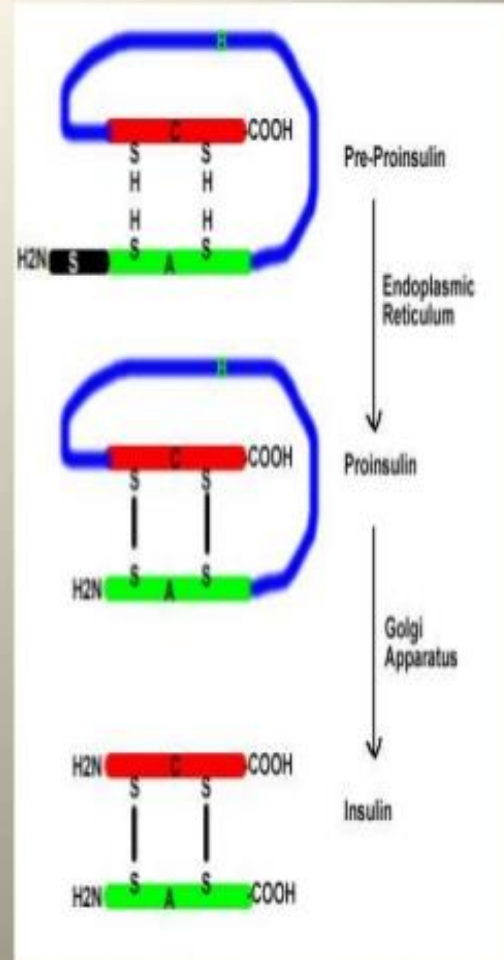


Figure 2.17: Structure and biosynthesis of insulin (Weiss *et al.*, 2000).

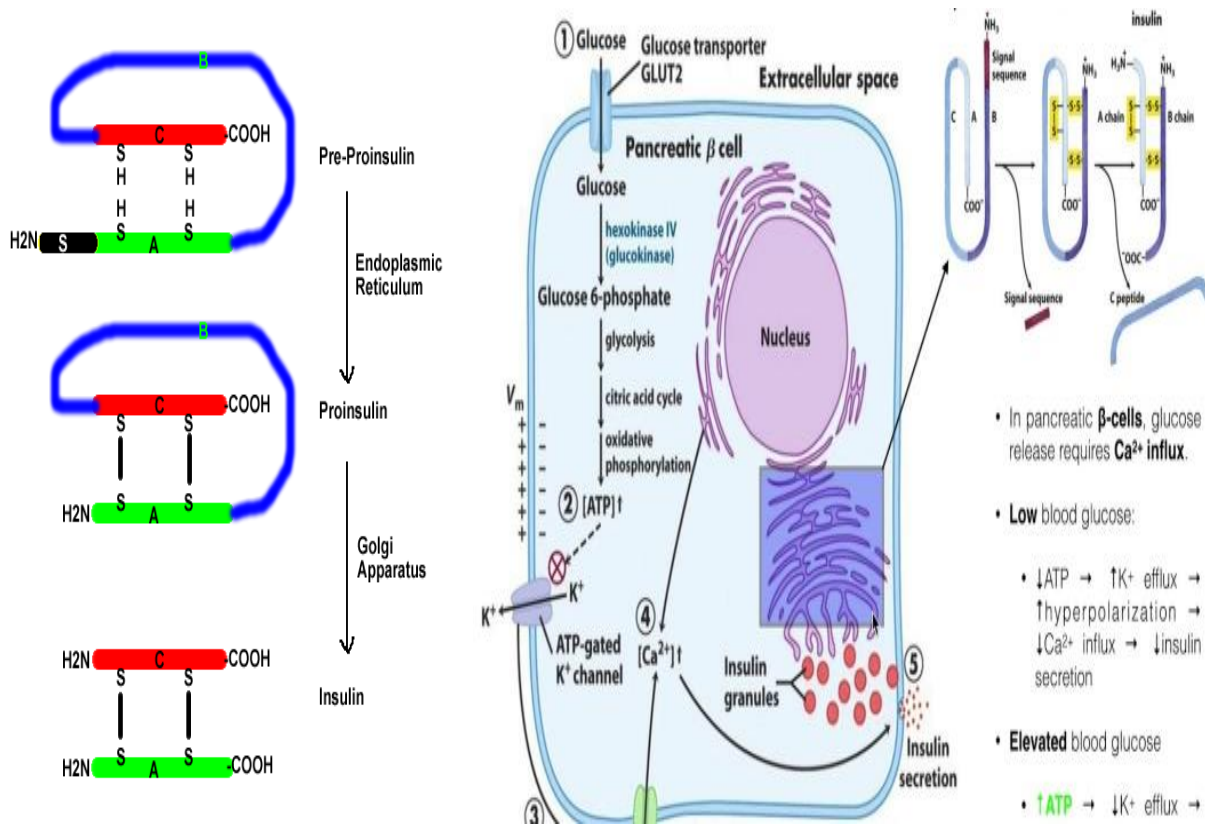


Figure 2.18: The mechanism of insulin biosynthesis by pancreatic β -cells (Fu *et al.*, 2012).

Key; ATP, adenosine triphosphate; V_m , voltage membrane; GLUT2, glucose transporter 2.

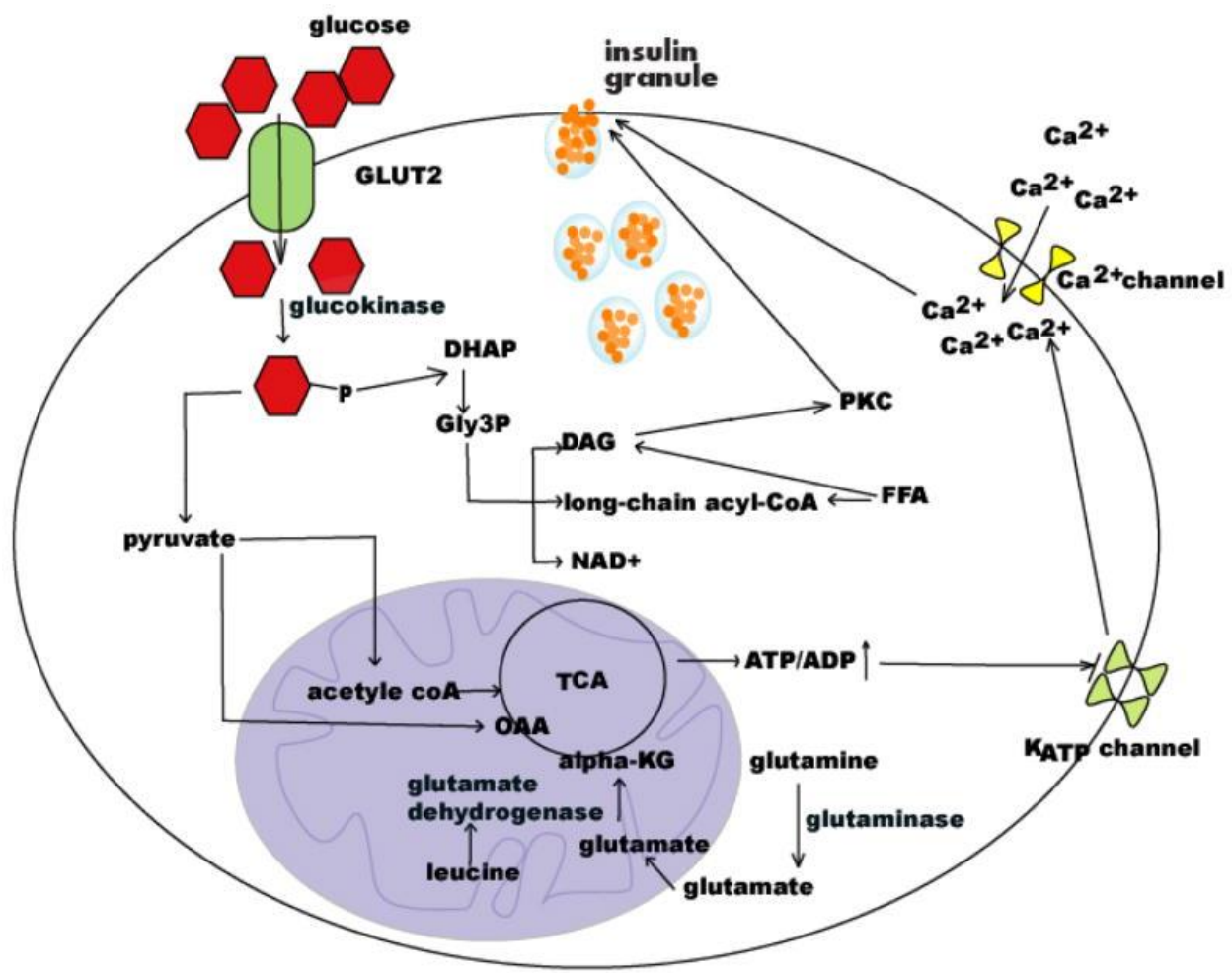


Figure 2.19: Schematic illustration of nutrient-regulated insulin secretion (Weiss *et al.*, 2000).

Key; ADP, adenosine diphosphate; ATP, adenosine triphosphate; DAG, diacylglycerol; DHAP, dihydroxyacetonephosphate; FFA, free fatty acid; GLUT2, glucose transporter 2; Gly3P, glycerol-3-phosphate; KG, ketoglutarate; NAD, nicotinamide adenine dinucleotide; OAA, oxaloacetic acid; PKC, protein kinase C; TCA, tricarboxylic acid.

2.11.1.4 Insulin Secretion

Glucose is the major regulator of insulin secretion by the pancreatic beta cells, although amino acids, ketones, various nutrients, gastrointestinal peptides, and neurotransmitters also influence insulin secretion. Glucose levels >3.9 mmol/l (70 mg/dl) stimulate insulin synthesis, primarily by enhancing protein translation and processing. Glucose stimulation of insulin secretion begins with its transport into the beta cells by a facilitative glucose transporter. Glucose phosphorylation by glucokinase is the rate-limiting step that controls glucose-regulated insulin secretion. Further metabolism of glucose-6-phosphate via glycolysis generates adenosine triphosphate (ATP), which inhibits the activity of an ATP-sensitive K^+ channel. This channel consists of two separate proteins: one is the binding site for certain oral hypoglycaemics (e.g., sulfonylureas, meglitinides); the other is an inwardly rectifying K^+ channel protein (Kir6.2). Inhibition of this K^+ channel induces beta-cell membrane depolarization, which opens voltage-dependent calcium channels and stimulates insulin secretion (Tarasov *et al.*, 2004; Bonora *et al.*, 2012). Insulin secretory profiles shows a pulsatile pattern of hormone release occurring in phases, with small secretory bursts occurring about every 10 minutes and greater amplitude oscillations of about 80–150 minutes. Incretins are released from neuroendocrine cells of the GIT following food ingestion, and they amplify glucose-stimulated insulin secretion while also suppressing glucagon secretion. Glucagon-like peptide 1 (GLP-1), the most potent incretin, is released from L cells in the small intestine and stimulates insulin secretion only when blood glucose is above the fasting level. Incretin analogues like exenatide and liraglutide are now used to enhance endogenous insulin secretion in the management of DM, primarily T2DM (Meier, 2012; Kalra *et al.*, 2016; Nauck *et al.*, 2021).

Ionic Control of Insulin Secretion

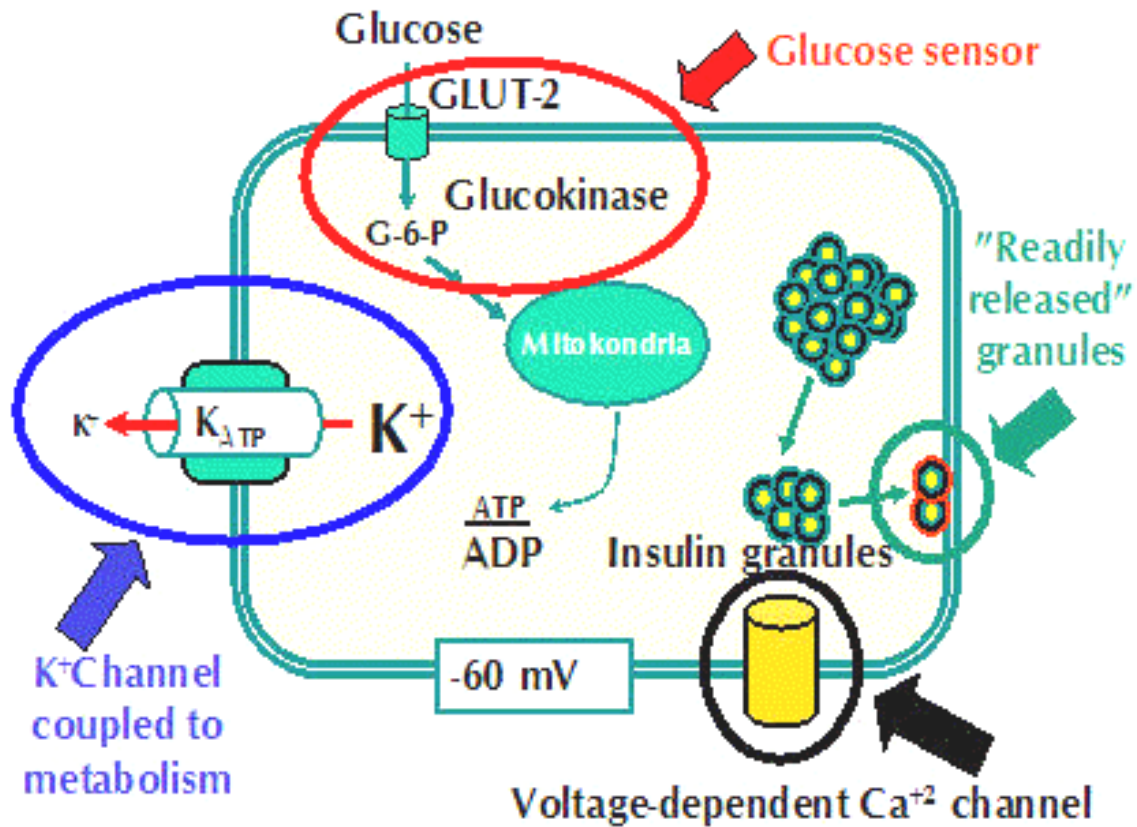


Figure 2.20: Mechanisms of glucose-stimulated insulin secretion and abnormalities in diabetes

(Fu *et al.*, 2012).

Key; ADP, adenosine diphosphate; ATP, adenosine triphosphate; G-6-P, glucose-6-phosphate;

GLUT 2, glucose transporter 2.

2.11.1.5 Insulin Degradation

Insulin has a short half-life in circulation following its release, estimated at 4–6 minutes. This allows minute-to-minute regulation of its metabolism. Circulating insulin is cleared by the liver as it passes through the portal circulation, which means that portal insulin levels are higher than those in the systemic circulation. The kidney is mainly responsible for insulin clearance in the systemic circulation, and delayed insulin clearance may cause problems with glycaemic control in people with kidney disease. Some degradation occurs within the insulin granules, and insulin is also degraded in other tissues after binding to the insulin receptor. In this receptor-mediated degradation, insulin-insulin receptor complexes come together on the plasma membrane of the target cell, forming groups that are sequestered in so-called coated pits. These invaginate to fuse with intracellular lysosomes, in which insulin is enzymatically degraded (Duckworth *et al.*, 1998).

2.11.2 Oral hypoglycaemic agents

Oral hypoglycaemic drugs are used for the management of T2DM. Some of them tend to improve insulin release (insulin secretagogues), some inhibit hepatic glucose production, while others improve the sensitivity of body tissues to insulin (insulin sensitizers). There are many distinct classes of hypoglycaemic agents, namely sulfonylureas (glimepiride, glipizide); biguanides (metformin); α -glucosidase inhibitors (acarbose, miglitol); thiazolidinediones (pioglitazone); meglitinides (repaglinide, nateglinide); incretin mimetics, which include GLP-1 agonists (exenatide, liraglutide) and dipeptidyl peptidase-4 (DPP-4) inhibitors (vildagliptin, sitagliptin, linagliptin); amylin analogue (pramlintide); and sodium glucose transporter-2 (SGLT2) inhibitors (empagliflozin, canagliflozin, dapagliflozin,) (Nathan *et al.*, 2007).

2.11.2.1 Metformin

Biguanides are derivatives of galegine, a guanidine isolated from *Galega officinalis* L. (also known as goat's rue or French lilac). The plant is used in the treatment of people with diabetes in Europe. Due to the toxic effects of galegine, a structural modification was introduced, which led to the development of metformin (dimethyl biguanide), with the synthetic analogue having improved efficacy, less toxicity, and better tolerability (Bailey and Day, 2004).



Figure 2.21A: *Galega officinalis* plant

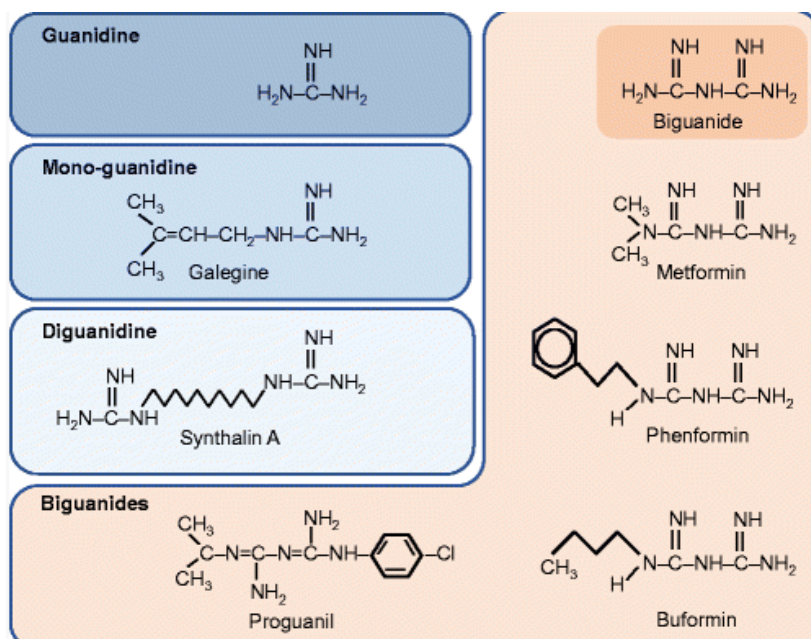


Figure 2.21B: Structures of the guanidine moiety compound from which metformin, and other biguanides are formed (Spasov *et al.*, 2017).

Metformin hydrochloride (N, N-dimethyl imido dicarbonimidic diamide hydrochloride) is an oral antihyperglycaemic drug used in the management of T2DM. It has become the most widely used medication in the treatment of T2DM since its approval in the United Kingdom in 1958 and the United States in 1995, with doses ranging from 500 to 2,500 mg/day. Metformin is a member of the biguanide class with an empirical formula of $C_4H_{11}N_5 \cdot HCl$.

It is a white to off-white crystalline powder that is freely soluble in water and is practically insoluble in acetone, ether, and chloroform. The pKa of metformin is 12.4, and the pH of 1% aqueous solution of metformin hydrochloride is 6.68 (Spasov *et al.*, 2017).

2.11.2.1.1 Mechanism of action

Metformin is an antihyperglycaemic agent that improves glucose tolerance in patients with T2DM, lowering both basal and postprandial plasma glucose. Its mechanisms of action are different from those of other classes of oral antihyperglycaemic agents. Metformin decreases hepatic glucose production, decreases intestinal absorption of glucose, and improves insulin sensitivity by increasing peripheral glucose uptake and utilization. Additionally, metformin can inhibit gluconeogenesis through activation of adenosine monophosphate (AMP)-activated protein kinase (AMPK). AMPK is an essential player in the regulation of energy metabolism, which plays a crucial role in DM and related metabolic diseases. It has been demonstrated that AMPK is required for maintaining glucose homeostasis (Wang *et al.*, 2017). Unlike sulfonylureas, metformin does not produce hypoglycaemia in either patient with T2DM or normal subjects and does not cause hyperinsulinaemia. With metformin therapy, insulin secretion remains unchanged although fasting plasma insulin levels and day-long plasma insulin response may decrease (Sanchez-Rangel and Inzucchi, 2017).

Since metformin has been in use worldwide for more than 50 years now, numerous studies concerning other potential indications for its use have emerged, which showed that metformin could also be used as an anticancer agent, an anti-aging agent, a cardiovascular protective agent, a neuroprotective agent, or an optional drug for the management of polycystic ovary syndrome (PCOS) (Wang *et al.*, 2017).

2.11.1.1.2 Side effects of metformin hydrochloride

a. Malaise:

Metformin intake may cause a feeling of ill-being in 10–25% of those who take it, shortly after its commencement, especially in women. Affected patients usually present with fatigue and occasional achiness that lasts for varying lengths of time.

b. Metformin-induced gastrointestinal disturbance

This is the most frequently reported side effect of metformin therapy, and it is characterised by nausea, occasional vomiting, diarrhoea, abdominal discomfort, and loss of appetite. This problem occurs more often after meals that are rich in fats or sugars. The symptoms subside over time or disappear after stopping or reducing the dose of the drug; hence it is recommended to start with a low dose and gradually increase it or take extended slow-release form, which rarely causes gastrointestinal upset (Suh, 2015).

c. Vitamin B12 malabsorption

Research has shown that 10–30% of patients who take metformin show evidence of reduced vitamin B absorption, especially after taking large doses for a long time, usually years (Filioussi *et al.*, 2003). The transfer of vitamin B12 from the gastrointestinal tract into the blood is aided by its combination with a substance formed in the stomach, called intrinsic factor.

Metformin interferes with the ability of body cells to absorb this intrinsic factor-vitamin B complex, leading to vitamin B12 insufficiency. Vitamin B12 is needed for the proper growth and functioning of every cell in the body (Adam *et al.*, 1983). It is also required for the synthesis of DNA and many other crucial biochemical functions. Hence, prolonged use of metformin may predispose to megaloblastic anaemia from malabsorption of vitamin B12. Sub-acute combined degeneration of the spinal cord with cognitive dysfunction may rarely be associated with deficiency of vitamin B12 caused by metformin therapy (Liu *et al.*, 2006).

d. Metformin-induced hepatotoxicity

Although hepatotoxicity induced by metformin use is rare, idiosyncratic drug-induced hepatotoxicity due to metformin use is still possible (Miralles-Linares *et al.*, 2012). This toxicity presents as serious symptoms of cholestatic hepatitis such as jaundice, severe itching, and abnormal liver enzymes. In patients with liver or kidney diseases of any kind, metformin can pose a problem because it alters liver function and is excreted through the kidneys. Hence, liver and kidney functions should be assessed before starting metformin and re-assessed at least once a year while taking it.

e. Anaemia

Megaloblastic anaemia occurs when the bone marrow does not have enough B vitamins to manufacture red blood cells. The bone marrow then releases immature and dysfunctional red blood cells into circulation. Although anaemia is not common among people taking metformin, it remains a risk for those whose vitamin B12 and folic acid levels were already low when metformin therapy was started.

f. Metformin-induced pancreatitis

Metformin has been associated with pancreatitis, either due to an overdose of the drug or in case of renal insufficiency. This may be characterised by an increase in serum amylase and lipase levels, but these normalize with discontinuation of metformin (Alsubaie and Almalki, 2013).

g. Hair loss

Metformin may contribute to male pattern hair loss at the temples and scalp of the head. Although the evidence seems small, some women have reported that hair loss was made worse by metformin.

h. Metformin-induced lactic acidosis

This occurs in about 3–10/100,000 people who take high concentrations of metformin, especially in diabetic patients with risk factors, including renal insufficiency or liver disease. It may also be secondary to pathological events like dehydration, sepsis or severe chronic stress, and any contraindication to metformin, hence causing metformin-associated lactic acidosis (MALA) (Suh, 2015; DeFronzo *et al.*, 2016). Symptoms of lactic acidosis include deep and rapid breathing; slow, irregular pulse; weakness; muscle pain; sleepiness, and malaise. Treatment requires intravenous administration of sodium bicarbonate.

i. Bile abnormalities

Bile is produced by the liver, stored in the gall bladder, and secreted into the intestines to facilitate absorption of fats into the bloodstream. It has been shown that metformin reduces the normal reabsorption of bile from the intestines back into the bloodstream, which causes elevated bile salt concentrations in the colon (Scarpello *et al.*, 1998).

j. Metformin-induced hypoglycemia

Although rare, high doses of metformin can lead to severe hypoglycaemia, even if taken alone without taking other anti-diabetic medications, due to decreased gluconeogenesis by the liver, reduced glucose uptake, and inadequate oral intake (Al-Abri *et al.*, 2013).

k. Metformin-induced coagulation alterations

It is quite common that metformin, even when taken as indicated, may suppress factors affecting the coagulation system and promote fibrinolysis in non-insulin dependent diabetes mellitus (Akinici *et al.*, 2008).

2.11.3. COMPLICATIONS OF OTHER ANTI-DIABETIC AGENTS

A. Insulin

Insulin therapy is associated with hypoglycaemia, which manifests as adrenergic and neuroglycopenic symptoms. These include hunger pangs, palpitation, excessive sweating, anxiety, confusion, lack of concentration, and coma.

Other complications include weight gain, hypersensitivity reactions, lipodystrophy at injection site, insulin resistance, and hypokalaemia.

B. Sulfonylureas

Hyperinsulinaemia and hypoglycaemia occur more commonly with chlorpropamide and glibenclamide but less with tolbutamide. Hypoglycaemia occurs more in the elderly and patients with renal disease. Other side effects include; weight gain due to an increase in appetite, gastrointestinal upset, dilutional hyponatraemia and water intoxication (chlorpropamide) due to vasopressin effect, disulfiram-like reaction with alcohol (chlorpropamide), and tachyphylaxis (secondary failure)

C. Meglitinides

The meglitinides have a lower incidence of hypoglycaemia compared to sulfonylureas; they also may lead to weight gain and drug interactions. It is contraindicated in hepatic and renal impairment.

D. Biguanides

Side effects are a metallic taste in the mouth, gastrointestinal disturbances (nausea, vomiting, and diarrhoea), lactic acidosis: common in patients with renal, liver, pulmonary, or cardiac disease and finally, long-term use interferes with vitamin B12 absorption.

Contraindications to its use include; pregnancy, renal disease, liver disease, alcoholism and conditions that predispose to hypoxia, such as cardiopulmonary dysfunction.

E. Thiazolidinediones

Use of this drug may be associated with fluid retention (oedema), weight gain, headache, abnormal liver function tests for 1st year of therapy and failure of oestrogen-containing oral contraceptives. Contraindications include; heart failure, pregnancy and significant liver disease.

F. A-glucosidase inhibitors

Common side effects affect mainly the gastrointestinal tract: flatulence, diarrhoea, abdominal pain, bloating, elevated liver enzymes. Use of the medication is contraindicated in inflammatory bowel disorders, renal disease, hepatic disease (used with caution) and intestinal obstruction.

G. Dipeptidyl peptidase-4 (dpp-4) inhibitors

Side effects are nausea, abdominal pain, and diarrhoea with nasopharyngitis.

H. Glucagon-like peptide-1 (glp-1) agonists

This class of drugs is associated with diarrhoea, nausea, vomiting, increased sweating, dizziness and indigestion.

I. SGLT2 inhibitors

They cause euglycaemic ketoacidosis, urinary tract infection, dehydration and non-sexually transmitted genital infections, such as yeast infections.

2.12. PREVENTION AND CURRENT TREATMENT OF DCAN

Prevention of DCAN focuses mainly on lifestyle modifications and tight glucose control. Early optimization of glucose control in patients with T1DM and a multifactorial approach targeting glycaemia, among other cardiovascular risk factors in individuals with T2DM, are proposed to help prevent or retard the onset of DCAN development (Pop-Busui *et al.*, 2017).

DCAN treatment is a complex process that includes: Lifestyle modification, reducing insulin resistance, achieving glycemic control, and treating other co-morbidities. Once DCAN is established, a unified algorithm and known disease-modifying treatment are still lacking (Serhiyenko and Serhiyenko, 2018). The DCCT study showed that intensive glucose control reduced CAN development by 45%, while in the Epidemiology of Diabetes Interventions and Complications (EDIC) study, this risk was reduced by 31%. Lifestyle modification, tight glycemic control and targeting cardiovascular disease risk factors reduced the risk of CAN development by 60% in patients with T2DM (Pop-Busui *et al.*, 2017).

Lifestyle modifications include rational nutrition and optimal level of physical activity, and correction of obesity. An active lifestyle is accompanied by three times less risk of increased mortality than a sedentary lifestyle (less than 1000 kcal/wk). Specific diet have not been recommended over another for DM patients, although three diet pattern are advised for individuals with or who are at risk of having DM (low-carbohydrate, low-fat calorie-restricted or Mediterranean diet). (Deli *et al.*, 2013; Fisher and Tahrani, 2017).

Hyperlipidemia in the presence of hyperglycemia generates additional reactive oxygen species (OS) that are also implicated in cell dysfunction. OS has been implicated in causing nerve damage in several animals, humans, and experimental models of DM. Antioxidants have been used due to their benefits as a physiological defence mechanism. The antioxidant systems (enzymatic component) such as zinc, copper, manganese and selenium, superoxide dismutase and catalase, may remove the reactive OS directly or sequentially, preventing their excessive accumulation and consequent adverse effects. Non-enzymatic antioxidants systems consist of scavenging molecules that are endogenously produced, such as GSH and uric acid or dietary derivatives such as vitamins A, C and E, carotenoids, lipoic acid (LA); coenzyme Q10 (CoQ10); and cofactors like albumin, vitamins B1, B3, folic and uric acid. In patients with macrovascular or microvascular complications, the antioxidant therapy is beneficial together with BP control, management of atherogenic dyslipidaemia and optimal glucose control (Fisher and Tahrani, 2017).

Other new possible perspective areas of CAN treatment are the administration of thromboxane A2 blockers and prostacyclin analogues, phosphodiesterase (PDE) 5 inhibitors, alpha-lipoic acid (ALA), omega 3 polyunsaturated fatty acid (ω -3 PUFAs), dihomo- γ -linolenic acid (DGLA) and the combined prescription of ALA, DGLA and ω -3 PUFAs. In addition, the combined administration of ALA, ω -3 PUFAs and benfotiamine promotes the reduction of chronic inflammation markers and increases of HRV parameters that might be useful in preventing the development and progression of CAN (Serhiyenko and Serhiyenko, 2018).

2.13. BETA BLOCKERS IN AUTONOMIC NEUROPATHY

There have been several controversies about the use of β blockers in patients with diabetes. Earlier studies showed that they worsen insulin resistance, impair glucose control, and cause metabolic derangements (Sica, 2005; Sirenko, 2017). Recently, some investigators have shown that third generation β blockers, such as carvedilol, a nonselective β -adrenoceptor blocker and selective α 1-adrenoceptor blocker do not have the effects stated above. Carvedilol has been shown to improve insulin sensitivity and cardiovascular outcomes in DM patients (Bakris *et al.*, 2004; Huang *et al.*, 2005; Sica, 2005; Huang *et al.*, 2007; McGill, 2009; Ahmad, 2017). Moreover, it has been shown consistently to improve mortality in patients with heart failure. By counteracting the exaggerated sympathetic activity that is the hallmark of the disease, it improves cardiac autonomic functions in chronic heart failure. Several guidelines have included β blockers as first-line drugs in the management of heart failure, especially after the congestive phase is over (Packer *et al.*, 2002; Poole-Wilson *et al.*, 2003). However, the effective role of carvedilol in the management of DCAN is yet to be established.

2.9.1. Pharmacokinetics and structure of carvedilol

Carvedilol is a nonselective beta-blocker and selective alpha-1 blocker. It has extensive first pass metabolism with resultant bioavailability of about 25–35% after oral dose. It is metabolized by liver enzymes, CYP2D6 and CYP2C9, through the process of aromatic ring oxidation and glucuronidation, after which it is then conjugated by glucuronidation and lastly sulfation. The three active metabolites exhibit only one-tenth of the vasodilating effect of the parent compound. However, the 4'-hydroxyphenyl metabolite is about 13-fold more potent in β -blockade than the parent drug (Morgan, 1994; Antepohl *et al.*, 1997).

Carvedilol has a mean half-life of 7 to 10 hours after oral administration. It has a mix of two enantiomorphs, R (+) -carvedilol and S (-) -carvedilol, with varying metabolic properties. The R (+) -carvedilol has preferential selection for metabolism as compared with the S (-) -carvedilol fraction (Selselehjonban *et al.*, 2019).

The majority of carvedilol (98%) is bound to plasma proteins, mainly to albumin. Carvedilol has a steady-state volume of distribution of about 115 L with a plasma clearance ranging from 500 to 700 mL/min. It is a basic, hydrophobic compound with a slow absorption when administered with food; however, this has not been associated with significant difference in bioavailability (Zhang *et al.*, 2021).

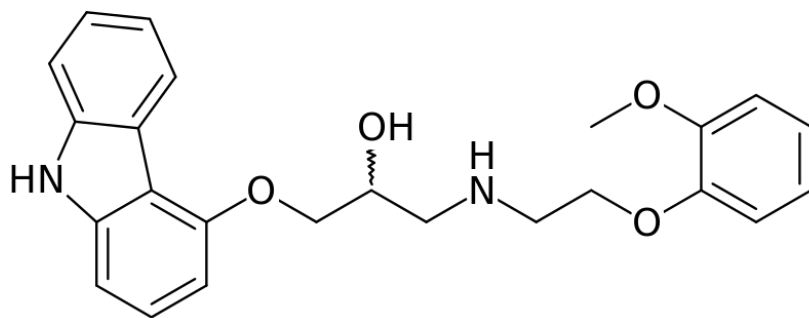


Figure 2.22: Structure of carvedilol (Wu *et al.*, 2007).

CHAPTER THREE

3.0 RESEARCH METHODOLOGY

3.1. Animals

The study was carried out following the guiding principles of the University of Ilorin Ethical Review Committee (UERC). The same ethical review committee approved the research with approval number UERC/ASN/2019/1912. Every effort was made to minimise the number of animals used and their suffering. The study was carried out between November 2017 and June 2020. Male Wistar rats were used for the experiment. All animals were acclimatized to their environment for two weeks before the commencement of the experiments. The rats were fed *ad libitum* while being housed in pairs in wooden cages.

3.2. Model development and surgical procedures

The study is a prospective, randomized, controlled experimental study done in the university-affiliated animal research facility.

3.2.1 Experimental groups

3.2.2 Phase 1:

Eighty-four male Wistar rats were divided into two broad groups; the first was the T1DM model, and the second, the T2DM model.

T1DM model: Forty-two male Wistar rats were grouped into two: sham control group (n=10) and diabetic group (n=32). T1DM was induced with intra-peritoneally administered single high dose STZ at 50 mg/kg under ketamine anaesthesia. Rats with plasma glucose >16 mmol/l on the third day after STZ-injection were randomized into different groups. Whole blood sample was taken from the proximal ventral tail vein for glucose measurement using a glucometer (Accu-Chek II Boehringer Mannheim Canada, Dorval, Quebec). Fasting plasma glucose levels, water

intake, and body weights of the animals were recorded at weekly intervals, while other parameters were determined at the beginning and end of the experiment.

T2DM model: T2DM was induced with eight weeks feeding with a high-fat diet (composition shown in Table 3.1 below); after that, low dose STZ at 25 mg/kg/day was administered intraperitoneally under ketamine anaesthesia over five days. Ten days after STZ-injection, rats with plasma glucose >16 mmol/l were randomized into different groups. Whole blood sample was taken from the proximal ventral tail vein for glucose measurement using a glucometer (Accu-Chek II Boehringer Mannheim Canada, Dorval, Quebec). Fasting plasma glucose levels, water consumption, and body weights were determined weekly, while other parameters were determinedmeasured at the beginning and end of the experiment.

Table 3.1: Compositions of the control and high-fat diets

Dietary components	Control diet	High-fat diet
Energy (Kcal/g)	3.00	6.4
Calorie percentage		
Carbohydrate	60	30
Fat	15	65
Protein	25	5
Weight percentage		
Carbohydrate	10	40
Fat	25	40
Protein	60	15
Materials	standard chow	maize, wheat offal, groundnut cake, soya meal, palm kernel cake/oil, bone meal, methionne, lysine, salt, finisher premix, cluppled

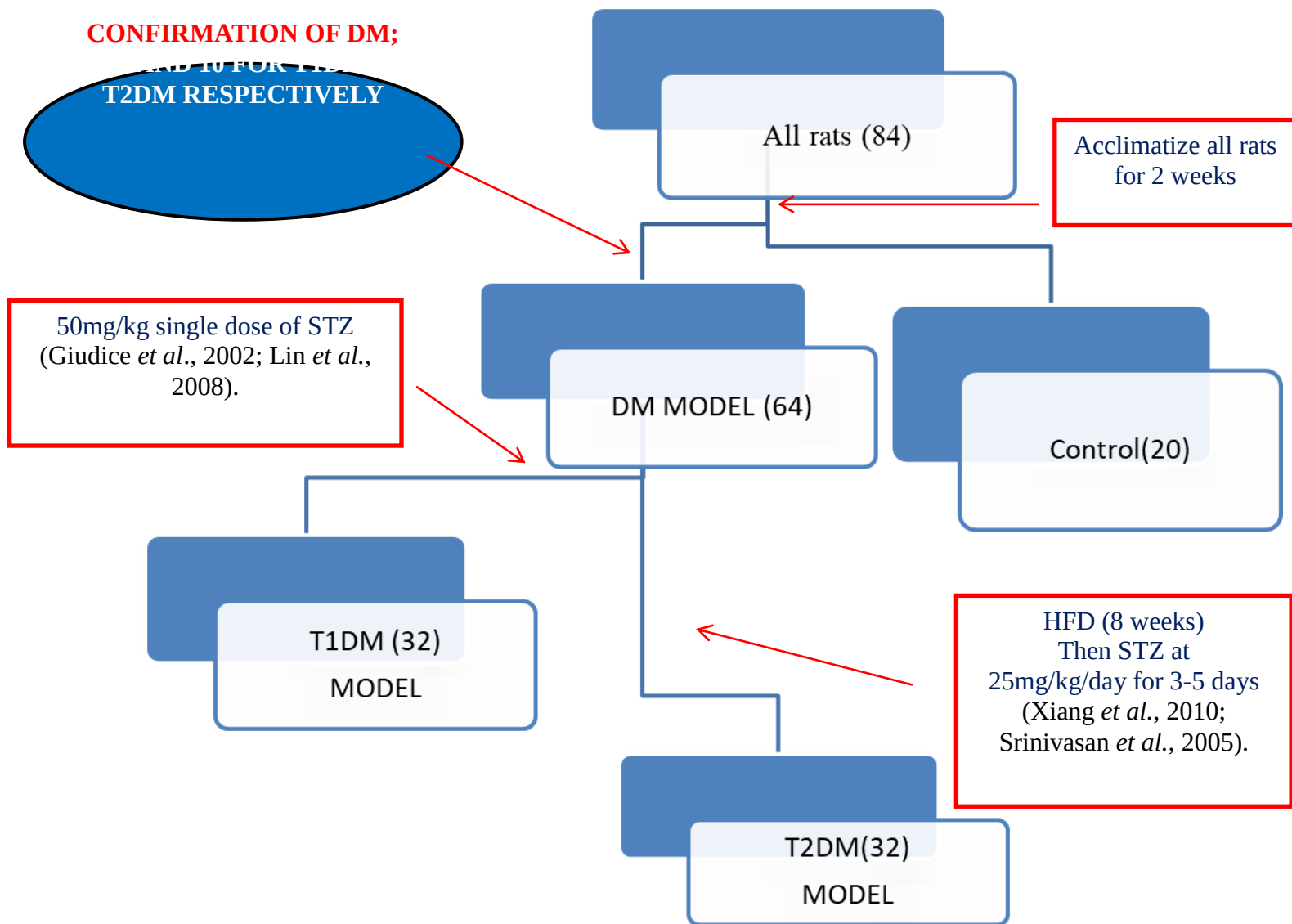


Figure 3.1: Schematic diagram showing the summary of phase 1 induction.

Key; DM, diabetes mellitus; HFD, high-fat diet; STZ, streptozotocin; T1DM, type 1 diabetes mellitus; T2DM, type 2 diabetes mellitus.

3.2.3 Phase 2: Each DM group was divided into four subgroups, as shown below.

The second phase consisted of the intervention phase, which lasted for 28 days. Carvedilol was administered in varying doses of 0.1 mg/kg, 1 mg/kg, and 10 mg/kg (to rats in DCAN A, B, and C groups, respectively). A DCAN control group (DCAN D) had only distilled water and no carvedilol administered. The effects of DCAN on all the subgroups of the model were studied using both non-invasive and invasive assessments of autonomic neuropathy. Time-varying, nonlinear and non-invasive methods were used to assess cardiac autonomic dysregulation from ECG records using Holter ECG.

TREATMENT GROUPS

- Group A (Control): 10 ml/kg of water
- Group B (DCAN 1A): 0.1 mg/kg of carvedilol
- Group C (DCAN 1B): 1 mg/kg of carvedilol
- Group D (DCAN 1C): 10 mg/kg of carvedilol
- Group E (DCAN 1D): 10 ml/kg of water
- Group F (Control): 10 ml/kg of water
- Group G (DCAN 2A): 0.1 mg/kg of carvedilol
- Group H (DCAN 2B): 1 mg/kg of carvedilol
- Group I (DCAN 2C): 10 mg/kg of carvedilol
- Group J (DCAN 2D): 10 ml/kg of water

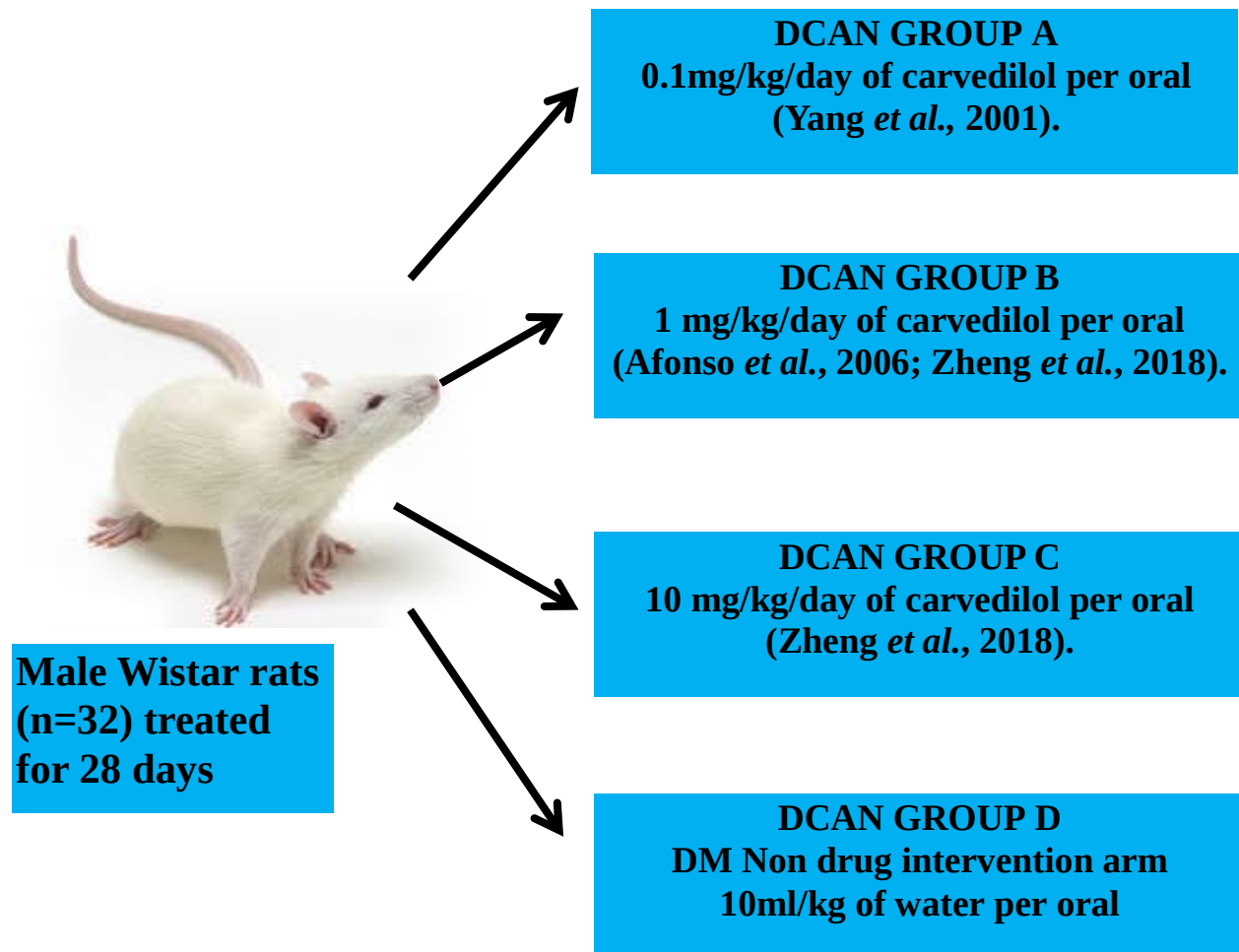


Figure 3.2: Diagrammatic representation of phase 2 of the experiment.

Key; DCAN, diabetic cardiac autonomic neuropathy; DM, diabetes mellitus

3.3. CHEMICALS (DRUGS) USED

The following drugs and chemicals were used in the experiment:

(i) Carvedilol is available in different dosage forms of 3.125 mg, 6.25 mg, 12.5 mg, and 25 mg.

It was administered orally through oral cannula after conversion to different doses of 0.1 mg/kg, 1 mg/kg, and 10 mg/kg, respectively.

(ii) Streptozotocin (powder) manufactured by (Sigma, St. Louis MO, USA).

(iii) Normal saline and distilled water

3.3.1 Sundry consumables and other materials

Animal cages and animal feeds, measuring cylinders, scissors, cotton wool, gloves, antiseptics, normal saline, distilled water, disposable syringes and needles (1ml, 2mls, 5mls, and 10mls), animal pellet feeds.

3.4 GENERAL ASSESSMENT

3.4.1 Bodyweight and food and water consumption

The body weights of the rats were monitored weekly with an electronic weighing balance. Food and water consumed by the animals were monitored daily. To know the amount of food consumed by the rats, a known weight of food was put in each cage, and the weight of the leftover food was subtracted from the known weight after 24 hours. Also, a known volume of water was put in each cage, and the volume of the leftover water was measured and subtracted from the known weight after 24 hours, using a glass cylinder, to know the volume of water consumed by the rats. Water and food intake were also adjusted for body weight.

3.4.2. Collection of blood and extraction of plasma

Blood samples were collected using a heart puncture method with a needle and a syringe after opening the thoracic cage and exposing the heart. The blood sample was collected in a heparin-coated sample tube. The blood was kept standing for ten minutes at room temperature, and then it was centrifuged at 3,000 rpm for fifteen minutes. The plasma was removed with a Pasteur pipette and stored in the freezer (about -4°C) until analysis.

3.4.3 Heart weight

The heart was first excised and immediately placed in a Petri dish containing phosphate buffer solution (PBS). It was also flushed with PBS to dislodge blood from its chambers, and then it was weighed. The heart weight/body weight ratio (relative weight) was then calculated.

3.4.4. Kidney weight

After exposing the kidneys, the adrenal glands, perirenal fat, and kidney capsule were removed. The kidneys were excised by severing the renal vessels, and they were immediately placed in a Petri dish containing PBS. They were then weighed, and their weight was adjusted for body weight by dividing the kidneys' weight by the bodyweight (relative weight).

3.4.5. Oral glucose tolerance test and intra-peritoneal glucose tolerance test

Glucose challenge test was performed 48 hours before the end of each phase of the experiment. The rats had a 12-hour overnight fast. Glucose at a dose of 2 g/kg of body weight was then administered per oral. Blood samples were collected from the rat tail before glucose load (oral and intra-peritoneal) and then sequentially after 30, 60, 90, and 120 minutes. Blood glucose levels were determined with a glucometer (Accu-Chek II and fine test, Boehringer Mannheim Canada).

Glucose tolerance was expressed as a function of the area under the OGTT, and intra-peritoneal glucose tolerance test (IPGTT) curves (AUC) as previously described and post-load glucose responses were also documented (Olatunji *et al.*, 2016).

3.4.6 Invasive biochemical assay

For the invasive assay, protein expression markers, which had been correlated with CAN in previous studies and also recommended by the CAN subcommittee of the Toronto Autonomic Neuropathy Working Group, were assayed. These include nerve growth factor (NGF), plasma catecholamines (CAT), and choline acetyltransferase (Choactase), which are markers of the sympathetic and parasympathetic nerves (Porojan *et al.*, 2010; Bernardi *et al.*, 2011; Rolim *et al.*, 2013; Dimitropoulos *et al.*, 2014; Aslam *et al.*, 2015).

3.4.7. Estimation of plasma parameters

3.4.7.1. Insulin

The estimation of plasma insulin was done with the enzyme-linked immunosorbent assay (ELISA) kit for rats (RayBiotech Inc., GA, USA), following the manufacturer's protocol. After preparation of the reagent, it was brought together with the samples to room temperature for the assay procedure. Each standard and sample (100 µl respectively) were added to the appropriate wells and incubated at room temperature for 2.5 hours with gentle shaking. The solution was discarded, and the wells were washed four times with 1X wash solution. This was done by filling each well with wash buffer (300 µl) using a multi-channel pipette. Complete removal of the liquid was done at each step for good performance. After the last wash, the remaining wash buffer was removed by decanting it and blotting the plate against clean paper towels. Prepared biotinylated antibody (100 µl) was then added to each well and incubated at room temperature

with gentle shaking for 1 hour, after which the solution was discarded. The wells were again washed with the buffer solution. Prepared streptavidin solution (100 µl) was then added to each well, incubated for 45 minutes at room temperature with gentle shaking, and then the solution discarded. The wells were rewashed with the buffer solution. Tetramethyl benzidine (TMB) one-step substrate reagent (100 µl) was then added to each well and incubated at room temperature for 30 minutes in the dark with gentle shaking. After that, 50 µl of stop solution was added to each well, and the set-up was read immediately with a microplate reader set to 450 nm. The mean absorbance for each sample was calculated from a standard curve using SigmaPlot software (SPSS Inc.).

3.4.7.1. Insulin resistance and pancreatic β-cell function

Insulin resistance (IR) was estimated using the homeostasis model assessment for insulin resistance ($\text{HOMA-IR} = \text{fasting glucose (mmol/l)} * \text{fasting insulin (}\mu\text{IU/l)}/22.5$), whereas $\text{HOMA-}\beta$ ($20 * \text{fasting insulin (}\mu\text{IU/l)} / \text{fasting glucose}-3.5$) and 1-hour post-load glucose level were used to determine pancreatic β-cell function. Elevated 1-hour post-load glucose level is a predictor of IR, pancreatic β-cell function, atherosclerotic cardiovascular disease, and renal dysfunction (Succurro *et al.*, 2010; Sciacqua *et al.*, 2011; Marini *et al.*, 2012; Bianchi *et al.*, 2013).

3.4.7.2. Lipids and lipoproteins

Total cholesterol, triglyceride, high-density lipoprotein and low-density lipoprotein cholesterol levels were determined by the enzymatic colourimetric method. The determination was based on the formation of colour after enzymatic hydrolysis and oxidation. The indicator quinone imine that was used was formed from H2O2 and 4-amino-antipyrine in the presence of phenol. The assay kit was obtained from Fortress Diagnostics (Co Atrim UK; product code: BXC0272).

Contents of the kit were standard cholesterol (5.17 mM), phosphate buffer (0.1 M), chloro-4-phenol (which is a chromogen; 5.0 mM), enzymes, cholesterol oxidase (100.0 IU/l), cholesterol esterase (170 IU/l), peroxidase (1200 IU/l), cholic acid sodium salt (2.0 mM), and 4-amino-antipyrine (0.3 mM). Equal volumes of the enzymes and buffer were mixed gently after standing for 5–10 min and served as a working reagent. Then, 10 µl of the samples were added to 1.0 ml of the working reagent. Working reagent (1.0 ml) was taken as a blank. The standard solution was prepared by mixing 10 µl of the standard with 1.0 ml of the working reagent. The preparation was mixed and incubated for 5 min at 37°C. The spectrophotometer (Boehringer Mannheim) was set at zero with the blank, while the absorbance values of the sample (ASAMPLE) and standard (ASTD) were read against the blank at 520 nm.

$$\text{Concentration (mM)} = (\text{ASAMPLE} / \text{ASTD}) \times \text{standard concentration}$$

Low-density lipoprotein cholesterol was estimated according to Friedewald's formula (Krishnaveni and Gowda, 2015).

3.4.7.3 Glutathione Assay

Glutathione (-glutamyl cysteinyl glycine or GSH) is a naturally occurring tri-peptide whose nucleophilic and reducing properties play a central role in metabolic pathways and the antioxidant system of most aerobic cells. GSH is required as a coenzyme by various enzymes, including glutathione peroxidase, glutathione S-transferase, and thiol-transferase. It also plays a significant role in drug metabolism, calcium metabolism, the -glutamyl cycle, cell membrane, and blood platelet functions. GSH is crucial to various life processes, including the detoxification of xenobiotics, thiol-disulfide exchange, maintenance of the –SH level of proteins, removal of hydroperoxides, free radicals and amino acid transport across membranes. The normal

physiological values for the concentration of intracellular GSH generally range from 1 to 10 mM.

Principles of procedure

The spectrophotometric procedures used in this study are based on the method of Ellman (Ellman *et al.*, 1959; Komersová *et al.*, 2007), who reported that 5,5'-dithiobis(2-nitrobenzoic acid) is reduced by SH groups to form 1 mole of 2-nitro-5-mercaptobenzoic acid per mole of SH. The nitromercaptobenzoic acid anion has an intense yellow colour, which, when analysed at a wavelength of 412nm, can be used to measure SH groups.

Sample pre-treatment

Plasma and serum sample

Protein precipitation reagent (20µl) was added to 0.2ml of plasma/serum sample in a centrifuge tube. The solution was then mixed thoroughly and centrifuged at 3,000rpm for 10 minutes. The clear supernatant was used for the assay.

Standard curve preparation

The 2 mM GSH standard was diluted with distilled water in ratio 1:20 by adding 20 µl of 2 mM GSH to 3.8ml of distilled water to make 100 µM GSH standard stock. The solution was prepared immediately before use.

Standard	GSH concentration (μM)	Volume of distilled water (μl)	Volume of 100 μM GSH stock (μL)
S1	0	1000	0
S2	20	800	200
S3	40	600	400
S4	60	400	600
S5	80	200	800
S6	100	-	1000

Assay procedure

1. 1ml of standard and samples were placed into a clean glass tube.
2. 2 ml GSH dilution buffer was added to the glass tube.
3. 0.05ml GSH chromogen was then added to the tube and mixed well.
4. 2ml of the solution was transferred into a cuvette and read at 412nm within 5 minutes.

Calculation

A standard curve was plotted using the absorbance value for each standard versus the GSH concentration for each standard. The equation of the line was found using a linear fit method. The concentration of GSH in each sample was then extrapolated from the graph.

3.4.7.4 Total Antioxidant Capacity Assay

PRINCIPLES OF PROCEDURE

The reduction potential of the sample and standard effectively converts Cu^{2+} to Cu^{+} , therefore changing the ion's absorption characteristics. The reduced form of copper will selectively form a stable 2:1 complex with the chromogenic reagent, with maximum absorption at ≈ 450 nm. A known concentration of Trolox was used to create the calibration curve. The data was expressed as mM Trolox equivalents or in μM copper reducing equivalents.

SAMPLE PREPARATION

Plasma: Blood was collected with a sodium citrate bottle and centrifuged at 3,000 rpm for 12 minutes. The plasma and aliquot were removed and stored at -70°C before protein determination, and further assays. Sample results can alternatively be expressed in ml of plasma. The sample was diluted in 5 folds with normal saline before assay.

ASSAY PROCEDURE

1. 50 μl of samples and each standard were placed into the cuvette supplied. The blank contained 50 μl of dilution buffer in place of standard or sample.
2. 1ml of assay buffer was added to the cuvettes that contained samples, standards, and blank.
3. The cuvette was read at 450 nm for reference measurement.
4. 50 μl of the copper solution was added and mixed.
5. 50 μl of chromogen was added and incubated for 5 minutes at room temperature.
6. The cuvette was then read a second time at 450 nm.

CALCULATIONS

1. The change in absorbance of sample and standard was calculated by subtracting the first absorbance readings from the second.
2. A calibration curve was drawn by plotting the absorbance of the standards (Y-axis) against the given concentration of the standards (X-axis).
3. The total antioxidant capacity of the samples was extrapolated from the calibration curve.
4. The total antioxidant capacity of the samples was expressed in mM Trolox equivalents.

3.4.7.5 Nerve Growth Factor ELISA

Assay Principle

The kit is an ELISA kit. The plate had been pre-coated with rat NGF antibody. NGF present in the sample was added and bound to antibodies coated on the wells. And then, a biotinylated rat NGF antibody was added and bound to NGF in the sample. Then streptavidin-horseradish peroxidase (HRP) was added and bound to the biotinylated NGF antibody. After incubation, unbound streptavidin-HRP was washed away during the washing step. Substrate solution was then added, and colour develops in proportion to the amount of rat NGF. The reaction was terminated by the addition of acidic stop solution, and absorbance was measured at 450 nm.

Assay Procedure

1. All reagents, standard solutions, and samples were prepared as instructed and brought to room temperature before use. The assay was performed at room temperature.

2. The number of strips required was determined for the assay. The strips were inserted in the frames for use. The unused strips were stored at 2–8°C.
3. 50 µl of the standard was added to standard well.
4. 40 µl of the sample was added to each sample well, then 10 µl anti-NGF antibody to each sample well, then 50 µl streptavidin-HRP to each sample well and standard well. The solution was then mixed well; the plated was covered with a sealer and incubated for 60 minutes at 37°C.
5. The sealer was removed, and the plate was washed five times with wash buffer. It was soaked well with at least 0.35 ml wash buffer for 30 seconds to 1 minute for each wash. The plate was then blotted onto paper towels or other absorbent material.
6. 50 µl substrate solution A was added to each well, and then 50 µl substrate solution B to each well. The plated cover was incubated with a new sealer for 10 minutes at 37°C in the dark.
7. 50 µl stop solution was added to each well, and the blue colour changed into yellow immediately.
8. The optical density (OD) of the solution in each well was determined immediately using a microplate reader set to 450 nm within 10 minutes after adding the stop solution.

Calculation of Result

A standard curve was constructed by plotting the average OD for each standard on the vertical (Y) axis against the concentration on the horizontal (X) axis and drawing a best-fit curve through the points on the graph. These calculations were performed with computer-based curve-fitting software, and the best-fit line was determined by regression analysis.

3.4.7.6 Noradrenaline ELISA

Assay Principle

This kit is an ELISA kit. The plate had been pre-coated with rat noradrenaline (NA) antibody. NA present in the sample was added and bound to antibodies coated on the wells. And then, a biotinylated rat NA antibody was added and bound to NA in the sample. Then streptavidin-HRP was added and bound to the biotinylated NA antibody. After incubation, unbound streptavidin-HRP was washed away during a washing step. Substrate solution was then added, and colour develops in proportion to the amount of rat NA. The reaction was terminated by the addition of acidic stop solution, and absorbance was measured at 450 nm.

Assay Procedure

1. All reagents, standard solutions, and samples were prepared as instructed and brought to room temperature before use. The assay was performed at room temperature.
2. Sample and ELISA reagent were added into each well and incubated for 1 hour at 37°C.
3. The sealer was removed, and the plate was washed five times with wash buffer.
4. Substrate solutions A and B were added and incubated for 10 minutes at 37°C.
5. Stop solution was added to each well, and the colour change was observed immediately.
6. The OD of the solution in each well was determined using a microplate reader set to 450 nm within 10 minutes after adding the stop solution.

Calculation of Result

A standard curve was constructed by plotting the concentration on the horizontal (X) axis against the average OD of the standard on the vertical (Y) axis and drawing a best-fit curve through the points on the graph. These calculations were performed with computer-based curve-fitting software, and the best-fit line was determined by regression analysis.

3.4.7.7. Choline Acetyltransferase ELISA

Assay Principle

This kit is an ELISA kit. The plate had been pre-coated with rat choline acetyltransferase (CHAT) antibody. CHAT present in the sample was added and bound to antibodies coated on the wells. And then biotinylated rat CHAT antibody was added and bound to CHAT in the sample. Then streptavidin-HRP was added and bound to the biotinylated CHAT antibody. After incubation, unbound streptavidin-HRP was washed away during a washing step. The substrate solution was then added, and colour develops in proportion to the amount of rat CHAT. The reaction was terminated by the addition of acidic stop solution, and absorbance was measured at 450 nm.

Assay Procedure

1. All reagents, standard solutions, and samples were prepared as instructed and brought to room temperature before use. The assay was performed at room temperature.
2. Sample and ELISA reagent were added into each well and incubated for 1 hour at 37°C.
3. The sealer was removed, and the plate was washed five times with wash buffer.
4. Substrate solutions A and B were added and incubated for 10 minutes at 37°C.
5. Stop solution was added to each well, and the colour change was observed immediately.
6. The OD of the solution in each well was determined using a microplate reader set to 450 nm within 10 minutes after adding the stop solution.

Calculation of Result

A standard curve was constructed by plotting the average OD of the standard on the vertical (Y) axis against the concentration on the horizontal (X) axis and drawing a best-fit curve through the

points on the graph. These calculations were performed with computer-based curve-fitting software, and the best-fit line was determined by regression analysis.

3.4.7.8. C-peptide assay

Reagent preparation

Standards: The lyophilized standard was reconstituted with 2.0 ml distilled water. It was then allowed to remain undisturbed until completely dissolved and then mixed by gentle inversion.

Wash Buffer: 1X wash buffer was prepared by adding the contents of the bottle (25 ml, 20X) to 475 ml of distilled water. The buffer was stored at room temperature (20–25°C).

Assay Procedure

All reagents, serum references, and controls were taken to room temperature (20–25°C) before commencing the assay.

1. The microplate wells for each reference, control, and patient specimen were formatted to be assayed in duplicate. Unused microwell strips were placed back into the aluminum bag, sealed and stored at 2–8°C.
2. 50 µl of the appropriate standard, control, and specimen were pipetted into the assigned wells.
3. 100 µl enzyme conjugate was also pipetted into each well.
4. The plate was gently mixed for 15–20 seconds and then incubated for 60 minutes at room temperature.
5. Liquid was removed from all wells. The wells were washed three times with 300 µl of 1X wash buffer (see “Reagent Preparation” section), after which it was blotted on absorbent paper towels.

6. 100 μ l of substrate was added to all wells and then incubated for 15 minutes at room temperature.

7. 50 μ l of stop solution was added to each well and gently mixed for 15–20 seconds.

10. The absorbance was read on the ELISA reader of each well at 450nm within 15 minutes after adding the stop solution.

Calculation of results

The standard curve was constructed as follows:

1. C-peptide standard value was checked on each standard vial.
2. To construct the standard curve, a plot of the OD for each C-peptide standard point (vertical axis) versus the C-peptide standard concentrations (horizontal axis) was made on a linear graph paper. The best-fit curve was drawn through the points.
3. The concentrations (ng/ml) for controls and each unknown sample were read from the curve.

3.4.7.9. Sorbitol dehydrogenase assay

Principle

The change in absorbance was measured at 340 nm according to the following reaction.

D-sorbitol + NAD + D-fructose + NADH + H⁺

Unit Definition

One unit of activity was defined as the amount of sorbitol dehydrogenase (SDH) that formed 1 μ mol of reduced nicotinamide adenine dinucleotide (NADH) per minute at 30 °C.

Solutions

I) Buffer solution: 100 mM tris aminomethane hydrochloride (Tris-HCl) buffer, pH 9.0

II) NAD⁺ solution: 20 mM (133 mg NAD⁺ free acid/10 ml distilled water)

III) D-sorbitol solution: 500mM (911 mg D-sorbitol/10 ml 100 mM Tris-HCl buffer), pH 9.0

Preparation of Enzyme Solution

The lyophilized enzyme was dissolved with distilled water and diluted to 5 to 10 U/ml with 50mM Tris-HCl buffer containing 1 mg/ml BSA, pH 8.0.

Procedure

1. Each of the following reaction mixtures was dissolved, and 3.00 ml of the reaction mixture was pipetted into a cuvette.

Solution I: 24.00 ml

Solution II: 3.00 ml

Solution III: 3.00 mL

2. The solution was incubated at 30°C for about 3 minutes.

3. Enzyme solution (0.01 mL) was added into the cuvette and mixed.

4. The absorbance change was read at 340 nm per minute (ΔAbs_{340}) in the linear portion of the curve.

Calculation

$$\frac{(\Delta\text{Abs}_{340}) \times (3.00 + 0.01)}{\text{Volume activity (U/mL)} = 6.22 \times 0.01 \times \text{d.f.}}$$

$$\text{Volume activity (U/mL)} = 6.22 \times 0.01 \times \text{d.f.}$$

$$\text{Specific activity (U/mg protein)} = \frac{\text{Volume activity (U/mL)}}{\text{Protein concentration (mg/mL)}}^*$$

$$\text{Protein concentration (mg/mL)}^*$$

d.f.: dilution factor

6.22: millimolar extinction coefficient of NADH ($\text{cm}^2/\mu\text{mol}$)

*Protein concentration; determined by Bradford's method (Kielkopf *et al.*, 2020).

3.4.7.10. Advanced glycated end product assay

Test principle

This ELISA kit uses the sandwich ELISA method. The micro ELISA plate provided in this kit had been pre-coated with an antibody specific to advanced glycated end products (AGEs). Standards or samples are added to the appropriate micro ELISA plate wells and combined with the specific antibody. A biotinylated detection antibody specific for AGEs and the avidin-HRP conjugate was added to each microplate well successively and incubated. Free components were washed away. The substrate solution was added to each well. Only those wells that contained AGEs, biotinylated detection antibody and avidin-HRP conjugate appeared blue. The enzyme-substrate reaction was terminated by adding a sulphuric acid solution, and the colour turned yellow. The OD was measured spectrophotometrically at a wavelength of $450 \text{ nm} \pm 2 \text{ nm}$. The OD value is proportional to the concentration of AGEs. The concentration of AGEs in the samples was calculated by comparing the OD of the samples to the standard curve.

Assay procedure: All reagents and samples were brought to room temperature before use. The samples were centrifuged again after thawing, before the assay. All the reagents were then mixed thoroughly by gently swirling before pipetting. Foaming was avoided, and all samples and standards were assayed in duplicate as recommended.

1. Sample: 100 μl each of standard, blank, and sample were added per well. Reference standard and sample diluent were also added to the blank well. Solutions were added to the micro ELISA plate's bottom, avoiding the inside wall and foaming as much as possible. The solution was then mixed gently while covering the plate with the sealer provided. After that, incubation was done for 90 minutes at 37°C .

2. Biotinylated detection antibody: The liquid in each well was removed. Immediately following that was the addition of 100 µl of biotinylated detection antibody working solution to each well. The plate was covered with a plate sealer while gently tapping the plate to ensure thorough mixing. Incubation was done for 1 hour at 37°C.
3. Wash: The solution in each well was aspirated, and the well was washed, repeating the process three times. Washing was done by filling each well with wash buffer (approximately 350µL). Complete removal of the liquid at each step was ensured. After the last wash, the remaining wash buffer was removed by aspirating or decanting. The plate was then inverted and patted against thick, clean, absorbent paper.
4. HRP conjugate: The addition of 100 µl of HRP conjugate working solution to each well was done, after which the plate was covered with the plate sealer. The solution was incubated for 30 minutes at 37°C.
5. Wash: The wash process was repeated five times as conducted in step 3.
6. Substrate: 90 µl of substrate solution was added to each well. Then the plate was covered with a new plate sealer. Incubation was done for about 15 minutes at 37°C while protecting the plate from light. The reaction time was shortened or extended according to the actual colour change, but it was not more than 30 minutes. The reaction was terminated when an apparent gradient appeared in the standard wells.
7. Stop: 50 µl of stop solution was added to each well, causing a colour change to yellow immediately.
8. OD measurement: Determination of the OD of the solution in each well was done at once, using a microplate reader set to 450 nm.

3.4.8. Standard curve for the assays

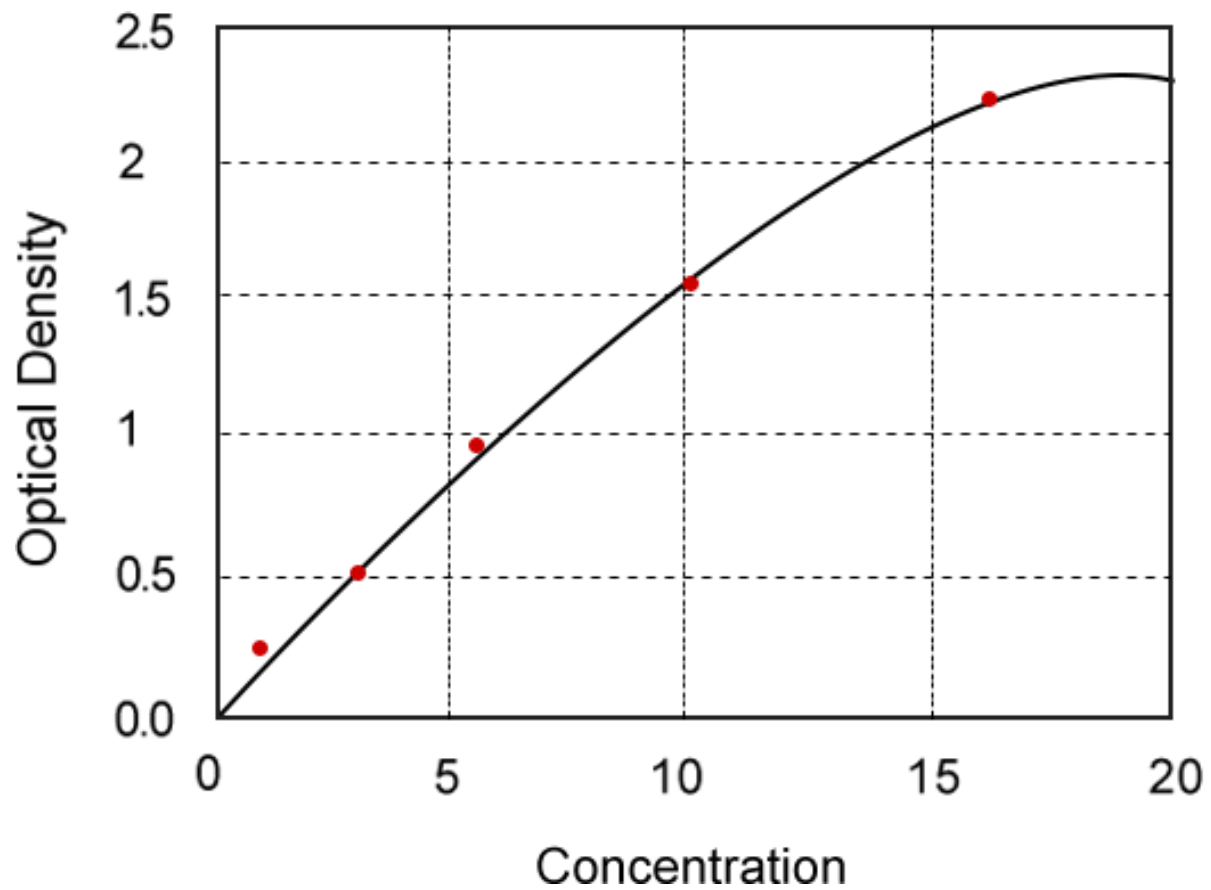


Figure 3.3: Graph showing the standard curve plotted from the optical density and concentration of the solutions during the assays.

3.5. Histological Studies

Histological studies were carried out on the epicardium of the heart to assay the nerve density. Paraffin sections from the heart (3 μ m thickness) were cut and evaluated using standard staining protocol for haematoxylin and eosin (H&E) and Beilchowsky histochemical silver stain (Wu *et al.*, 2007;Hernández-Morera *et al.*, 2016; Saleh *et al.*, 2017). Morphometric analysis was done after histochemistry to quantify and evaluate the nerve density using Image-J computer software. Histology of the pancreas and kidney were also done using H&E stains.

3.6. Holter electrocardiography and Measurement of time and frequency domain parameters

The Wistar rats were anaesthetized with ketamine (75 mg/kg), administered subcutaneously to stabilize the animal. The ECG signals were recorded continuously for at least 15 minutes in the limb, augmented, and chest leads. These recordings were subsequently stored on a computer hard disk with custom-made software for analysis as shown in appendix I-IV. The analysis focused on the delineation technique using lead II of the ECG because it shows more robust P, R, S, and T waves. Lead II reading was obtained in the Wistar rat by placing the negative electrode near the right shoulder and the positive electrode to the left of the xiphoid space, in the same way as in the Einthoven triangle (right arm position in the negative electrode and left leg position in the positive electrode) (Konopelski and Ufnal, 2016; Arini *et al.*, 2018).

Frequency domain (low frequency (LF), high frequency (HF), and LF: HF) and time-domain parameters, including mean heart rate (HR), the standard deviation of normal-to-normal R-R intervals (SDNN), root mean square of successive differences in normal-to-normal R-R intervals (RMSSD), and percentage of successive normal sinus R-R intervals >50 ms (PNN50), were

calculated from the HRV data. SDNN provided an estimate of overall HRV and sympathetic balance, while RMSSD yielded an estimate of the short-term components of HRV and parasympathetic functions.

3.7. STATISTICAL ANALYSIS

Data analysis was conducted with Statistical Product and Service Solutions version 24 (SPSS Inc, IL., USA) for Windows. Results were expressed as mean $\pm$ standard error of mean (SEM). Comparisons among more than two groups were performed with one-way analysis of variance (ANOVA), and *post hoc* analyses were done with Bonferonni test for pairwise comparison. Group means were compared for two independent samples using Student's *t*-test. P-value less than 0.05 was taken as statistically significant.

CHAPTER FOUR

4.0 RESULTS

4.1 Mean food and fluid intake

The mean feed and water intakes of each experimental animal were measured daily throughout the study duration. The T2DM group with HFD had significantly ($p<0.05$) higher water intake (400.50 ± 10.50 mls) compared with the control group (200.50 ± 5.00 mls). Few days after DM induction with STZ, a significant decrease in food (180.00 ± 5.50 g) intake was observed among the STZ-induced groups when compared with the normoglycaemic control (NC) group (240.50 ± 10.50 g). However, few days after developing DM, the feed intake increased significantly (300.00 ± 10.50 g) among the DM groups compared with the NC group (240.00 ± 10.00 g). From 1 week of treatment till the completion of the study (day 28), it was observed that the feed intake among DM rats treated with carvedilol was significantly ($p<0.05$) lower (260.50 ± 10.50 g) when compared with the untreated DM group (300.50 ± 10.00 g), but higher than in the NC group (240.50 ± 10.50 g). There was no significant difference in the food intake among the various treated groups.

There was an initial decline following T1DM induction but after a week post induction, feed intake was similar between both NC and DCAN group.

4.2 Diabetes induction and rat weight

This study showed that STZ-induced DM rats developed significantly ($p < 0.05$) higher blood glucose levels (29.56 ± 7.03 vs. 4.68 ± 0.59) mmol/L in the T1DM model compared with NC, with a similar trend in T2DM model (28.50 ± 6.51 vs. 4.56 ± 0.59) mmol/L.

Type 1 DM model: In the type 1 arm of the study, there was weight loss after administering a single dose of 50 mg/kg of STZ compared to the control animals that had sustained weight gain, as shown in Figure 4.1. Seventy-six percent (76%) of the animals in the T1DM group developed DM (blood glucose >16 mmol/L) as confirmed three days after DM induction.

Type 2 DM model: Animals in this arm of the study showed initial progressive weight gain following the administration of a high-fat diet in the DCAN group for the first eight weeks, compared with the control group. The weight, however, was noticed to have a downward trend after STZ induction in the DCAN group, compared to the control animals that continued to increase in weight slowly, as shown in Figure 4.2. Eighty-nine percent (89%) of the animals in the T2DM group developed DM (blood glucose >16 mmol/L) as confirmed 7–10 days after STZ induction.

Carvedilol significantly improved the glycaemic level in both T1DM and T2DM models as shown in figure 4.3.

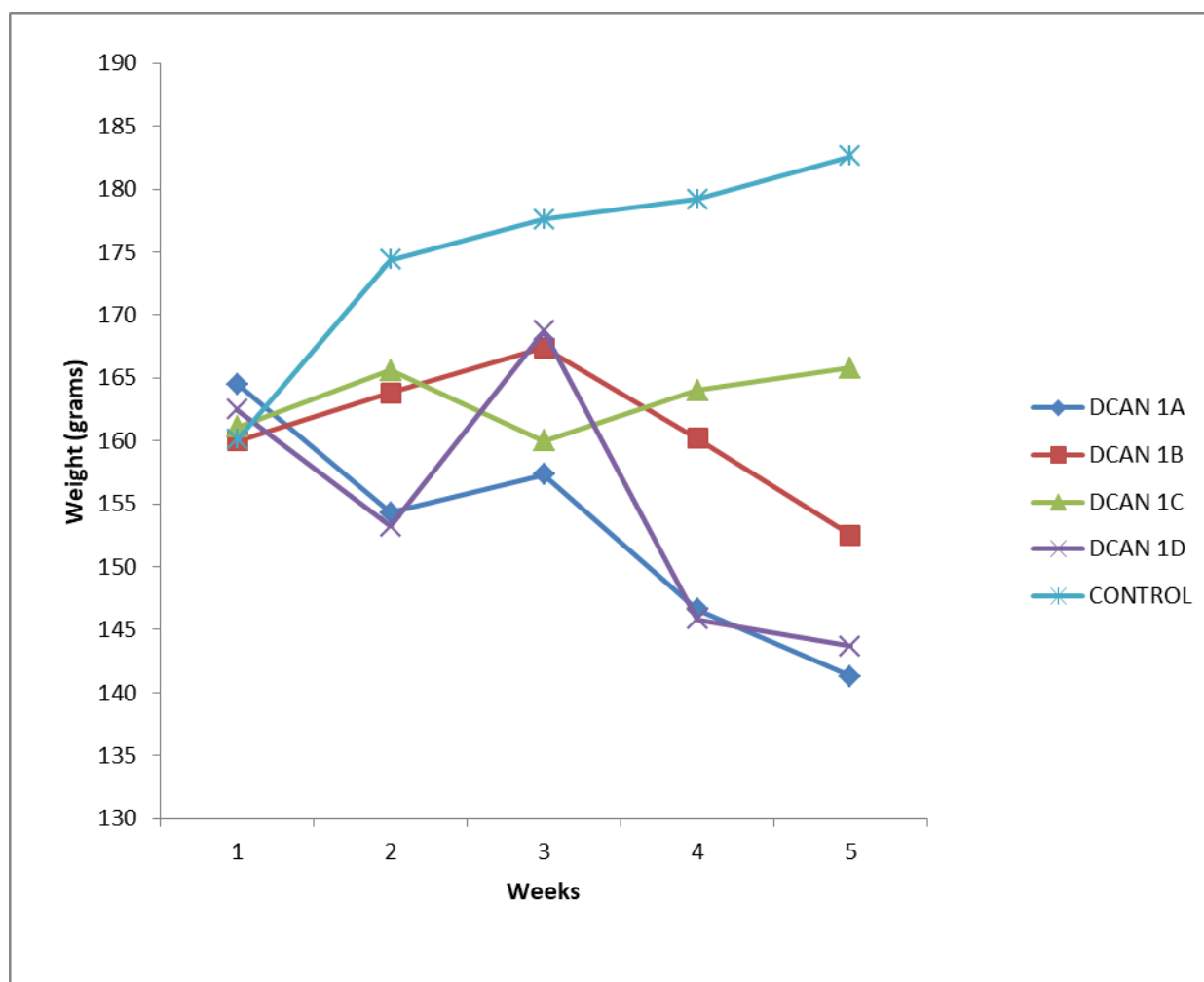


Figure 4.1: Weight changes in the control and type 1 diabetic rat models. There was weight loss after administration of single dose of 50 mg/kg of streptozotocin in the experimental groups, compared to the control animals that had sustained weight gain. Values are expressed as mean $\pm$ standard error of mean (n=8).

Key

DCAN 1A: DM 1 (+0.1 mg/kg carvedilol); DCAN 1B: DM 1(+1 mg/kg carvedilol)

DCAN 1C: DM 1 (+10 mg/kg carvedilol); DCAN 1D: DM 1 (+10 ml/kg normal saline)

DM, diabetes mellitus; DCAN, diabetic cardiac autonomic neuropathy

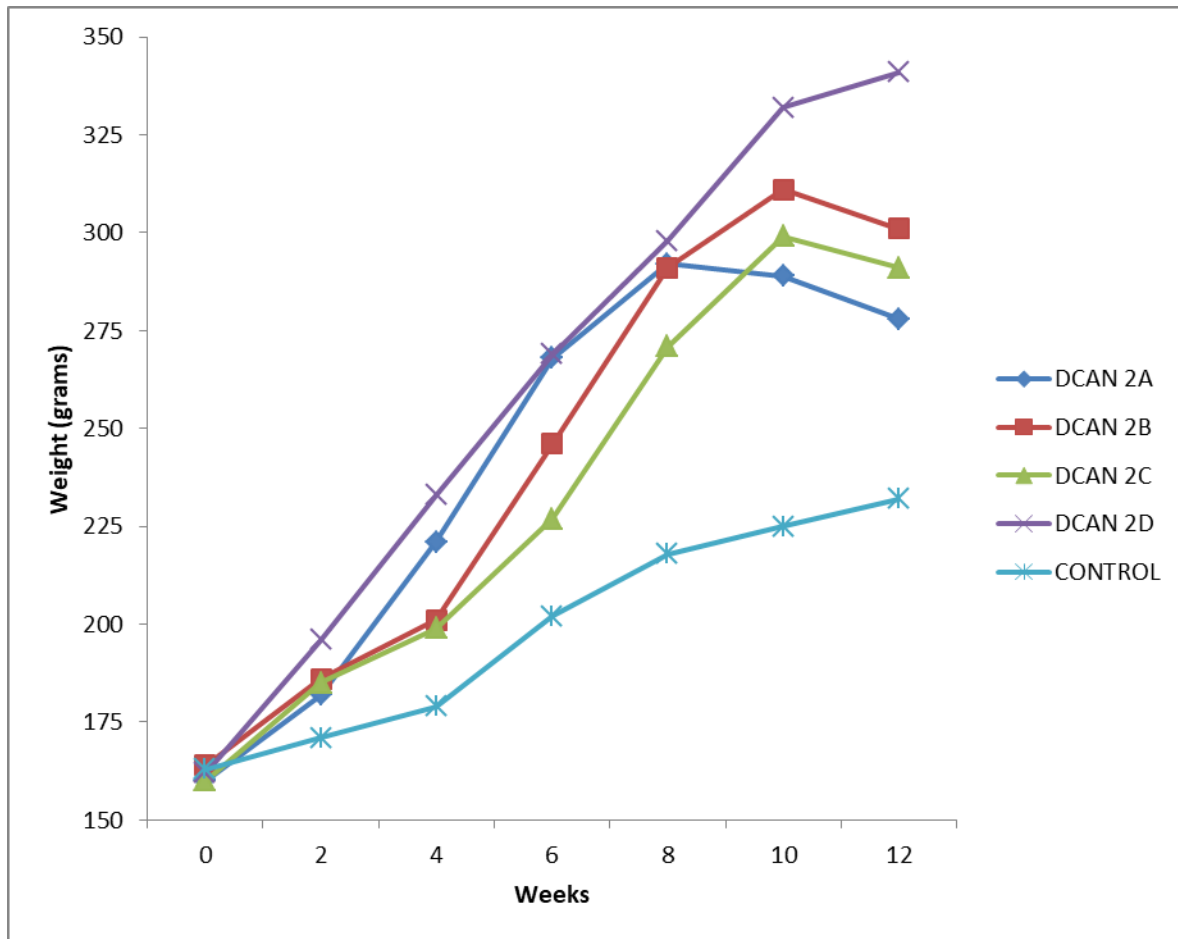


Figure 4.2: Weight changes in the control and type 2 diabetic rat models. Experimental animals (DCAN 2A, 2B, 2C, and 2D) progressively increased in weight while being fed high-fat diet for the first eight weeks, but later lost weight after streptozotocin administration and diabetes induction. In contrast, the control animals had sustained weight gain.

Key

DCAN 2A: DM 2 (+0.1 mg/kg carvedilol); DCAN 2B: DM 2 (+1 mg/kg carvedilol)

DCAN 2C: DM 2 (+10 mg/kg carvedilol); DCAN 2D: DM 2 (+10 ml/kg normal saline)

DM, diabetes mellitus; DCAN, diabetic cardiac autonomic neuropathy

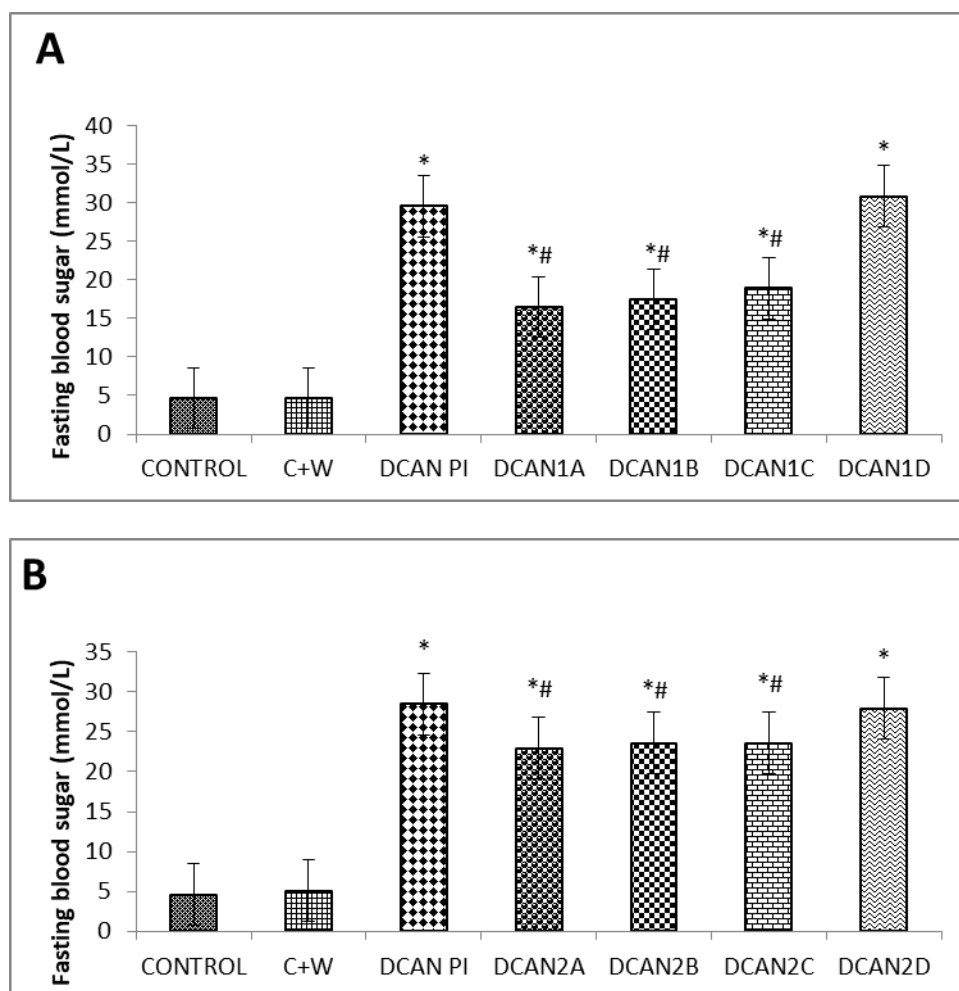


Figure 4.3: Pattern of fasting blood sugar concentration following T1DM induction (A) and T2DM induction (B). Seventy-six percent (76%) of animals in T1DM group were diabetic after administration of single high dose of streptozotocin (50 mg/kg), with fasting blood glucose higher in T1DM experimental groups compared to the control group. DM induction rate was 89% in T2DM group by using high-fat diet for 8 weeks, followed by intra-peritoneal streptozotocin administration (25 mg/kg), with fasting blood glucose higher in T2DM experimental groups compared to the control group. Values are expressed as mean $\pm$ standard error of mean (n=8). * $p < 0.05$ in DCAN groups vs. control; # $p < 0.05$ compared with diabetic untreated group.

Key

Control: Without intervention; C+W: Control (+10 ml/kg normal saline)

DCAN PI: DM induction pre-intervention

DCAN 1A: DM 1 (+0.1 mg/kg carvedilol); DCAN 1B: DM 1 (+1 mg/kg carvedilol)

DCAN 1C: DM 1 (+10 mg/kg carvedilol); DCAN 1D: DM 1 (+10 ml/kg normal saline)

DCAN 2A: DM 2 (+0.1 mg/kg carvedilol); DCAN 2B: DM 2 (+1 mg/kg carvedilol)

DCAN 2C: DM 2 (+10 mg/kg carvedilol); DCAN 2D: DM 2 (+10 ml/kg normal saline)

DM, diabetes mellitus; DCAN, diabetic cardiac autonomic neuropathy

4.3 Effect of DCAN induction on biochemical parameters

Table 4.1 shows the effect of DCAN induction on biochemical parameters. A similar trend was observed for both T1DM and T2DM for most of the parameters except for total cholesterol and HOMA-IR that were significantly elevated in the T2DM group (total cholesterol: 66.61 ± 8.02 vs. 69.28 ± 6.40 , $p=0.001$; HOMA-IR: 0.13 ± 0.08 vs. 0.25 ± 0.09 ; $p=0.016$) but not in the T1DM group ($p=0.317$ and 0.116 for total cholesterol and HOMA-IR respectively) following DM induction. Insulin, C-peptide, NGF, antioxidants, and choactase were all significantly reduced in both DM models compared to the controls, as shown in Table 4.1. This is in contrast to noradrenaline, AGEs, and lipids that were elevated following DM induction.

Table 4.1: Overall effect of DCAN induction on biochemical parameters of diabetic groups compared with controls

Parameters	Type 1 control	Type 1 post-DM induction	p value	Type 2 control	Type 2 Post-DM induction	p value
FBS (mmol/l)	5.18±0.46	17.46±7.84	<0.0001*	5.31±0.57	18.39±10.4 7	<0.0001 *
TC (mg/dl)	61.85±8.02	57.89±6.80	0.317	66.61±8.02	69.28±6.40	0.001*
TG (mg/dl)	66.07±5.64	81.64±4.2	0.470	66.07±5.64	94.02±7.6	0.121
AGEs (ng/ml)	23.04±5.17	63.54±2.09	<0.0001*	66.04±6.37	143.61±9.0 9	<0.0001 *
C-peptide (ng/ml)	1.13±0.60	0.63±0.53	0.160	1.20±0.67	0.73±0.43	0.001*
Insulin (µIU/ml)	2.24±1.01	0.56±0.47	0.003*	2.89±1.01	1.14±0.67	<0.0001 *
HOMA-IR	0.12±0.05	0.15±0.04	0.116	0.13±0.08	0.28±0.09	0.016*
NGF (pg/ml)#	270.16(111.1 8-318.34)	120.56(83.27- 160.58)	0.03*	234.16(91.1 8-311.34)	150.56(89.2 7-169.58)	<0.04*
NA (pg/ml)	119.62±15.5	589.74±99.48	0.010*	113.4±57.65	508.6±130.6	0.010*
Choactase (ng/ml)	1.24±0.04	0.81±0.03	0.02*	1.22±0.04	0.93±0.03	0.03*
GSH (Mm)	0.41 ± 0.03	0.36 ± 0.03	0.04*	0.32 ± 0.01	0.22 ± 0.01	0.03*
TAC (Mm)	1.37 ± 0.1	0.49 ± 0.01	0.013*	1.21 ± 0.08	0.82 ± 0.04	0.04*
SDH (u/L)	32.9 ± 1.6	12.3 ± 1.1	0.015*	34.1 ± 0.1	18.6 ± 1.9	0.03*

KEY: FBS, fasting blood sugar; TC, total cholesterol; TG, triglyceride; AGEs, advanced glycated end products; HOMA-IR, homeostasis model assessment for insulin resistance; NGF, nerve growth factor; NA, noradrenaline; Choactase, choline acetyl transferase; GSH, Gluthathione; TAC, Total antioxidant capacity; DM, diabetes mellitus; DCAN, diabetic cardiac autonomic neuropathy. Values are expressed as mean ± standard error of mean (n=8). *p<0.05 statistically significant compared with control group. #: Median (range) was used as data did not follow normal distribution pattern.

4.4 Effect of DCAN and carvedilol on biochemical parameters.

4.4.1. Effect of DCAN and carvedilol on Plasma insulin, C-peptide and insulin resistance

The plasma insulin level was significantly lower (0.56 ± 0.47 vs. 2.24 ± 1.01) following T1DM induction compared with the control group ($p=0.003$). Similarly, the T2DM DCAN group had significantly lower insulin (1.14 ± 0.67 vs. 2.89 ± 1.01 ; $p < 0.0001$). Carvedilol had no significant effect on insulin levels across the varying DCAN subgroups in both DM models (figure 4.4).

C-peptide concentration followed similar trend as insulin with a significantly lower levels in both T1DM and T2DM models as compared to the non-diabetic controls (figure 4.5).

HOMA-IR (which is a method for assessing β -cell function and insulin resistance (IR) from basal (fasting) glucose and insulin or C-peptide concentrations) was significantly higher in T2DM (0.28 ± 0.09 vs. 0.13 ± 0.08 ; $p=0.016$) compared with the non-diabetic control group, but not significantly higher in the T1DM model. Carvedilol at a dose of 10 mg/kg significantly improved HOMA-IR in the T2DM model as shown in Figure 4.6.

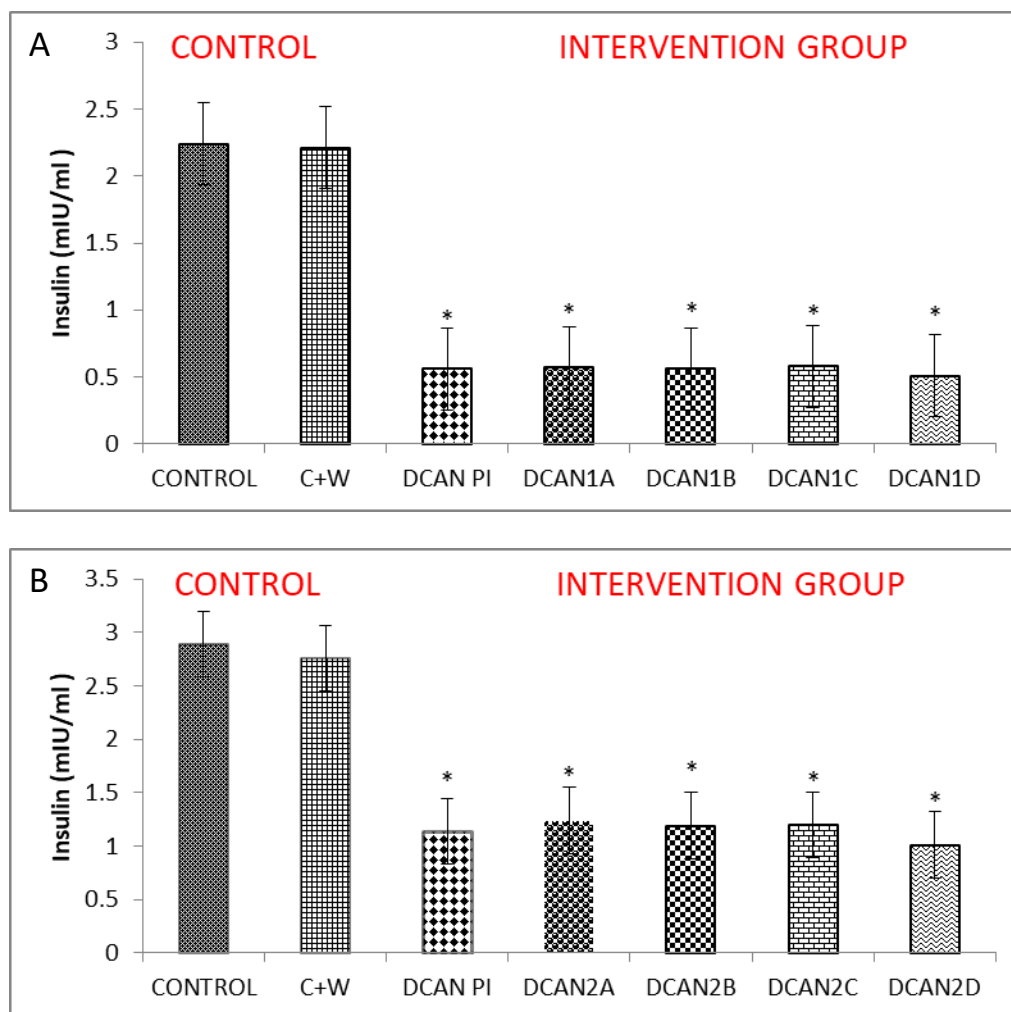


Figure 4.4 Effect of DCAN induction and carvedilol administration on insulin in T1DM (A) and T2DM (B) experimental animals compared to control groups. DCAN induction significantly reduced the blood level of insulin (more in T1DM than T2DM). Carvedilol had no significant effect on insulin. Values are expressed as mean $\pm$ standard error of mean (n=8). *p<0.05 in DCAN groups vs. control; *significance (p<0.05).

Key; Control: Without intervention; C+W: Control (+10 ml/kg normal saline)

DCAN PI: DM induction pre-intervention,

DCAN 1A: DM 1 (+0.1 mg/kg carvedilol), DCAN 1B: DM 1(+1 mg/kg carvedilol),

DCAN 1C: DM 1 (+10 mg/kg carvedilol), DCAN 1D: DM 1 (+10 ml/kg normal saline)

DCAN 2A: DM 2 (+0.1 mg/kg carvedilol), DCAN 2B: DM 2(+1 mg/kg carvedilol)

DCAN 2C: DM 2 (+10 mg/kg carvedilol), DCAN 2D: DM 2 (+10 ml/kg normal saline)

DM, diabetes mellitus; DCAN, diabetic cardiac autonomic neuropathy; HOMA-IR, homeostasis model assessment for insulin resistance.

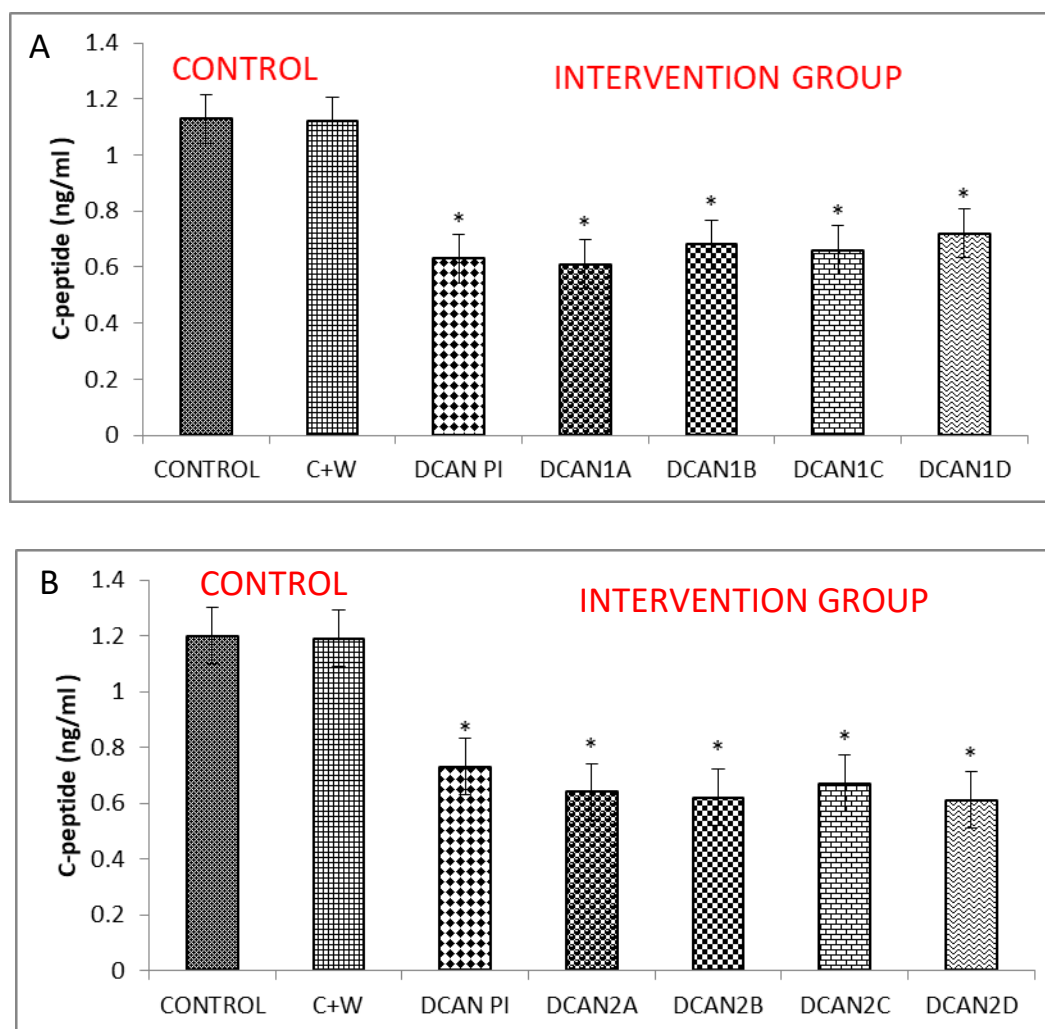


Figure 4.5 Effect of DCAN induction and carvedilol administration on C-peptide in T1DM (A) and T2DM (B) experimental animals compared to control groups. DCAN induction significantly reduced the blood level of C-peptide. Carvedilol had no significant effect on C-peptide the diabetic model groups. Values are expressed as mean $\pm$ standard error of mean (n=8). *p<0.05 in DCAN groups vs. control; *significance (p<0.05).

Key; Control: Without intervention; C+W: Control (+10 ml/kg normal saline)

DCAN PI: DM induction pre-intervention,

DCAN 1A: DM 1 (+0.1 mg/kg carvedilol), DCAN 1B: DM 1(+1 mg/kg carvedilol),

DCAN 1C: DM 1 (+10 mg/kg carvedilol), DCAN 1D: DM 1 (+10 ml/kg normal saline)

DCAN 2A: DM 2 (+0.1 mg/kg carvedilol), DCAN 2B: DM 2(+1 mg/kg carvedilol)

DCAN 2C: DM 2 (+10 mg/kg carvedilol), DCAN 2D: DM 2 (+10 ml/kg normal saline)

DM, diabetes mellitus; DCAN, diabetic cardiac autonomic neuropathy; HOMA-IR, homeostasis model assessment for insulin resistance.

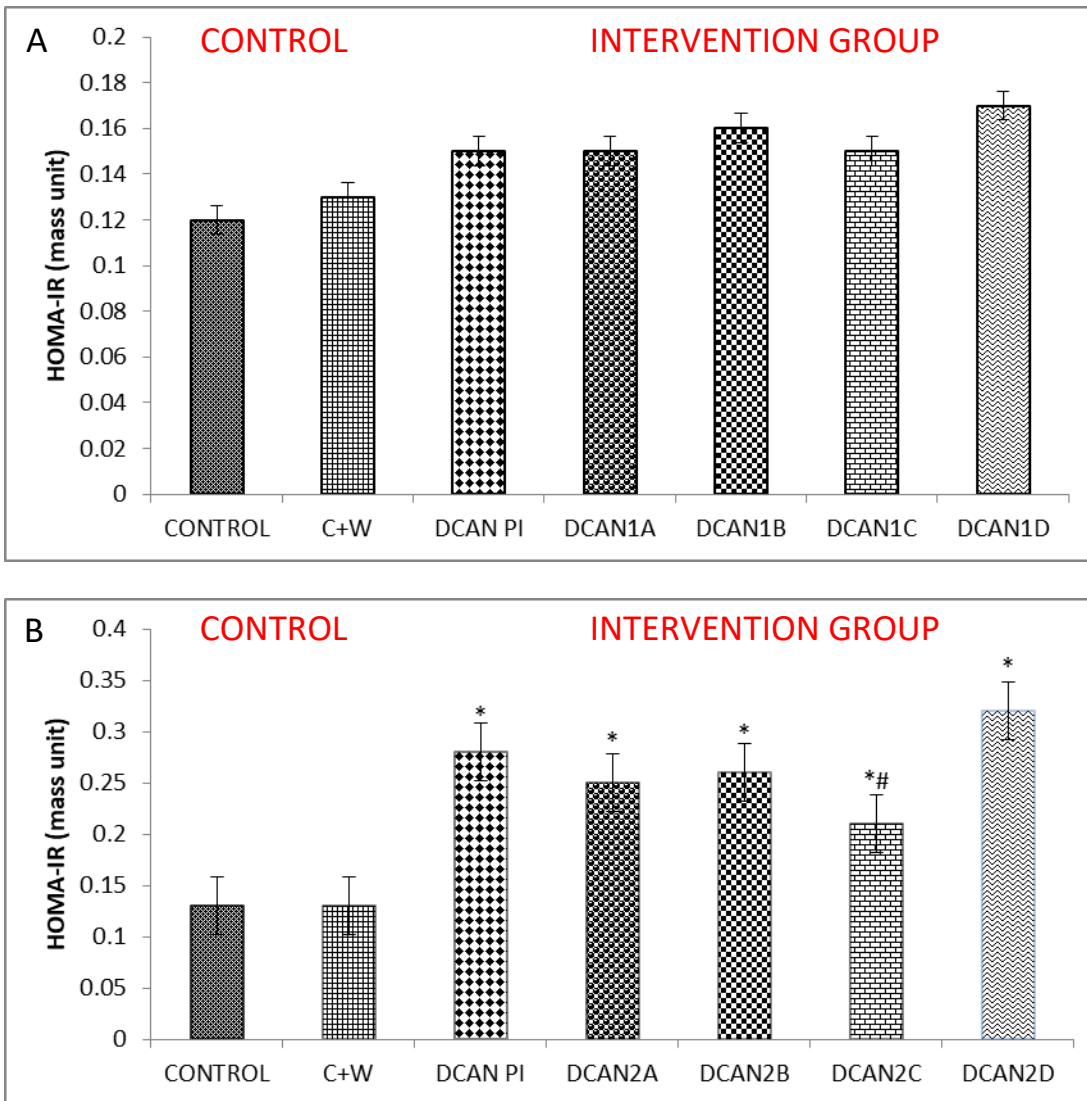


Figure 4.6: Effect of DCAN induction and carvedilol administration on HOMA-IR in T1DM (A) and T2DM (B) experimental animals compared to control groups. DCAN induction significantly increased HOMA-IR level in T2DM. Carvedilol at a dose of 10 mg/kg improved HOMA-IR in T2DM rat models. Values are expressed as mean $\pm$ standard error of mean (n=8).

* p <0.05 in DCAN groups vs. control; # p <0.05 compared with diabetic untreated group
*significance (p<0.05).

Key: Control: Without intervention; C+W: Control (+10 ml/kg normal saline)

DCAN PI: DM induction pre-intervention,

DCAN 1A: DM 1 (+0.1 mg/kg carvedilol), DCAN 1B: DM 1(+1 mg/kg carvedilol),

DCAN 1C: DM 1 (+10 mg/kg carvedilol), DCAN 1D: DM 1 (+10 ml/kg normal saline)

DCAN 2A: DM 2 (+0.1 mg/kg carvedilol), DCAN 2B: DM 2(+1 mg/kg carvedilol)

DCAN 2C: DM 2 (+10 mg/kg carvedilol), DCAN 2D: DM 2 (+10 ml/kg normal saline)

DM, diabetes mellitus; DCAN, diabetic cardiac autonomic neuropathy; HOMA-IR, homeostasis model assessment for insulin resistance.

4.4.2. Effect of DCAN and carvedilol on Plasma lipids, urea and creatinine

The plasma urea was observed to increase significantly following DCAN induction compared to the control groups in both T1DM (29.2 ± 5 vs. 12.3)mg/dl and T2DM (31.8 ± 5 vs. 9.2 ± 0.9) mg/l models. Carvedilol significantly improved urea levels in both the T1DM and T2DM models (figure 4.7).

The plasma creatinine concentration was observed to increase significantly in the DM groups (154.5 ± 5.9 vs. 89.4 ± 3.8) mmol/l in T1DM and (152.4 ± 5.9 vs. 72.1 ± 3.8) mmol/l in T2DM with no significant difference despite carvedilol administration in both T1DM and T2DM models (Figure 4.8).

The triglycerides were significantly higher in both DCAN models compared with the controls. Carvedilol reduced the triglyceride levels significantly in both models (figure 4.9).

However, total cholesterol was only significantly higher in the T2DM DCAN model ($p=0.001$). Carvedilol administered at 1 mg/kg and 10 mg/kg doses had significant improvement effect on the total cholesterol levels in T2DM model groups. Carvedilol at 1 mg/kg significantly decreased the total cholesterol level in the T1DM model groups (figure 4.10).

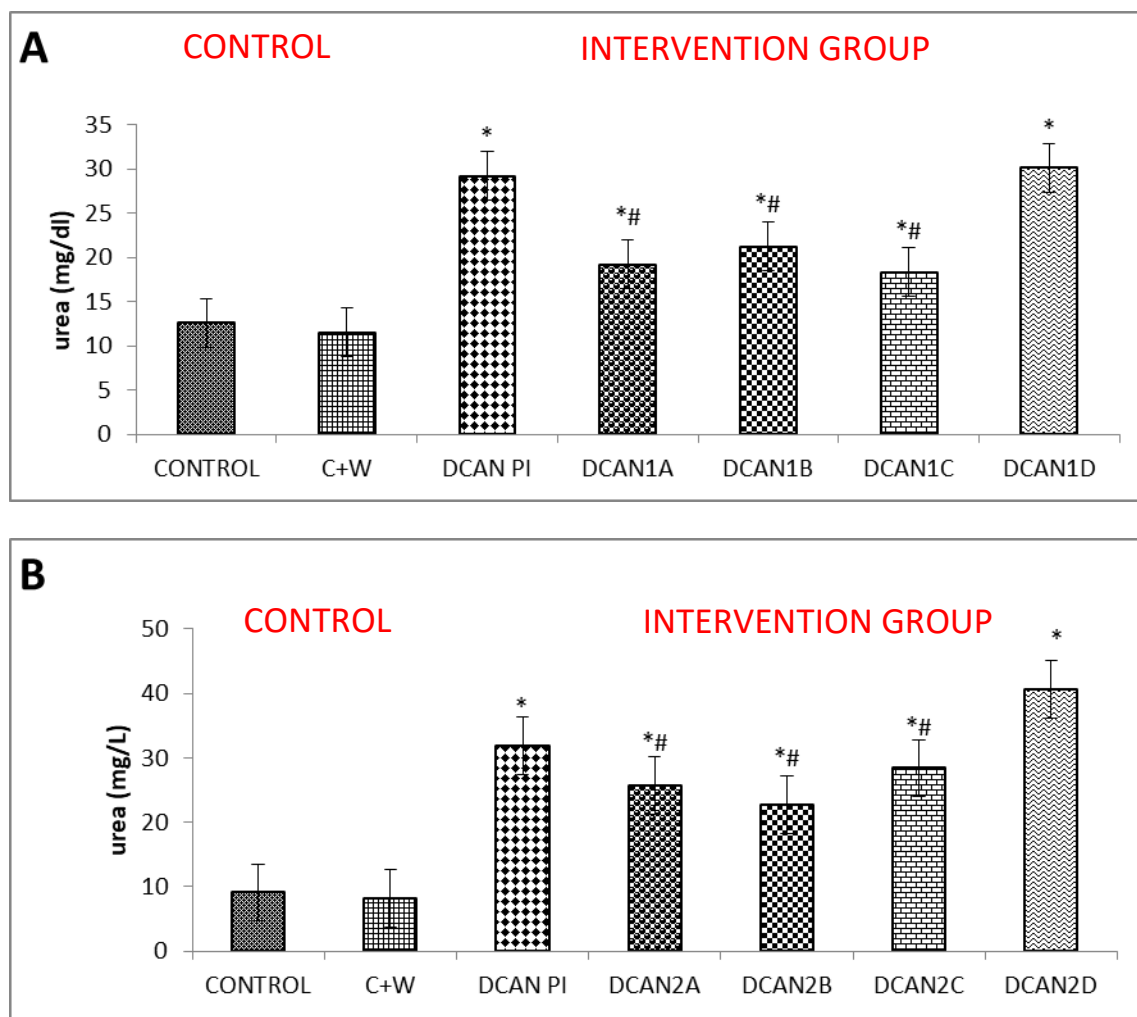


Figure 4.7: Effect of DCAN induction and carvedilol administration on plasma urea in T1DM (A) and T2DM (B). DCAN induction significantly increased the level of urea in both T1DM and T2DM model groups. Carvedilol administration significantly improved the urea level in T1DM and T2DM models. Values are expressed as mean $\pm$ standard error of mean (n=8). * $p < 0.05$ in DCAN groups vs. control; # $p < 0.05$ compared with untreated group (statistically significant at $p < 0.05$).

Key: Control: Without intervention; C+W: Control (+10 ml/kg normal saline)

DCAN PI: DM induction pre-intervention,

DCAN 1A: DM 1 (+0.1 mg/kg carvedilol), DCAN 1B: DM 1(+1 mg/kg carvedilol),

DCAN 1C: DM 1 (+10 mg/kg carvedilol), DCAN 1D: DM 1 (+10 ml/kg normal saline)

DCAN 2A: DM 2 (+0.1 mg/kg carvedilol), DCAN 2B: DM 2(+1 mg/kg carvedilol)

DCAN 2C: DM 2 (+10 mg/kg carvedilol), DCAN 2D: DM 2 (+10 ml/kg normal saline)

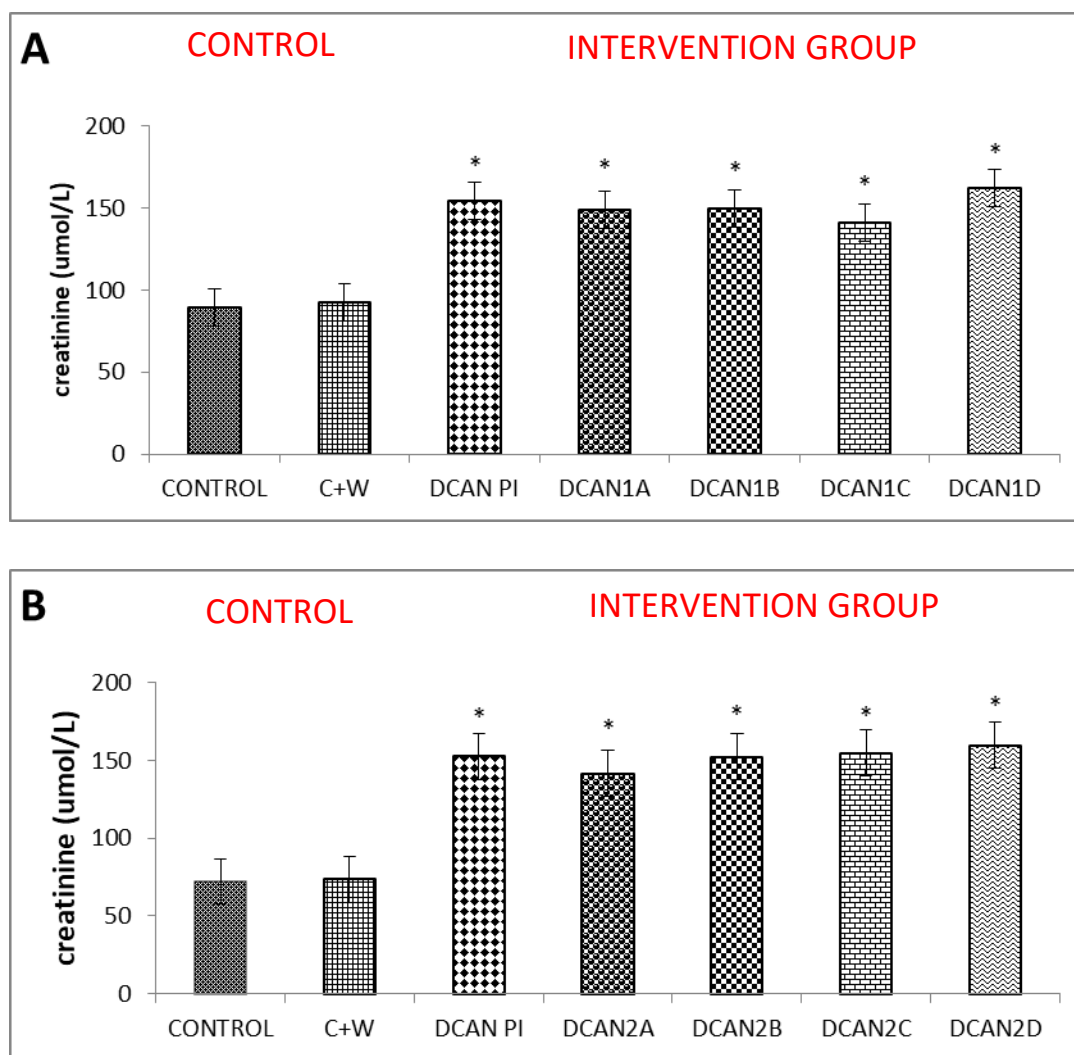


Figure 4.8: Effect of DCAN induction and carvedilol administration on creatinine in T1DM (A) and T2DM (B). DCAN induction significantly increased the level of creatinine in both T1DM and T2DM model groups. Carvedilol administration had no effect on creatinine level in both models. Values are expressed as mean $\pm$ standard error of mean (n=8). * $p < 0.05$ in DCAN groups vs. control (statistically significant at $p < 0.05$).

Key: Control: Without intervention; C+W: Control (+10 ml/kg normal saline)

DCAN PI: DM induction pre-intervention,

DCAN 1A: DM 1 (+0.1 mg/kg carvedilol), DCAN 1B: DM 1(+1 mg/kg carvedilol),

DCAN 1C: DM 1 (+10 mg/kg carvedilol), DCAN 1D: DM 1 (+10 ml/kg normal saline)

DCAN 2A: DM 2 (+0.1 mg/kg carvedilol), DCAN 2B: DM 2(+1 mg/kg carvedilol)

DCAN 2C: DM 2 (+10 mg/kg carvedilol), DCAN 2D: DM 2 (+10 ml/kg normal saline)

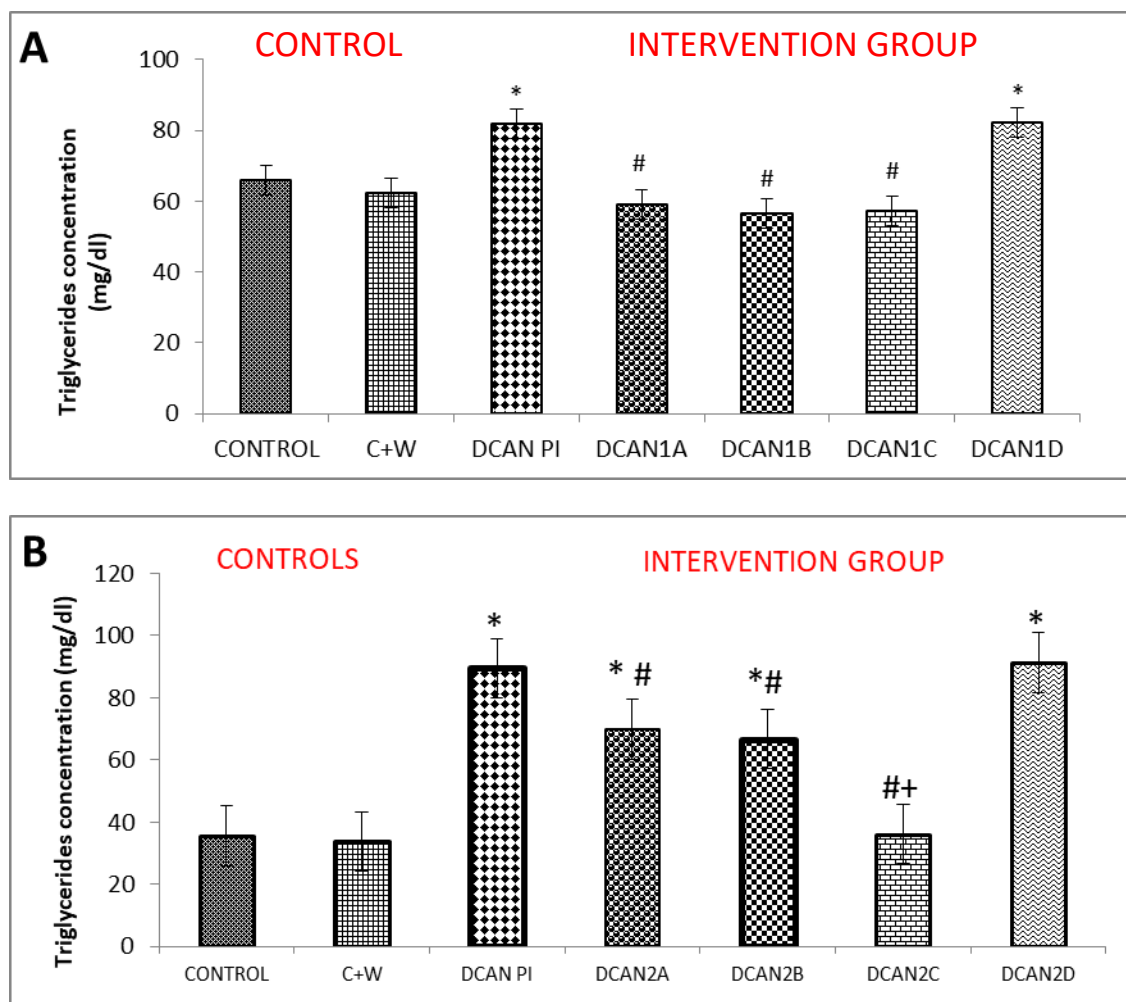


Figure 4.9: Effect of carvedilol on lipid profile in T2DM (A) and T1DM (B). DCAN induction increased TG levels in T2DM model groups. All doses of carvedilol administered significantly improved the TG level. DCAN induction significantly increased TG in T1DM model groups. Carvedilol administration significantly decreases the TG levels in type 1 model groups. TG, triglycerides; TC, total cholesterol; DM, diabetes mellitus; DCAN, diabetic cardiac autonomic neuropathy; T1DM, type 1 diabetes mellitus; T2DM, type 2 diabetes mellitus

Values are expressed as mean $\pm$ standard error of mean (n=8).

* p < 0.05 in DCAN groups vs. control; # p < 0.05 compared with untreated group; + p < 0.05 compared among varying carvedilol doses (statistically significant at p < 0.05).

Key: Control: Without intervention; C+W: Control (+10 ml/kg normal saline)

DCAN PI: DM induction pre-intervention,

DCAN 1A: DM 1 (+0.1 mg/kg carvedilol), DCAN 1B: DM 1 (+1 mg/kg carvedilol),

DCAN 1C: DM 1 (+10 mg/kg carvedilol), DCAN 1D: DM 1 (+10 ml/kg normal saline)

DCAN 2A: DM 2 (+0.1 mg/kg carvedilol), DCAN 2B: DM 2 (+1 mg/kg carvedilol)

DCAN 2C: DM 2 (+10 mg/kg carvedilol), DCAN 2D: DM 2 (+10 ml/kg normal saline)

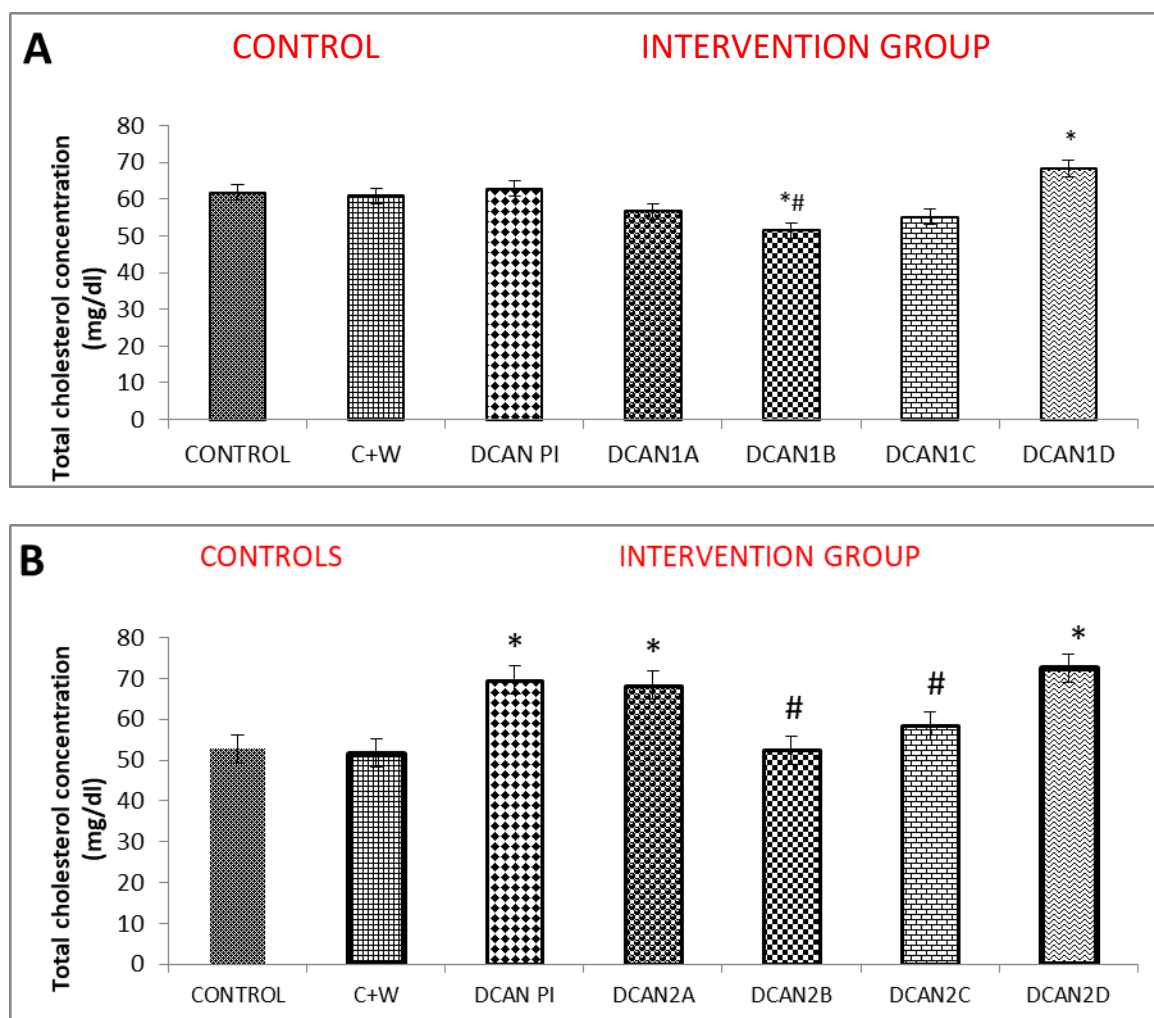


Figure 4.10: Effect of carvedilol on lipid profile in T1DM (A) and T2DM (B). DCAN induction increased TC levels in T2DM model groups. Carvedilol administered at 1 mg/kg and 10 mg/kg doses had significant improvement effect on the TC levels in T2DM model groups. Carvedilol at 1 mg/kg significantly decreased the TC level in the T1DM model groups.

TG, triglycerides; TC, total cholesterol; DM, diabetes mellitus; DCAN, diabetic cardiac autonomic neuropathy; T1DM, diabetes mellitus; T2DM, type 2 diabetes mellitus

Values are expressed as mean $\pm$ standard error of mean (n=8). * p <0.05 in DCAN groups vs. control; # p <0.05 compared with untreated group (statistically significant at p<0.05).

Key:

Control: Without intervention; C+W: Control (+10 ml/kg normal saline)

DCAN PI: DM induction pre-intervention,

DCAN 1A: DM 1 (+0.1 mg/kg carvedilol), DCAN 1B: DM 1(+1 mg/kg carvedilol),

DCAN 1C: DM 1 (+10 mg/kg carvedilol), DCAN 1D: DM 1 (+10 ml/kg normal saline)

DCAN 2A: DM 2 (+0.1 mg/kg carvedilol), DCAN 2B: DM 2(+1 mg/kg carvedilol)

DCAN 2C: DM 2 (+10 mg/kg carvedilol), DCAN 2D: DM 2 (+10 ml/kg normal saline)

4.4.3 Effect of DCAN and carvedilol on advanced glycated end products

Following DCAN induction in T1DM groups, there was a significant increase in the level of AGEs (63.5 ± 6.2) ng/ml compared to the non-diabetic control group (23.8 ± 0.8) ng/ml. It was observed that various carvedilol doses did not affect the blood level of AGEs in the T1DM model groups. Similarly, DCAN induction significantly increased the level of AGEs in the T2DM model groups (143.6 ± 9.1 ng/ml) compared to the non-diabetic control group (66.04 ± 6.4) ng/ml. However, there was significant improvement of the AGE levels in T2DM models following administration of the various doses of carvedilol, as shown in Figure 4.11.

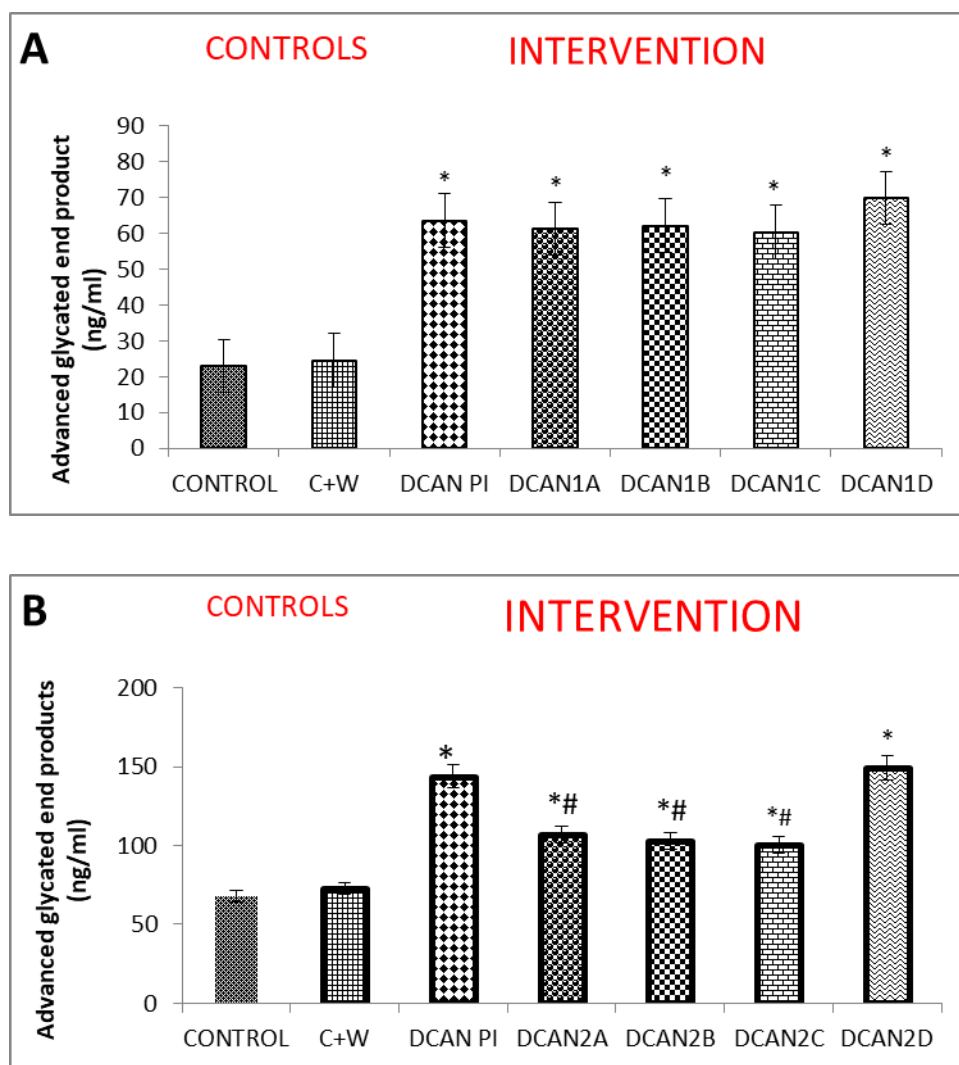


Figure 4.11: DCAN induction increased levels of AGEs in both T1DM (A) and T2DM (B) models. Carvedilol at all doses reduced AGEs in the T2DM model compared to the diabetic non-intervention group. Values are expressed as mean $\pm$ standard error of mean (n=8). * p < 0.05 in DCAN groups vs. control; # p < 0.05 compared with untreated group (statistically significant at p < 0.05).

AGEs, advanced glyated end products; DM, diabetes mellitus; DCAN, diabetic cardiac autonomic neuropathy; T1DM, diabetes mellitus; T2DM, type 2 diabetes mellitus

Key

Control: Without intervention; C+W: Control (+10 ml/kg normal saline)

DCAN PI: DM induction pre-intervention,

DCAN 1A: DM 1 (+0.1 mg/kg carvedilol), DCAN 1B: DM 1(+1 mg/kg carvedilol),

DCAN 1C: DM 1 (+10 mg/kg carvedilol), DCAN 1D: DM 1 (+10 ml/kg normal saline)

DCAN 2A: DM 2 (+0.1 mg/kg carvedilol), DCAN 2B: DM 2(+1 mg/kg carvedilol)

DCAN 2C: DM 2 (+10 mg/kg carvedilol), DCAN 2D: DM 2 (+10 ml/kg normal saline)

4.4.4. Effects of DCAN and carvedilol on plasma antioxidants activity

STZ-induced DCAN significantly reduced antioxidant activity. Both total antioxidant capacity (0.49 ± 0.01 Mm Trolox equivalent) and glutathione levels (0.36 ± 0.03 Mm) were reduced after DCAN induction in T1DM groups compared to the non-diabetic control group (1.37 ± 0.1 Mm Trolox equivalent and 0.41 ± 0.03 Mm respectively). Carvedilol administration for 28 days significantly increased the glutathione level at all doses given ($p < 0.003$), unlike the total antioxidant activity that only had a significant dose-related increase in the group that received 10 mg/kg of carvedilol (figure 4.12 and 4.13).

DCAN induction significantly reduced the glutathione (0.22 ± 0.01 Mm) and total antioxidant capacity (0.82 ± 0.04 Mm Trolox equivalent) in the T2DM model groups compared to the non-diabetic control group (0.32 ± 0.01 Mm and 1.21 ± 0.08 Mm Trolox equivalent respectively). Administration of carvedilol significantly improved the glutathione levels and the total antioxidant capacity across all categories of doses administered in the T2DM model as shown in figure 4.12 and 4.13.

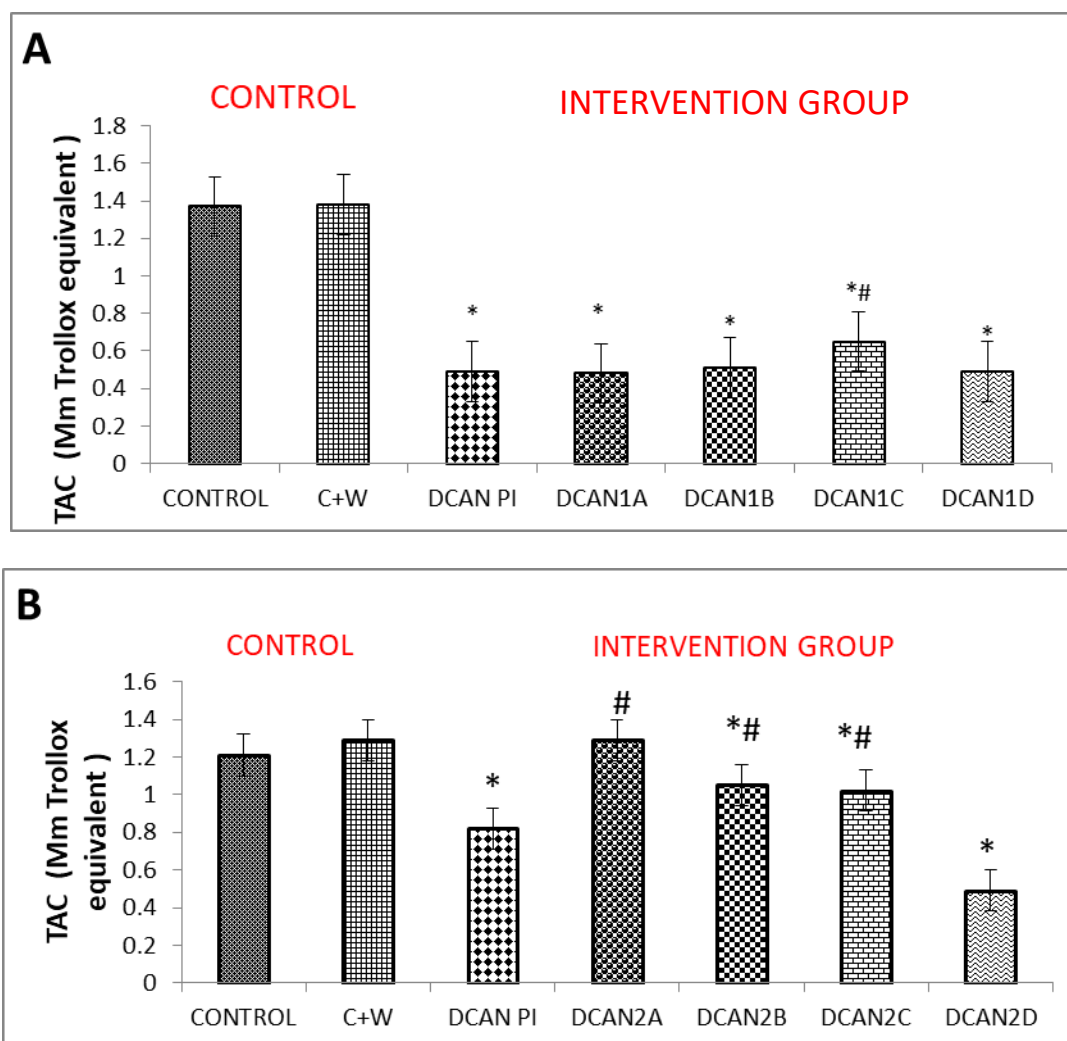


Figure 4.12: Effect of carvedilol on total antioxidant capacity (TAC) in T1DM (A) and T2DM (B). TAC concentration was improved with administration of 10 mg/kg carvedilol in T1DM model while all doses improved TAC in T2DM. Values are expressed as mean $\pm$ standard error of mean (n=8). * p < 0.05 in DCAN groups vs. control; # p < 0.05 compared with untreated group (statistically significant at p < 0.05).

DM, diabetes mellitus; DCAN, diabetic cardiac autonomic neuropathy; T1DM, diabetes mellitus; T2DM, type 2 diabetes mellitus

Key: Control: Without intervention; C+W: Control (+10 ml/kg normal saline)

DCAN PI: DM induction pre-intervention,

DCAN 1A: DM 1 (+0.1 mg/kg carvedilol), DCAN 1B: DM 1(+1 mg/kg carvedilol),

DCAN 1C: DM 1 (+10 mg/kg carvedilol), DCAN 1D: DM 1 (+10 ml/kg normal saline)

DCAN 2A: DM 2 (+0.1 mg/kg carvedilol), DCAN 2B: DM 2(+1 mg/kg carvedilol)

DCAN 2C: DM 2 (+10 mg/kg carvedilol), DCAN 2D: DM 2 (+10 ml/kg normal saline)

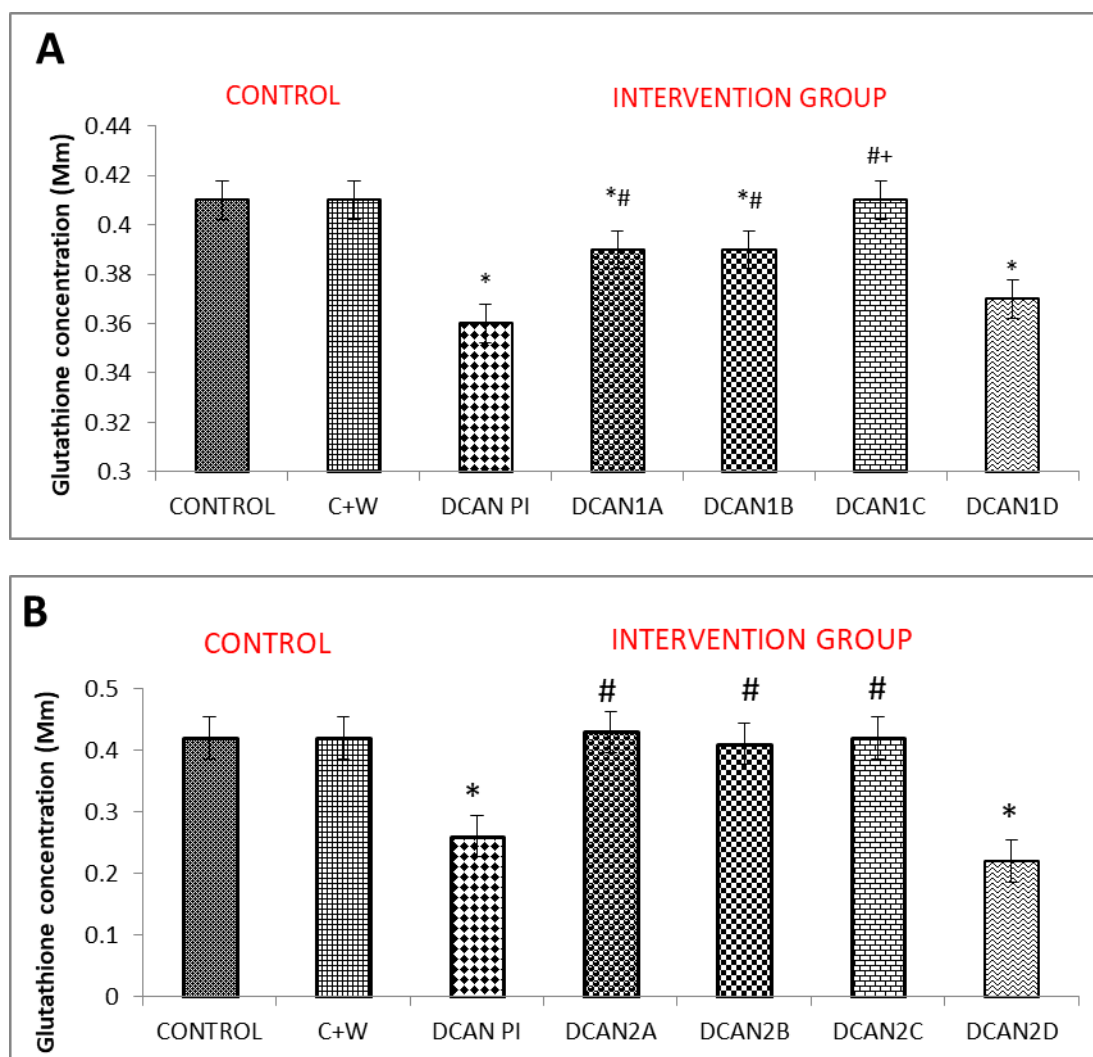


Figure 4.13: Effect of carvedilol on glutathione in T1DM (A) and T2DM (B). Carvedilol at all doses significantly improved glutathione concentration in both T1DM and T2DM models. Values are expressed as mean $\pm$ standard error of mean (n=8). * p < 0.05 in DCAN groups vs. control; # p < 0.05 compared with untreated group; + p < 0.05 compared among varying carvedilol doses (statistically significant at p < 0.05).

DM, diabetes mellitus; DCAN, diabetic cardiac autonomic neuropathy; T1DM, diabetes mellitus; T2DM, type 2 diabetes mellitus

Key; Control: Without intervention; C+W: Control (+10 ml/kg normal saline)

DCAN PI: DM induction pre-intervention,

DCAN 1A: DM 1 (+0.1 mg/kg carvedilol), DCAN 1B: DM 1(+1 mg/kg carvedilol),

DCAN 1C: DM 1 (+10 mg/kg carvedilol), DCAN 1D: DM 1 (+10 ml/kg normal saline)

DCAN 2A: DM 2 (+0.1 mg/kg carvedilol), DCAN 2B: DM 2(+1 mg/kg carvedilol)

DCAN 2C: DM 2 (+10 mg/kg carvedilol), DCAN 2D: DM 2 (+10 ml/kg normal saline)

4.4.5. Effect of DCAN and carvedilol on plasma sorbitol dehydrogenase

STZ-induced DCAN significantly reduced sorbitol dehydrogenase (SDH) activity compared to the non-diabetic control group (12.3 ± 1.1 vs. 32.9 ± 1.6) u/l and (18.6 ± 1.9 vs. 34.1 ± 0.1) u/l in T1DM and T2DM model respectively. SDH is an enzyme in the polyol pathway, which converts sorbitol to fructose. Studies have shown that sorbitol, along with other metabolites, mediates the neurotoxic effect of chronic hyperglycaemia. Carvedilol at 1mg/kg increased SDH level in T1DM, while all doses of carvedilol improved the SDH level significantly in T2DM models compared with the diabetic control, as shown in Figure 4.14.

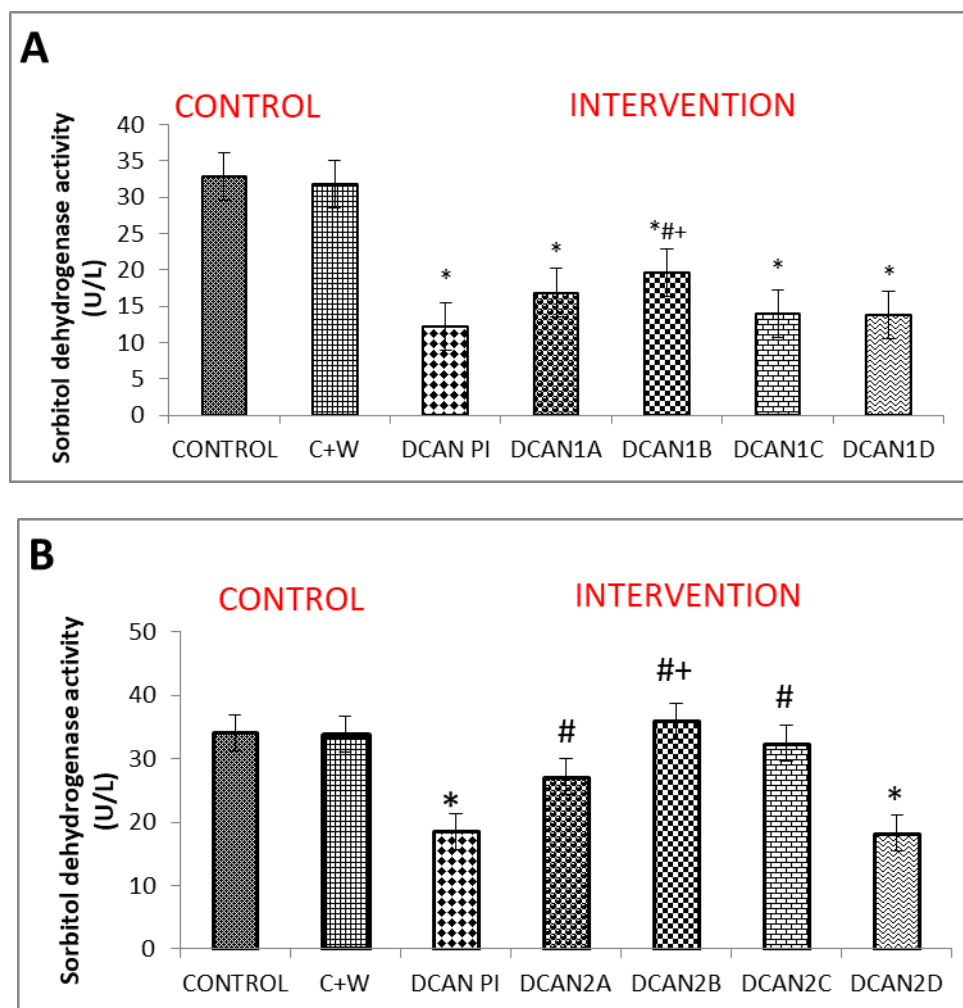


Figure 4.14: Carvedilol improved the level of sorbitol dehydrogenase activity in T1DM (A) and T2DM (B) DCAN rat models. Carvedilol at 1 mg/kg significantly improved sorbitol dehydrogenase in T1DM, while all doses had effect on T2DM model. Values are expressed as mean $\pm$ standard error of mean (n=8). * p < 0.05 in DCAN groups vs. control; # p < 0.05 compared with untreated group; + p < 0.05 compared among varying carvedilol doses (statistically significant at p < 0.05).

Key

Control: Without intervention; C+W: Control (+10 ml/kg normal saline)

DCAN PI: DM induction pre-intervention,

DCAN 1A: DM 1 (+0.1 mg/kg carvedilol), DCAN 1B: DM 1(+1 mg/kg carvedilol),

DCAN 1C: DM 1 (+10 mg/kg carvedilol), DCAN 1D: DM 1 (+10 ml/kg normal saline)

DCAN 2A: DM 2 (+0.1 mg/kg carvedilol), DCAN 2B: DM 2(+1 mg/kg carvedilol)

DCAN 2C: DM 2 (+10 mg/kg carvedilol), DCAN 2D: DM 2 (+10 ml/kg normal saline)

DM, diabetes mellitus; DCAN, diabetic cardiac autonomic neuropathy; T1DM, diabetes mellitus; T2DM, type 2 diabetes mellitus

4.4.6. Effect of DCAN and carvedilol on noradrenaline

There was a significant increase in noradrenaline level ($p=0.010$) following DCAN induction in both T1DM (589.7 ± 99.4 pg/ml) and T2DM models (508.6 ± 130.6 pg/ml) when compared to the respective control groups (119.6 ± 15.5 pg/ml and 113.4 ± 14.6 pg/ml). However, there was significantly reduced level of noradrenaline in the 1 mg/kg and 10 mg/kg carvedilol administration groups in the T1DM model. Similarly, carvedilol at all doses was observed to have a significant negative effect on noradrenaline activity after administration in T2DM model groups (figure 4.15).

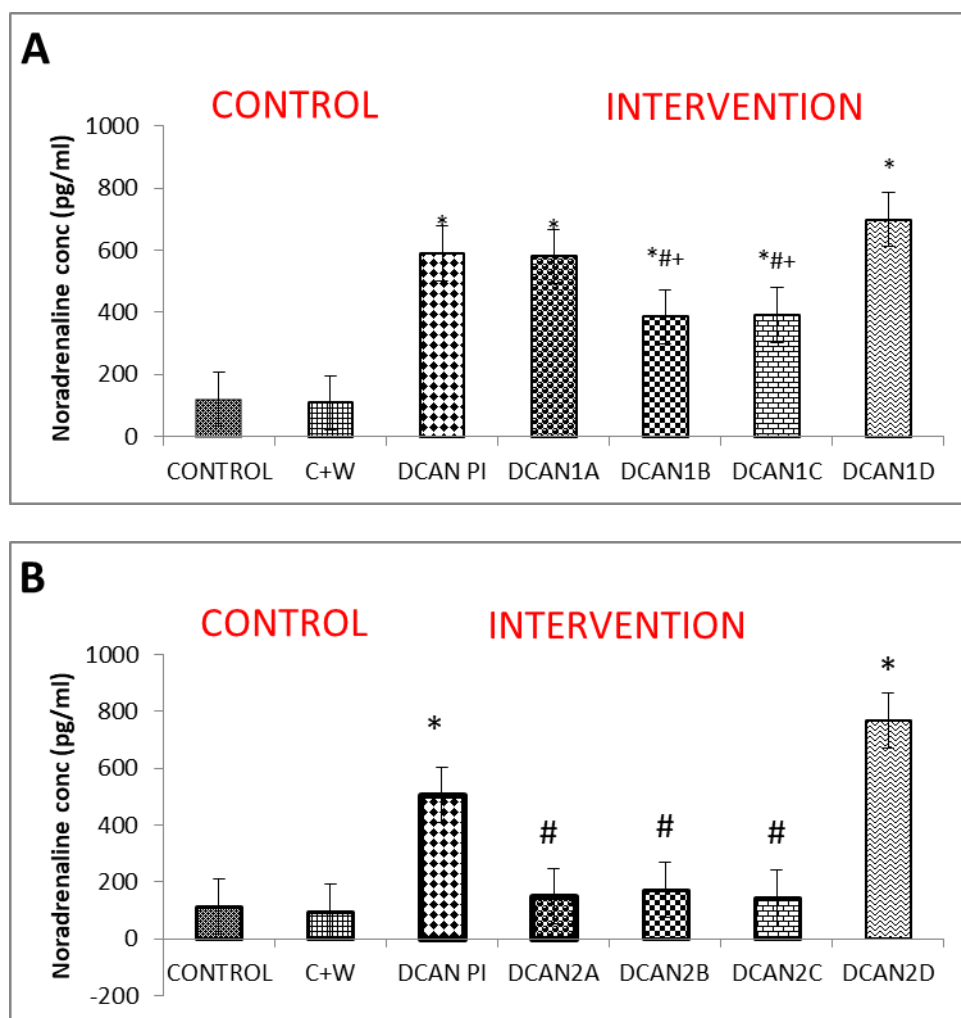


Figure 4.15: DM induction increased noradrenaline concentration, but this was ameliorated by carvedilol administration in T1DM (A) and T2DM (B) models. Carvedilol at 1 mg/kg and 10 mg/kg significantly reduced noradrenaline concentration in T1DM model. All doses were effective for reducing noradrenaline concentration in T2DM model. Values are expressed as mean $\pm$ standard error of mean (n=8). * $p < 0.05$ in DCAN groups vs. control; # $p < 0.05$ compared with untreated group; + $p < 0.05$ compared among varying carvedilol doses (statistically significant at $p < 0.05$).

Key

Control: Without intervention; C+W: Control (+10 ml/kg normal saline)

DCAN PI: DM induction pre-intervention,

DCAN 1A: DM 1 (+0.1 mg/kg carvedilol), DCAN 1B: DM 1(+1 mg/kg carvedilol),

DCAN 1C: DM 1 (+10 mg/kg carvedilol), DCAN 1D: DM 1 (+10 ml/kg normal saline)

DCAN 2A: DM 2 (+0.1 mg/kg carvedilol), DCAN 2B: DM 2(+1 mg/kg carvedilol)

DCAN 2C: DM 2 (+10 mg/kg carvedilol), DCAN 2D: DM 2 (+10 ml/kg normal saline)

DM, diabetes mellitus; DCAN, diabetic cardiac autonomic neuropathy; T1DM, diabetes mellitus; T2DM, type 2 diabetes mellitus

4.4.7. Effect of DCAN and carvedilol on nerve growth factor

The level of NGF was observed to be significantly reduced in the DCAN group for both T1DM (120.56(83.27-160.58) vs. 270.16(111.18-318.34) pg/ml and T2DM (150.56(89.27-169.58) vs. 234.16(91.18-311.34) pg/ml models when compared to the respective non-diabetic control groups. Carvedilol had a significant trophic effect on NGF expression, thus increasing its concentration at all doses administered both in the T1DM and T2DM models (figure 4.16).

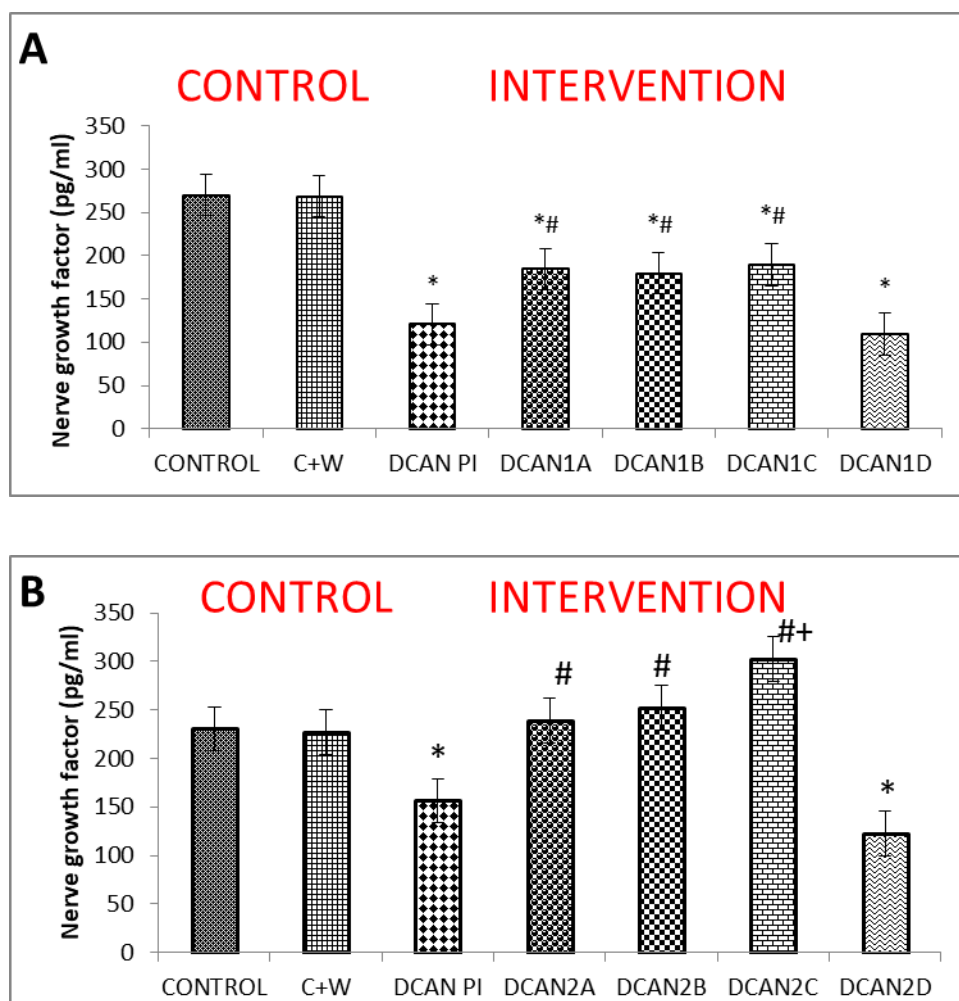


Figure 4.16: Carvedilol increased nerve growth factor in T1DM (A) and T2DM (B) models. Carvedilol administration significantly increased the concentration of nerve growth factor at all doses administered both in the T1DM and T2DM models. Values are expressed as median (range) (n=8).* * p <0.05 in DCAN groups vs. control; # p <0.05 compared with untreated group; + p < 0.05 compared among varying carvedilol doses (statistically significant at p<0.05).

Key

Control: Without intervention; C+W: Control (+10 ml/kg normal saline)

DCAN PI: DM induction pre-intervention,

DCAN 1A: DM 1 (+0.1 mg/kg carvedilol), DCAN 1B: DM 1(+1 mg/kg carvedilol),

DCAN 1C: DM 1 (+10 mg/kg carvedilol), DCAN 1D: DM 1 (+10 ml/kg normal saline)

DCAN 2A: DM 2 (+0.1 mg/kg carvedilol), DCAN 2B: DM 2(+1 mg/kg carvedilol)

DCAN 2C: DM 2 (+10 mg/kg carvedilol), DCAN 2D: DM 2 (+10 ml/kg normal saline)

DM, diabetes mellitus; DCAN, diabetic cardiac autonomic neuropathy; T1DM, type 1 diabetes mellitus; T2DM, type 2 diabetes mellitus

4.4.8. Effect of DCAN and carvedilol on Choline acetyltransferase

DCAN induction significantly reduced the level of choline acetyltransferase (choactase), which is a nerve protein marker of parasympathetic function, in both T1DM (0.8 ± 0.03 ng/ml) and T2DM (0.9 ± 0.02 ng/ml) model groups compared with the respective control groups (1.2 ± 0.05 ng/ml and 1.2 ± 0.03 ng/ml). However, following administration of varying doses of carvedilol, there was a significant increase in choactase level across all interventional sub-groups in both T1DM and T2DM DCAN models (figure 4.17).

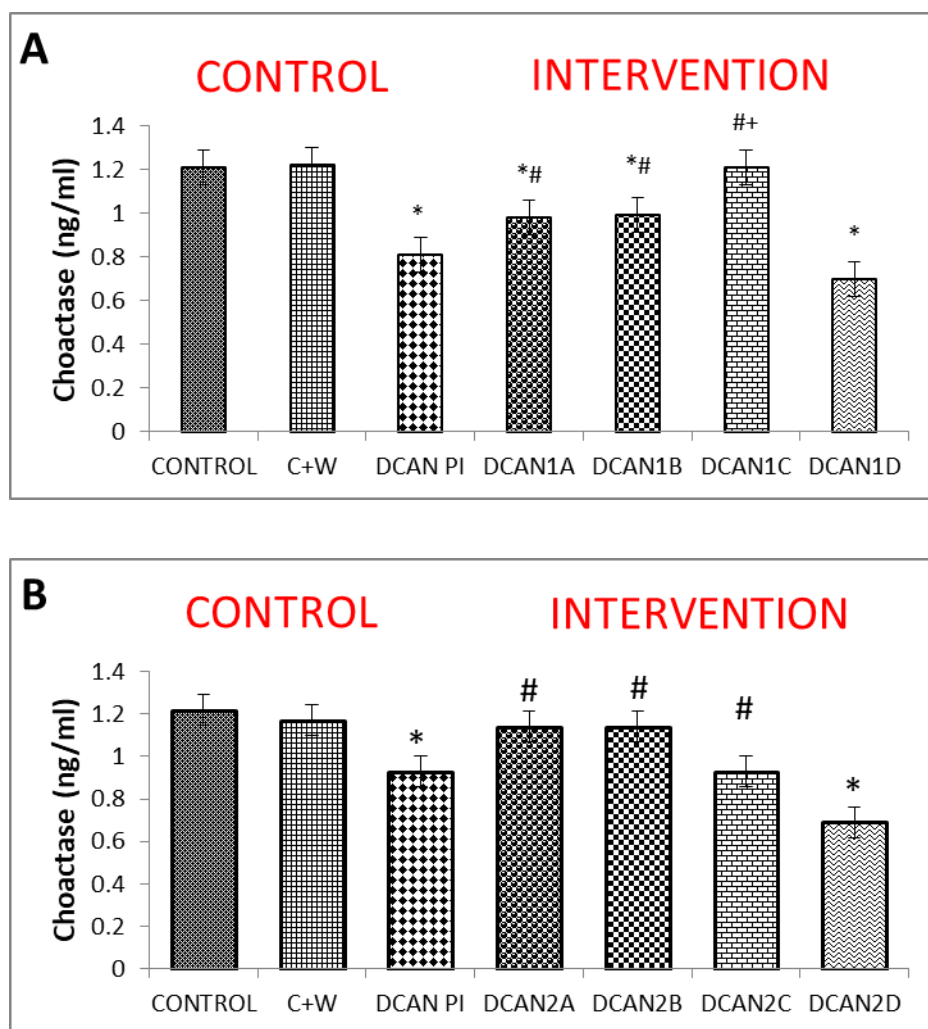


Figure 4.17: Carvedilol improved the expression of choline acetyl transferase (choactase) level in both T1DM (A) and T2DM (B) models. Values are expressed as mean $\pm$ standard error of mean (n=8). * $p < 0.05$ in DCAN groups vs. control; # $p < 0.05$ compared with untreated group; + $p < 0.05$ compared among varying carvedilol doses (statistically significant at $p < 0.05$).

Key

Control: Without intervention; C+W: Control (+10 ml/kg normal saline)

DCAN PI: DM induction pre-intervention,

DCAN 1A: DM 1 (+0.1 mg/kg carvedilol), DCAN 1B: DM 1(+1 mg/kg carvedilol),

DCAN 1C: DM 1 (+10 mg/kg carvedilol), DCAN 1D: DM 1 (+10 ml/kg normal saline)

DCAN 2A: DM 2 (+0.1 mg/kg carvedilol), DCAN 2B: DM 2(+1 mg/kg carvedilol)

DCAN 2C: DM 2 (+10 mg/kg carvedilol), DCAN 2D: DM 2 (+10 ml/kg normal saline)

DM, diabetes mellitus; DCAN, diabetic cardiac autonomic neuropathy; T1DM, type 1 diabetes mellitus; T2DM, type 2 diabetes mellitus

4.5. Oral and Intra-peritoneal glucose tolerance test

At the end of the study, both oral and intraperitoneal glucose tolerance tests were done to observe and compare the different routes of glucose administration. There was an immediate increase in the blood glucose level during OGTT in the DCAN and control groups for T2DM models. There was significant impairment of glucose handling at 30 min post-glucose ingestion and further after that for the subgroup that received no carvedilol (DCAN 2D) and the subgroup given 0.1 mg/kg carvedilol (DCAN 2A). Blood sugar level decreased at the 60, 90, and 120 min time points in the control group, unlike in the DCAN subgroups (Figure 4.18). Moreover, the DCAN subgroups had a glucose tolerance defect that could be a sign of impaired absorption and response to glucose (which further corroborates DM induction). The intraperitoneal administration of glucose also showed significant impairment in glucose homeostasis; however, more derangement was observed than when glucose was administered through the oral route.

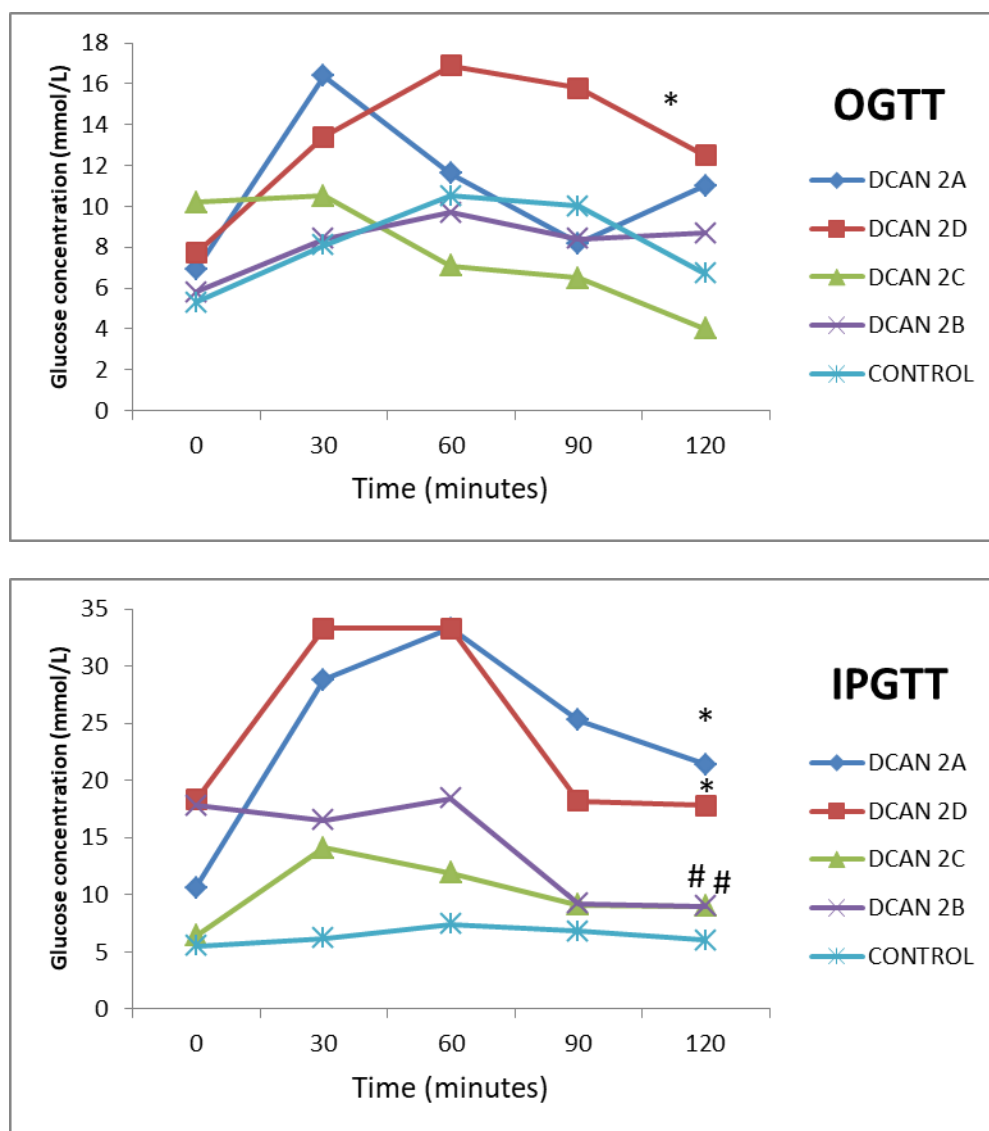


Figure 4.18: Oral and intra-peritoneal glucose tolerance tests profiles in T2DM model. Carvedilol improved glucose tolerance with more effects in the oral than the intra-peritoneal test. Values are expressed as mean $\pm$ standard error of mean (n=8). * $p < 0.05$ compared with control; # $p < 0.05$ compared with diabetic control.

IPGTT, intra-peritoneal glucose tolerance test; OGTT, oral glucose tolerance test; DM, diabetes mellitus; DCAN, diabetic cardiac autonomic neuropathy; T2DM, type 2 diabetes mellitus

Key

Control: Control (+10 ml/kg normal saline)

DCAN 2A: DM 2 (+0.1 mg/kg carvedilol); DCAN 2B: DM 2 (+1 mg/kg carvedilol)

DCAN 2C: DM 2 (+10 mg/kg carvedilol); DCAN 2D: DM 2 (+10 ml/kg normal saline)

4.6 Effect of DCAN and carvedilol on heart rate variability indices

The evaluation of autonomic function using indices of HRV (frequency and time-domain spectra) showed a similar pattern with the biochemical laboratory markers. DCAN induction impaired both low frequency (LF) and high frequency (HF) activities.

4.6.1 Effect of DCAN and carvedilol on low and high frequency spectral activity:

Induction of DCAN significantly increased LF activity in the T1DM model ($13,716.6 \pm 237.1$ ms*ms) compared with the non-diabetic control group ($10,075.2 \pm 152.9$ ms*ms). A similar pattern was observed for the T2DM ($10,720.3 \pm 198.8$ ms*ms) model compared to the non-diabetic control group ($7,090.5 \pm 105.2$ ms*ms). Following carvedilol administration, there was no significant improvement in the LF spectral across all the different doses, as shown in Figure 4.19. The diabetic non-interventional group for T2DM had higher LF spectral at the end of the intervention.

The reverse was seen for the HF domain, in which DCAN induction significantly decreased the HF spectral for both T1DM ($9,525.8 \pm 122.4$ vs. $17,981 \pm 276.2$ ms*ms) and T2DM ($5,400.6 \pm 103.3$ vs. $12,083.9 \pm 199.2$ ms*ms) models as compared to their respective non-diabetic control groups. Following administration of carvedilol for 28 days, at all interventional doses of carvedilol in the T1DM models, there was a significant improvement in the HF activity, while only the subgroups that received 1 mg/kg and 10 mg/kg of carvedilol in the T2DM model had significant improvement in their HF domain activities as shown in Figure 4.20.

DCAN induction also increased the low frequency to high frequency (LF/HF) ratio in both the T1DM (0.188 ± 0.004) and T2DM (0.139 ± 0.002) models compared to the respective non-diabetic control groups (0.07 ± 0.005 and 0.09 ± 0.005). Carvedilol administration significantly improved the LF/HF ratio across all subgroups in the T2DM model. However, only the

subgroups that had 1 mg/kg and 10 mg/kg administered showed significant improvement in the T1DM model compared to the diabetic control group, as shown in Figure 4.21.

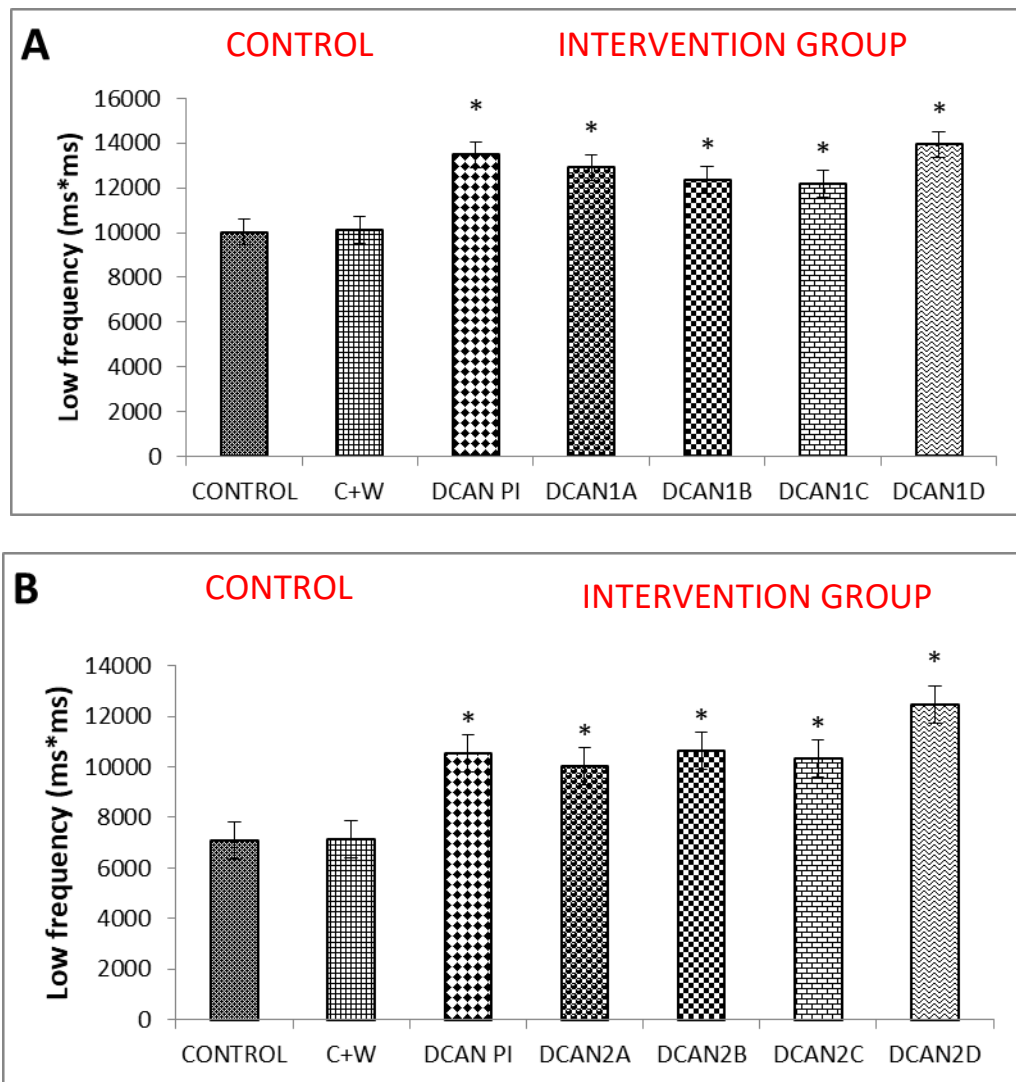


Figure 4.19; Effect of DCAN induction and carvedilol treatment on low frequency activity spectral in T1DM (A) and T2DM (B) models across various dosage groups. Carvedilol did not have significant effect on the low frequency activity spectral. Values are expressed as mean $\pm$ standard error of mean (n=8). * p < 0.05 in DCAN groups vs. control (statistically significant at p < 0.05).

DM, diabetes mellitus; DCAN, diabetic cardiac autonomic neuropathy; T1DM, type 1 diabetes mellitus; T2DM, type 2 diabetes mellitus

Key

Control: Without intervention; C+W: Control (+10 ml/kg normal saline)

DCAN PI: DM induction pre-intervention,

DCAN 1A: DM 1 (+0.1 mg/kg carvedilol), DCAN 1B: DM 1(+1 mg/kg carvedilol),

DCAN 1C: DM 1 (+10 mg/kg carvedilol), DCAN 1D: DM 1 (+10 ml/kg normal saline)

DCAN 2A: DM 2 (+0.1 mg/kg carvedilol), DCAN 2B: DM 2(+1 mg/kg carvedilol)

DCAN 2C: DM 2 (+10 mg/kg carvedilol), DCAN 2D: DM 2 (+10 ml/kg normal saline)

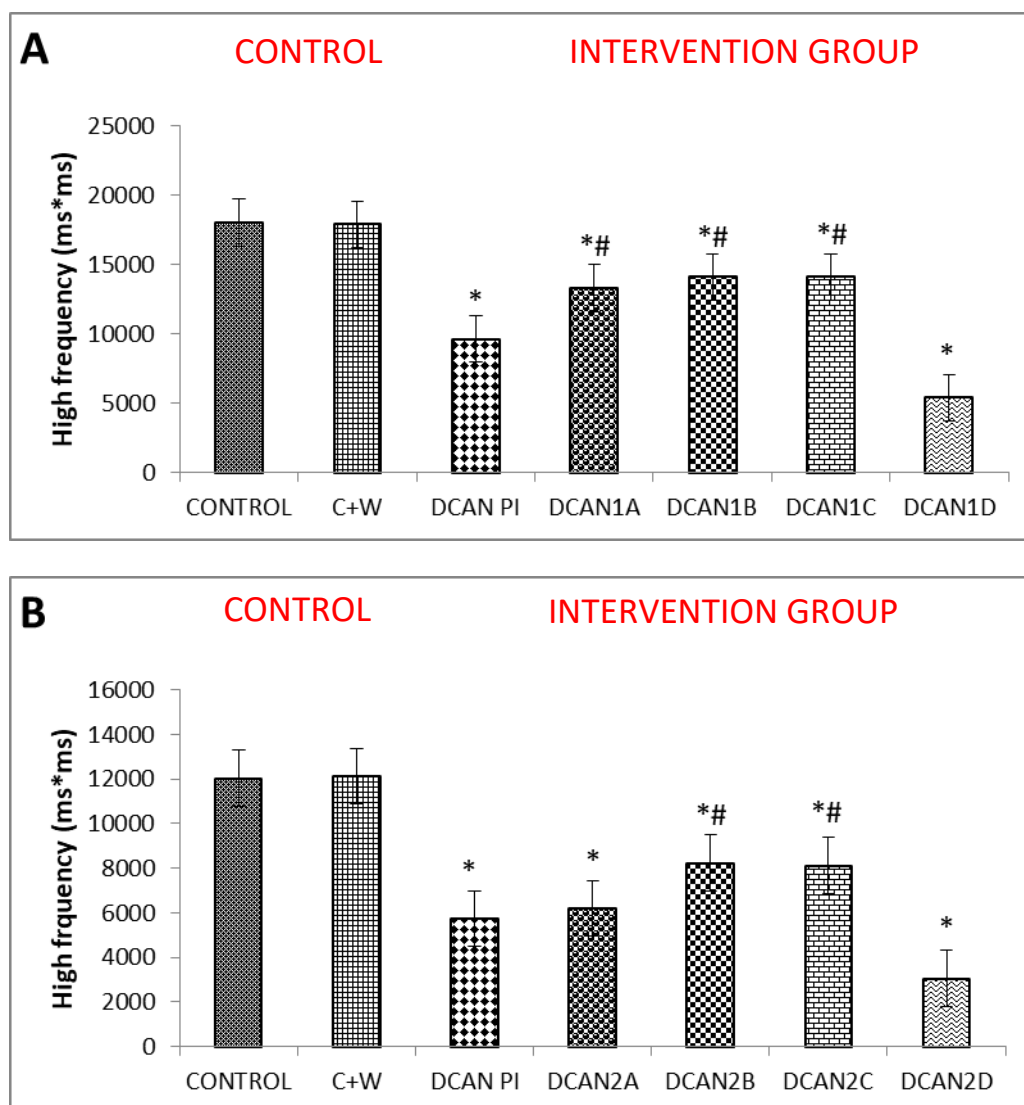


Figure 4.20; Effect of DCAN induction and carvedilol treatment on high frequency spectral activity in T1DM (A) and T2DM (B) models. Carvedilol significantly improved the high frequency activity in both type 1 and type 2 DM models. Values are expressed as mean $\pm$ standard error of mean (n=8). * p <0.05 in DCAN groups vs. control; # p <0.05 compared with untreated group (statistically significant at p<0.05).

DM, diabetes mellitus; DCAN, diabetic cardiac autonomic neuropathy; T1DM, type 1 diabetes mellitus; T2DM, type 2 diabetes mellitus

Key; Control: Without intervention; C+W: Control (+10 ml/kg normal saline)

DCAN PI: DM induction pre-intervention,

DCAN 1A: DM 1 (+0.1 mg/kg carvedilol), DCAN 1B: DM 1(+1 mg/kg carvedilol),

DCAN 1C: DM 1 (+10 mg/kg carvedilol), DCAN 1D: DM 1 (+10 ml/kg normal saline)

DCAN 2A: DM 2 (+0.1 mg/kg carvedilol), DCAN 2B: DM 2(+1 mg/kg carvedilol)

DCAN 2C: DM 2 (+10 mg/kg carvedilol), DCAN 2D: DM 2 (+10 ml/kg normal saline)

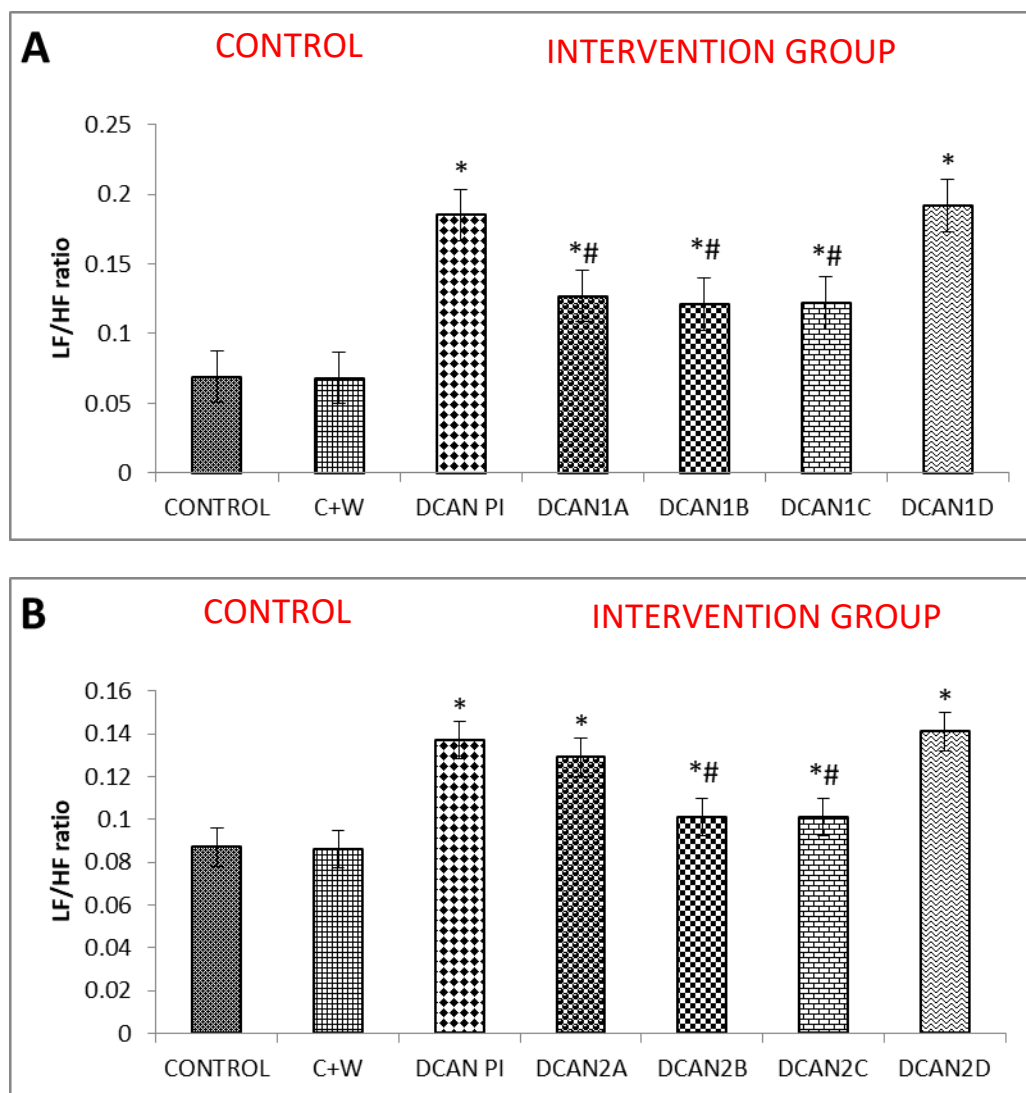


Figure 4.21: Effect of DCAN induction and carvedilol treatment on low frequency/high frequency ratio among T2DM (A) and T1DM (B) models. Carvedilol significantly improved the low/high frequency activity in both T1DM and T2DM models. Values are expressed as mean $\pm$ standard error of mean (n=8). * p <0.05 in DCAN groups vs. control; # p <0.05 compared with untreated group (statistically significant at p<0.05).

DM, diabetes mellitus; DCAN, diabetic cardiac autonomic neuropathy; LF/HF; low frequency/high frequency; T1DM, type 1 diabetes mellitus; T2DM, type 2 diabetes mellitus

Key: Control: Without intervention; C+W: Control (+10 ml/kg normal saline)

DCAN PI: DM induction pre-intervention,

DCAN 1A: DM 1 (+0.1 mg/kg carvedilol), DCAN 1B: DM 1(+1 mg/kg carvedilol),

DCAN 1C: DM 1 (+10 mg/kg carvedilol), DCAN 1D: DM 1 (+10 ml/kg normal saline)

DCAN 2A: DM 2 (+0.1 mg/kg carvedilol), DCAN 2B: DM 2(+1 mg/kg carvedilol)

DCAN 2C: DM 2 (+10 mg/kg carvedilol), DCAN 2D: DM 2 (+10 ml/kg normal saline)

4.6.2. Effect of DCAN and carvedilol on time-domain RR parameters

DCAN induction significantly increased the minimum RR interval in both T1DM (409.6 ± 10.1 ms vs. 302.8 ± 9.2 ms) and T2DM models (410.2 ± 10.1 ms vs. 354.7 ± 9.5 ms) compared to their respective non-diabetic control groups. Carvedilol intervention improved the heart rate across all subgroups of varying doses in T1DM and T2DM groups compared to the diabetic control groups, as shown in Figure 4.22.

Similarly, the maximum RR interval was observed to be increased in the DCAN groups in both T1DM ($1,926.4 \pm 20.7$ ms vs. $1,345.2 \pm 20.1$ ms) and T2DM ($1,906.9 \pm 19.3$ ms vs. $1,434.7 \pm 18.6$ ms) models compared to their respective non-diabetic control groups. Administration of carvedilol was associated with significant improvement across all subgroups in the T1DM model, but only the 1 mg/kg and 10 mg/kg subgroups showed significant improvement in the T2DM model (figure 4.23).

Accordingly, DCAN induction significantly increased the average RR interval in both T1DM (894.1 ± 16.4 ms vs. 689.5 ± 15.5 ms) and T1DM (901.2 ± 17.2 ms vs. 703.8 ± 15.9 ms) models compared to their respective non-diabetic control groups. Carvedilol administration at all the doses showed significant improvement in both DCAN models compared with the DM controls (figure 4.24).

4.6.3. Effect of DCAN and carvedilol on other time-domain parameters

DCAN induction with STZ significantly decreased the mean of the standard deviations of all the NN intervals for each 5 min segment of a 24 h HRV recording (SDNN) in both T1DM (109.5 ± 3.2 ms vs. 289.4 ± 5.1 ms) and T2DM (118.3 ± 3.4 ms vs. 334.2 ± 5.9 ms) models compared to their respective non-diabetic controls. Carvedilol administration at 1 mg/kg and 10 mg/kg

showed significant improvement of SDNN in both T1DM and T2DM DCAN models compared to the diabetic control groups (figure 4.25).

Similarly, STZ-induced DCAN significantly decreased the HRV index in both T1DM (52.3 ± 5.1 ms vs. 91.2 ± 7.6 ms) and T2DM (47.5 ± 5.0 ms vs. 100.8 ± 7.9 ms) models compared to the respective non-diabetic control groups. Carvedilol administration was associated with significant improvement in HRV index across all subgroups in both T1DM and T2DM models compared to the diabetic controls, as shown in Figure 4.26.

The root mean square of successive RR interval differences (RMSSD) was observed to be lower in both T1DM (135.2 ± 6.2 ms vs. 352.8 ± 9.5 ms) and T2DM (172.6 ± 8.1 ms vs. 442.2 ± 10.8 ms) models compared to their respective non-diabetic control groups. Carvedilol administration showed improvement across all groups in the T1DM model, but only the sub-group given 10 mg/kg showed significant improvement in the T2DM model (figure 4.27).

DCAN induction significantly decreased the percentage of successive RR intervals that differed by more than 50 ms (PNN50) in both T1DM ($54.9 \pm 8.1\%$ vs. $78.1 \pm 10.2\%$) and T2DM2 ($62.8 \pm 8.4\%$ vs. $81.6 \pm 10.4\%$) models compared to the respective non-diabetic control groups. Following carvedilol administration at 1 mg/kg and 10 mg/kg, there was a significant improvement in PNN50 in the T1DM model and all subgroups of the T2DM, compared to the diabetic controls as shown in Figure 4.28.

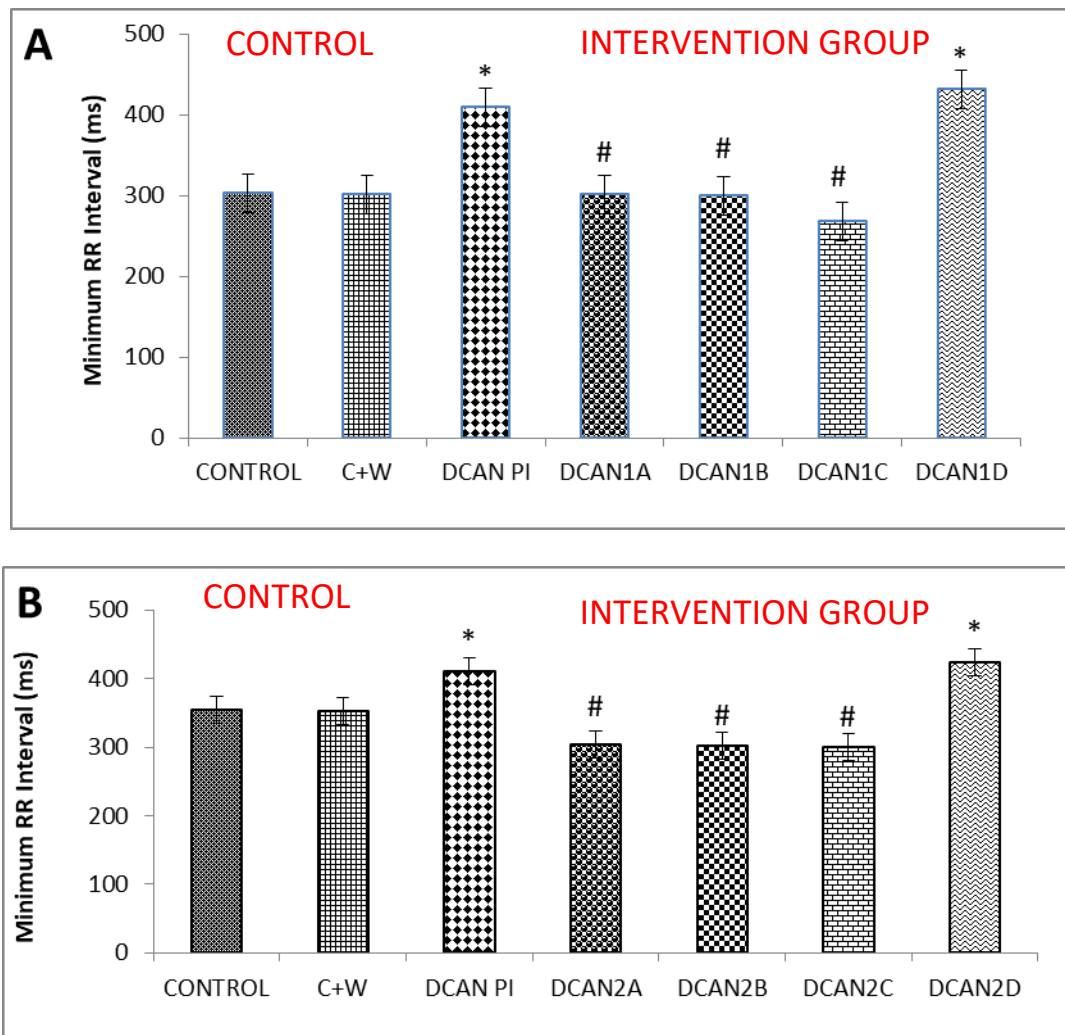


Figure 4.22: Time domain parameters in T1DM and T2DM models. There was significant increase in the minimum RR following DCAN induction in T1DM (A) and T2DM (B) models respectively. Carvedilol significantly improved the time domain parameters in both T1DM and T2DM models. Values are expressed as mean $\pm$ standard (n=8). * $p < 0.05$ in DCAN groups vs. control; # $p < 0.05$ compared with untreated group (statistically significant at $p < 0.05$).

DM, diabetes mellitus; DCAN, diabetic cardiac autonomic neuropathy; T1DM, type 1 diabetes mellitus; T2DM, type 2 diabetes mellitus

Key:

Control: Without intervention; C+W: Control (+10 ml/kg normal saline)

DCAN PI: DM induction pre-intervention,

DCAN 1A: DM 1 (+0.1 mg/kg carvedilol), DCAN 1B: DM 1 (+1 mg/kg carvedilol),

DCAN 1C: DM 1 (+10 mg/kg carvedilol), DCAN 1D: DM 1 (+10 ml/kg normal saline)

DCAN 2A: DM 2 (+0.1 mg/kg carvedilol), DCAN 2B: DM 2 (+1 mg/kg carvedilol)

DCAN 2C: DM 2 (+10 mg/kg carvedilol), DCAN 2D: DM 2 (+10 ml/kg normal saline)

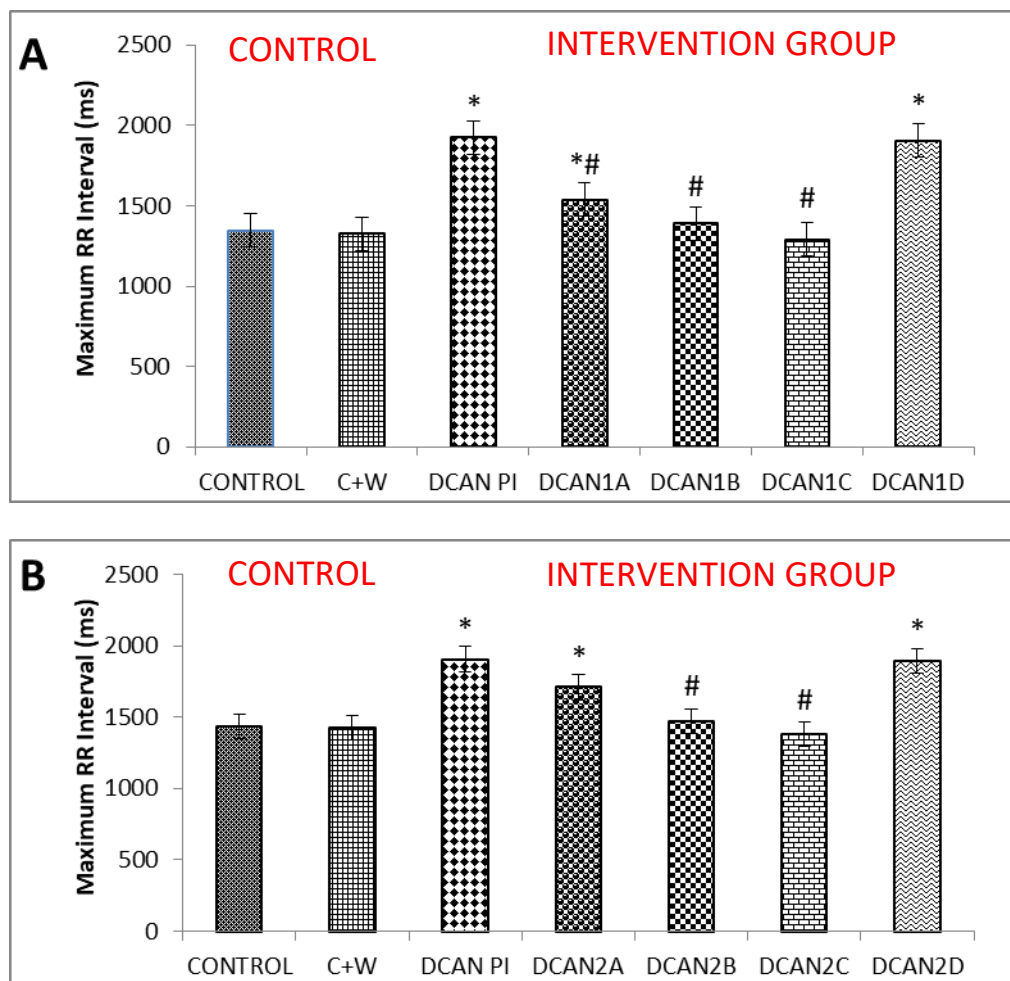


Figure 4.23: Time domain parameters in T1DM and T2DM models. There was significant increase in the maximum RR following DCAN induction in T1DM (A) and T2DM (B) models respectively. Carvedilol significantly improved the time domain parameters in both T1DM and T2DM models. Values are expressed as mean $\pm$ standard (n=8). * $p < 0.05$ in DCAN groups vs. control; # $p < 0.05$ compared with untreated group (statistically significant at $p < 0.05$).

DM, diabetes mellitus; DCAN, diabetic cardiac autonomic neuropathy; T1DM, type 1 diabetes mellitus; T2DM, type 2 diabetes mellitus

Key:

Control: Without intervention; C+W: Control (+10 ml/kg normal saline)

DCAN PI: DM induction pre-intervention,

DCAN 1A: DM 1 (+0.1 mg/kg carvedilol), DCAN 1B: DM 1 (+1 mg/kg carvedilol),

DCAN 1C: DM 1 (+10 mg/kg carvedilol), DCAN 1D: DM 1 (+10 ml/kg normal saline)

DCAN 2A: DM 2 (+0.1 mg/kg carvedilol), DCAN 2B: DM 2 (+1 mg/kg carvedilol)

DCAN 2C: DM 2 (+10 mg/kg carvedilol), DCAN 2D: DM 2 (+10 ml/kg normal saline)

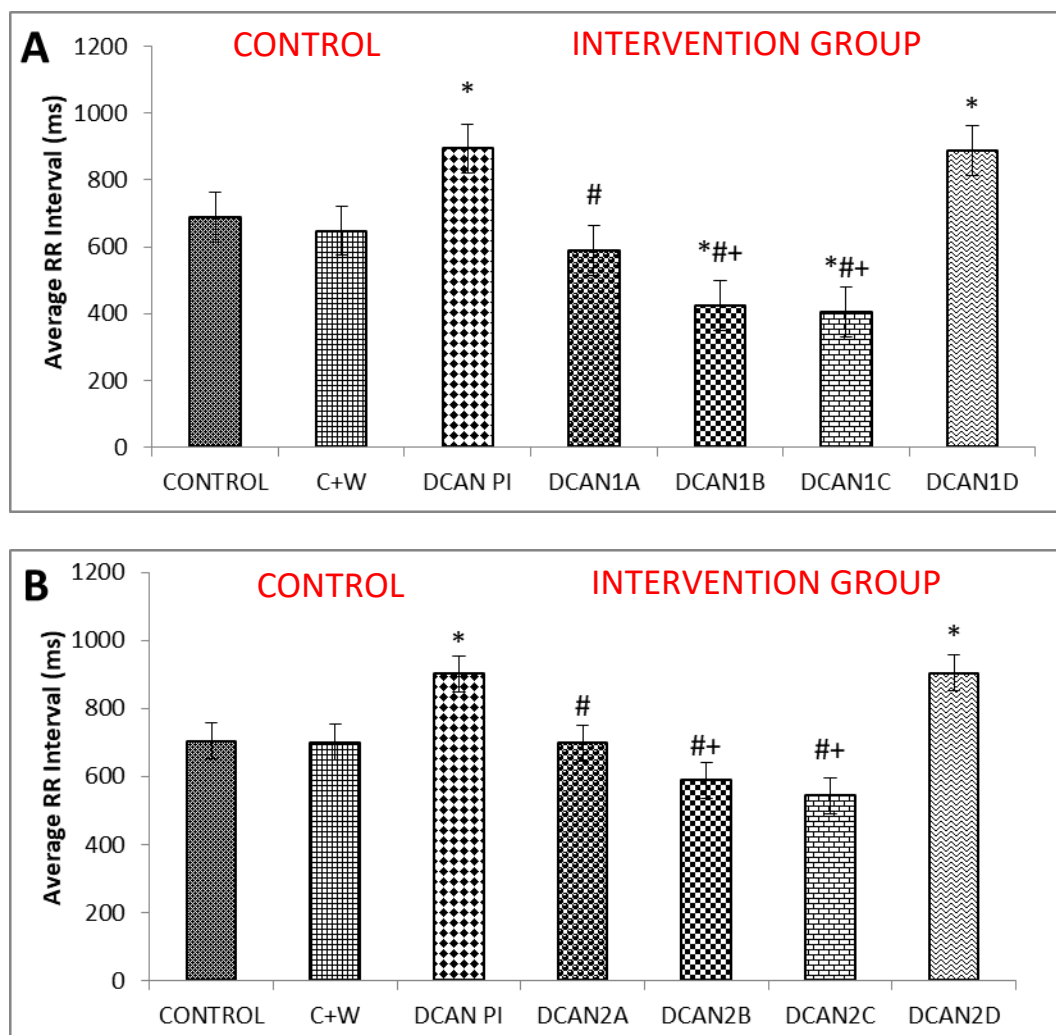


Figure 4.24: Time domain parameters in T1DM and T2DM models. There was significant increase in the average RR intervals following DCAN induction in T1DM (A) and T2DM (B) models respectively. Carvedilol significantly improved the time domain parameters in both T1DM and T2DM models. Values are expressed as mean $\pm$ standard (n=8). * $p < 0.05$ in DCAN groups vs. control; # $p < 0.05$ compared with untreated group; + $p < 0.05$ compared among varying carvedilol doses (statistically significant at $p < 0.05$).

DM, diabetes mellitus; DCAN, diabetic cardiac autonomic neuropathy; T1DM, type 1 diabetes mellitus; T2DM, type 2 diabetes mellitus

Key; Control: Without intervention; C+W: Control (+10 ml/kg normal saline)

DCAN PI: DM induction pre-intervention,

DCAN 1A: DM 1 (+0.1 mg/kg carvedilol), DCAN 1B: DM 1 (+1 mg/kg carvedilol),

DCAN 1C: DM 1 (+10 mg/kg carvedilol), DCAN 1D: DM 1 (+10 ml/kg normal saline)

DCAN 2A: DM 2 (+0.1 mg/kg carvedilol), DCAN 2B: DM 2 (+1 mg/kg carvedilol)

DCAN 2C: DM 2 (+10 mg/kg carvedilol), DCAN 2D: DM 2 (+10 ml/kg normal saline)

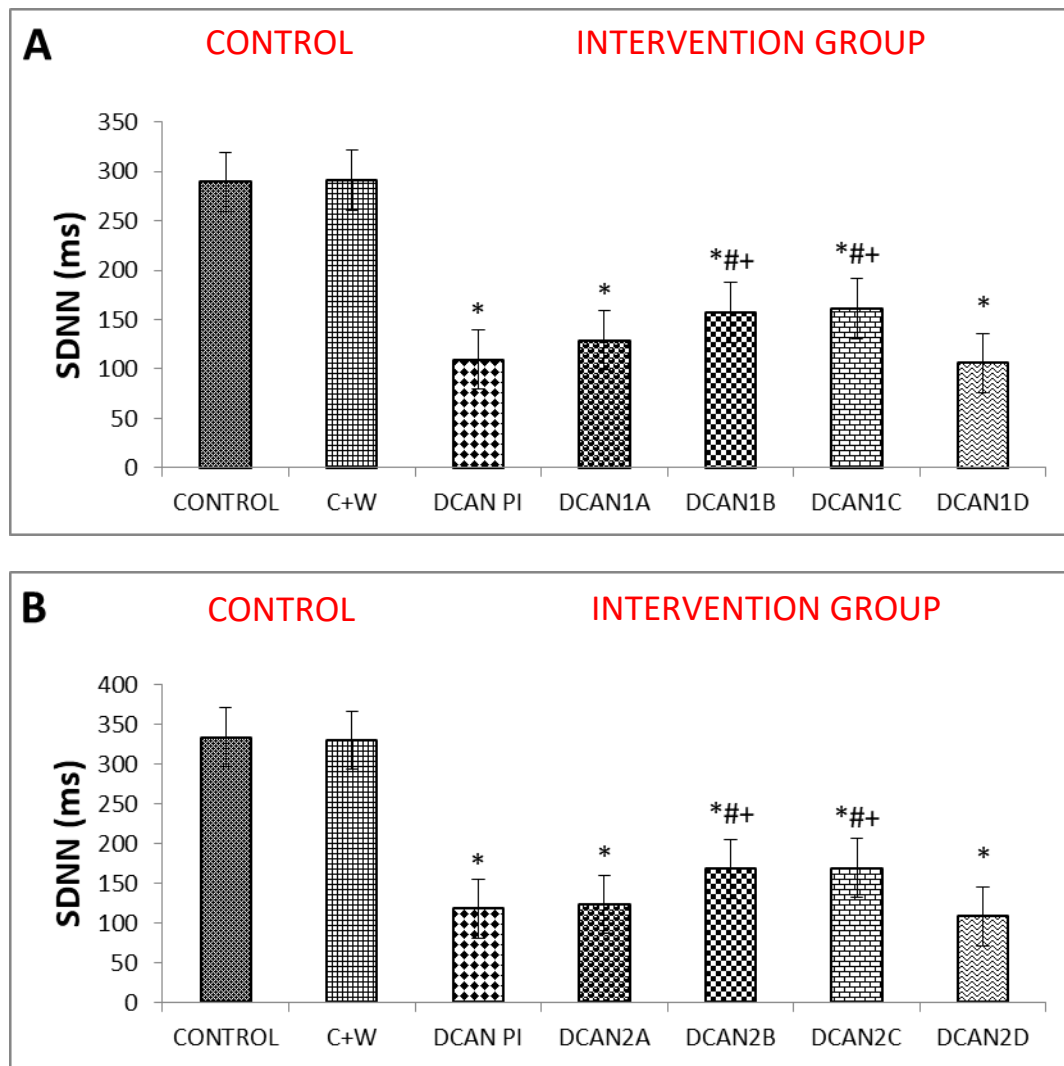


Figure 4.25: Time domain parameters in T1DM (A) and T2DM (B) DCAN models. Carvedilol significantly improved the SDNN parameters in both T1DM and T2DM models. Values are expressed as mean $\pm$ standard error of mean (n=8). * $p < 0.05$ in DCAN groups vs. control; # $p < 0.05$ compared with untreated group; + $p < 0.05$ compared among varying carvedilol doses (statistically significant at $p < 0.05$).

Key:

Control: Without intervention; C+W: Control (+10 ml/kg normal saline)

DCAN PI: DM induction pre-intervention,

DCAN 1A: DM 1 (+0.1 mg/kg carvedilol), DCAN 1B: DM 1 (+1 mg/kg carvedilol),

DCAN 1C: DM 1 (+10 mg/kg carvedilol), DCAN 1D: DM 1 (+10 ml/kg normal saline)

DCAN 2A: DM 2 (+0.1 mg/kg carvedilol), DCAN 2B: DM 2 (+1 mg/kg carvedilol)

DCAN 2C: DM 2 (+10 mg/kg carvedilol), DCAN 2D: DM 2 (+10 ml/kg normal saline)

DCAN, diabetic cardiac autonomic neuropathy; T1DM, type 1 diabetes mellitus; T2DM, type 2 diabetes mellitus; SDNN index (SDNNI), mean of the standard deviations of all the NN intervals for each 5 min segment of a 24 h HRV recording.

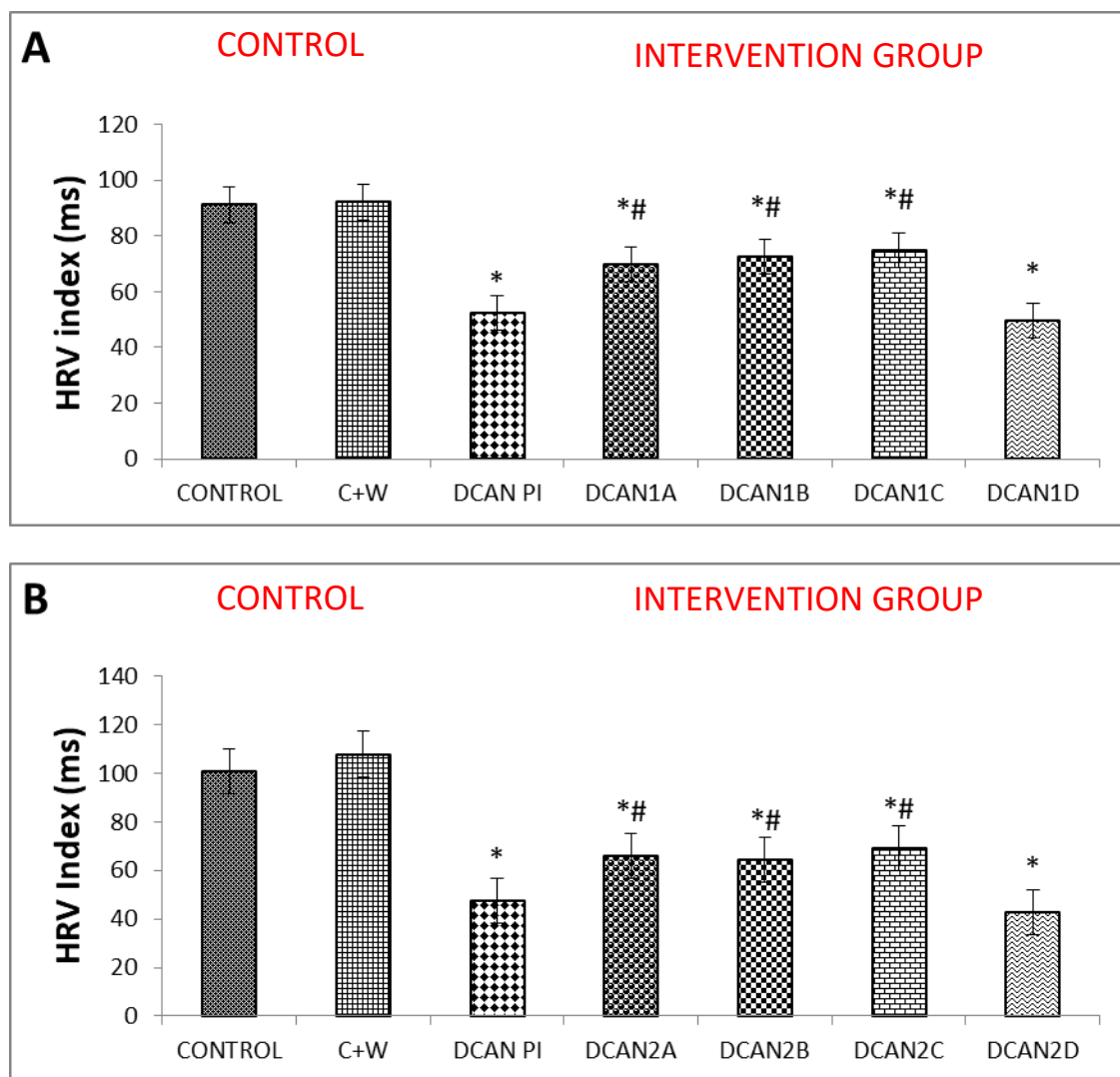


Figure 4.26: Time domain parameters in T1DM (A) and T2DM (B) DCAN models. Carvedilol significantly improved the HRVindex parameters in both T1DM and T2DM models. Values are expressed as mean $\pm$ standard error of mean (n=8). * p <0.05 in DCAN groups vs. control; # p <0.05 compared with untreated group (statistically significant at p<0.05).

Key:

Control: Without intervention; C+W: Control (+10 ml/kg normal saline)

DCAN PI: DM induction pre-intervention,

DCAN 1A: DM 1 (+0.1 mg/kg carvedilol), DCAN 1B: DM 1(+1 mg/kg carvedilol),

DCAN 1C: DM 1 (+10 mg/kg carvedilol), DCAN 1D: DM 1 (+10 ml/kg normal saline)

DCAN 2A: DM 2 (+0.1 mg/kg carvedilol), DCAN 2B: DM 2(+1 mg/kg carvedilol)

DCAN 2C: DM 2 (+10 mg/kg carvedilol), DCAN 2D: DM 2 (+10 ml/kg normal saline)

DCAN, diabetic cardiac autonomic neuropathy; T1DM, type 1 diabetes mellitus; T2DM, type 2 diabetes mellitus; HRV index, Integral of the density of the RR interval histogram divided by its height

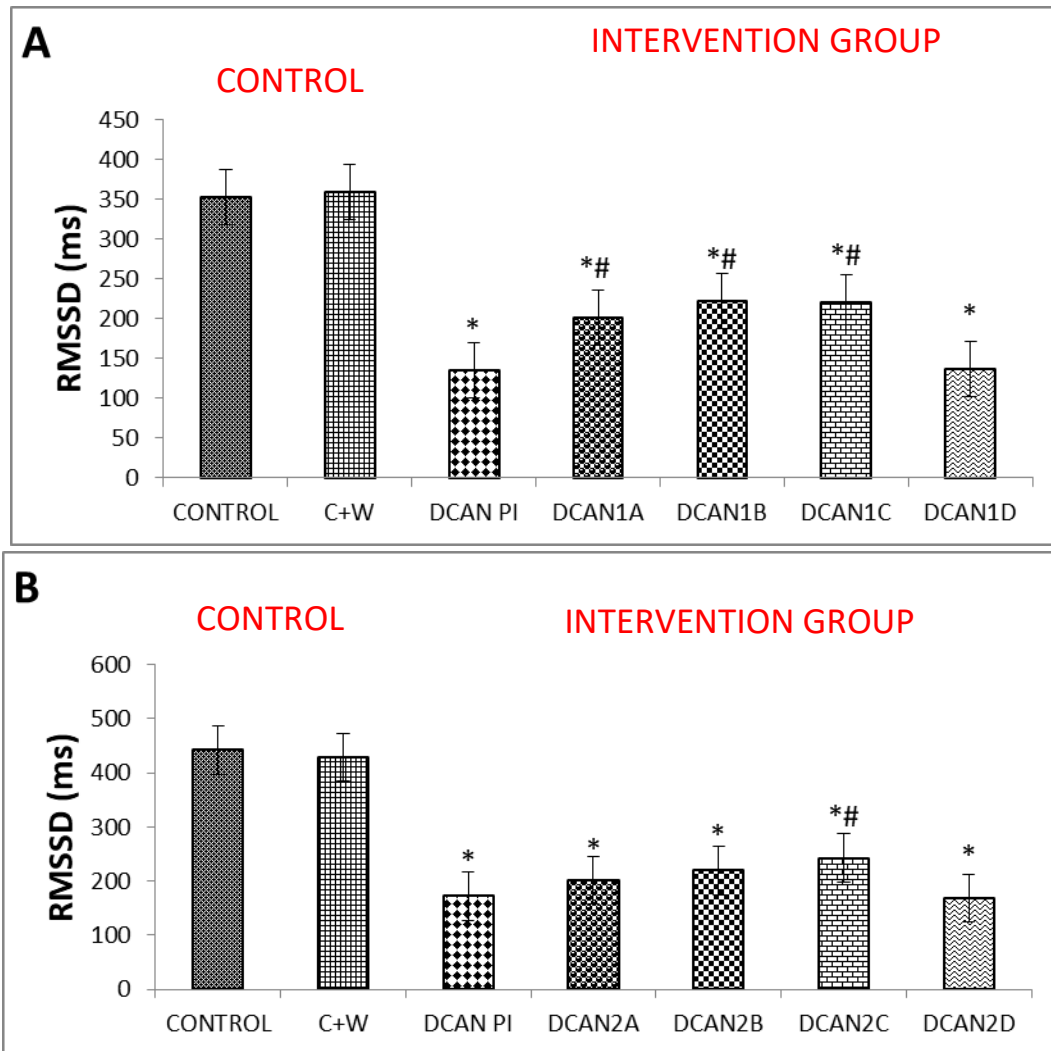


Figure 4.27: T1DM (A) and T2DM (B) DCAN time domain parameters. Carvedilol significantly improved the RMSSD domain parameters in both T1DM and T2DM models. Values are expressed as mean $\pm$ standard error of mean (n=8). * $p < 0.05$ in DCAN groups vs. control; # $p < 0.05$ compared with untreated group (statistically significant at $p < 0.05$).

Key:

Control: Without intervention; C+W: Control (+10 ml/kg normal saline)

DCAN PI: DM induction pre-intervention,

DCAN 1A: DM 1 (+0.1 mg/kg carvedilol), DCAN 1B: DM 1(+1 mg/kg carvedilol),

DCAN 1C: DM 1 (+10 mg/kg carvedilol), DCAN 1D: DM 1 (+10 ml/kg normal saline)

DCAN 2A: DM 2 (+0.1 mg/kg carvedilol), DCAN 2B: DM 2(+1 mg/kg carvedilol)

DCAN 2C: DM 2 (+10 mg/kg carvedilol), DCAN 2D: DM 2 (+10 ml/kg normal saline)

RMSSD, root mean square of successive RR interval differences; DCAN, diabetic cardiac autonomic neuropathy; T1DM, type 1 diabetes mellitus; T2DM, type 2 diabetes mellitus.

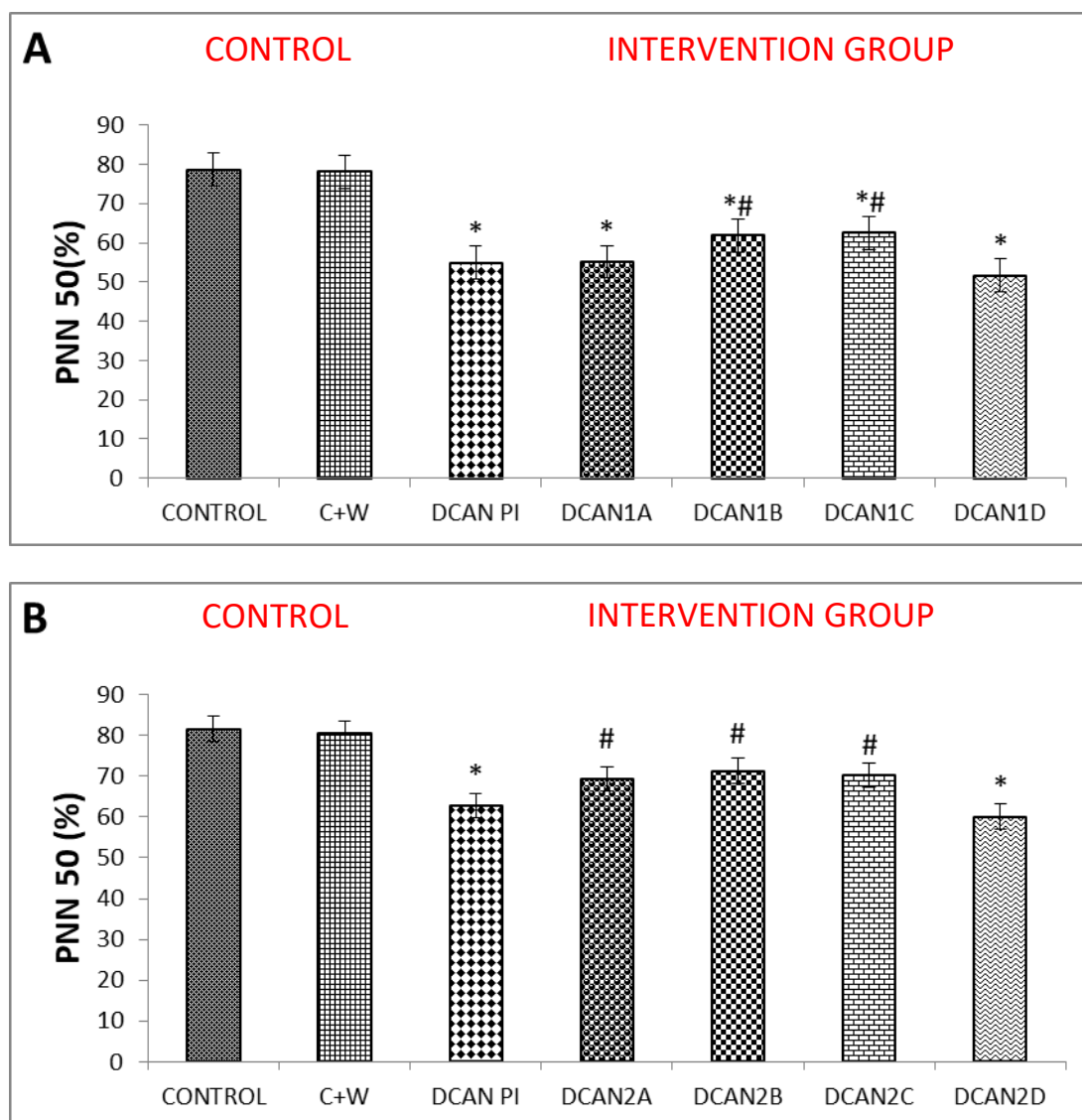


Figure 4.28: T1DM (A) and T2DM (B) DCAN time domain parameters. Carvedilol significantly improved the PNN50 domain parameters in both T1DM and T2DM models. Values are expressed as mean $\pm$ standard error of mean (n=8). * $p < 0.05$ in DCAN groups vs. control; # $p < 0.05$ compared with untreated group (statistically significant at $p < 0.05$).

Key; Control: Without intervention; C+W: Control (+10 ml/kg normal saline)

DCAN PI: DM induction pre-intervention,

DCAN 1A: DM 1 (+0.1 mg/kg carvedilol), DCAN 1B: DM 1(+1 mg/kg carvedilol),

DCAN 1C: DM 1 (+10 mg/kg carvedilol), DCAN 1D: DM 1 (+10 ml/kg normal saline)

DCAN 2A: DM 2 (+0.1 mg/kg carvedilol), DCAN 2B: DM 2(+1 mg/kg carvedilol)

DCAN 2C: DM 2 (+10 mg/kg carvedilol), DCAN 2D: DM 2 (+10 ml/kg normal saline)

PNN50, Percentage of successive RR intervals that differ by more than 50 ms; DCAN, diabetic cardiac autonomic neuropathy; T1DM, type 1 diabetes mellitus; T2DM, type 2 diabetes mellitus

4.7.1 Effect of DCAN on Histology of Pancreas

Histology sections of the pancreas using hematoxylin and eosin (H&E) stain showed normal pancreatic islet cells with deeply staining nuclei and normal-sized blood vessels and pancreatic ducts in the non-diabetic control group. The exocrine pancreas also showed normal glandular cells with deeply staining nuclei. The T1DM model specimens had significant shrinkage of the islet of Langerhans cells with degeneration of the beta-cell nuclei. This is in contrast to the T2DM model specimens, which showed oedematous pancreatic tissue with the widening of the inter-glandular spaces and engorged blood vessels with less shrinkage of the islets of Langerhans (Figure 4.29).

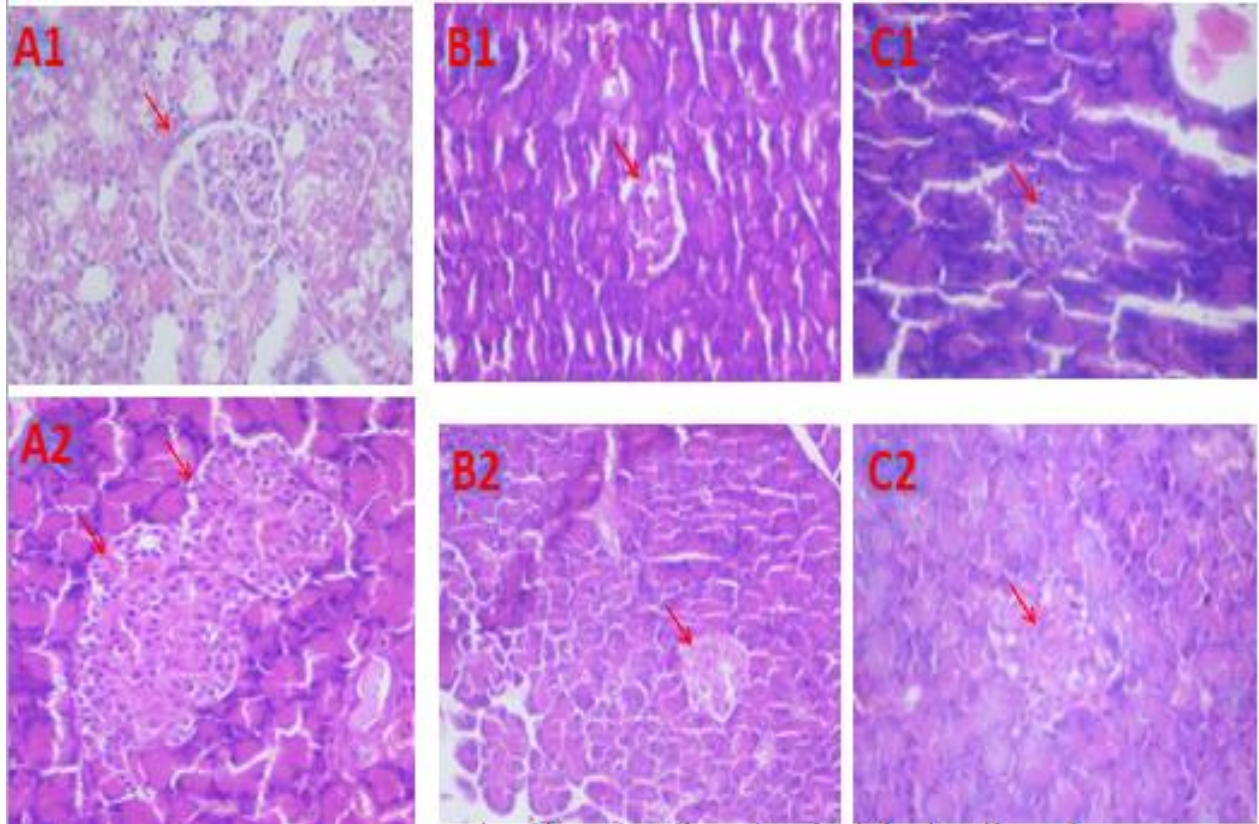


Figure 4.29: Photomicrographs of sections of rat pancreas stained with hematoxylin and eosin
A1&2= Control groups with normal glandular cells and pancreatic architecture; B1&2=T2DM group with shrinkage of the islet cells; C1&2=T1DM group with markedly shrunk islets cells, degeneration of B cell nuclei with edema and haemorrhage.

Red arrows= pancreatic islet cells

4.7.2 Effect of DCAN on kidney histology

Histology sections of the non-diabetic control group kidney using H&E stain showed well-demarcated cortex and medulla, normal-looking glomeruli, Bowman's capsule, Bowman's space, proximal and distal renal tubules, and blood vessels, with deeply staining nuclei of the glomeruli and tubular epithelium. All these were in keeping with normal renal morphology, as shown in Figure 4.30.

The section of specimens from the DCAN groups showed varying degree of renal parenchymal damage, including dilatation of the glomeruli, loss of Bowman's space, presence of pale-staining nuclei of the glomeruli and tubular epithelium, and dilatation of the proximal and distal tubules, all of which are features in keeping with renal injury. There were no significant morphological differences seen after 28 days of carvedilol intervention. Specimens from the DCAN diabetic control group also showed loss of normal kidney morphology evidenced by the crumpling of the glomeruli, presence of pale-staining nuclei of the glomeruli and tubular epithelium, dilatation of the Bowman's space, and dilatation of the proximal and distal tubules—features which were in keeping with renal injury.

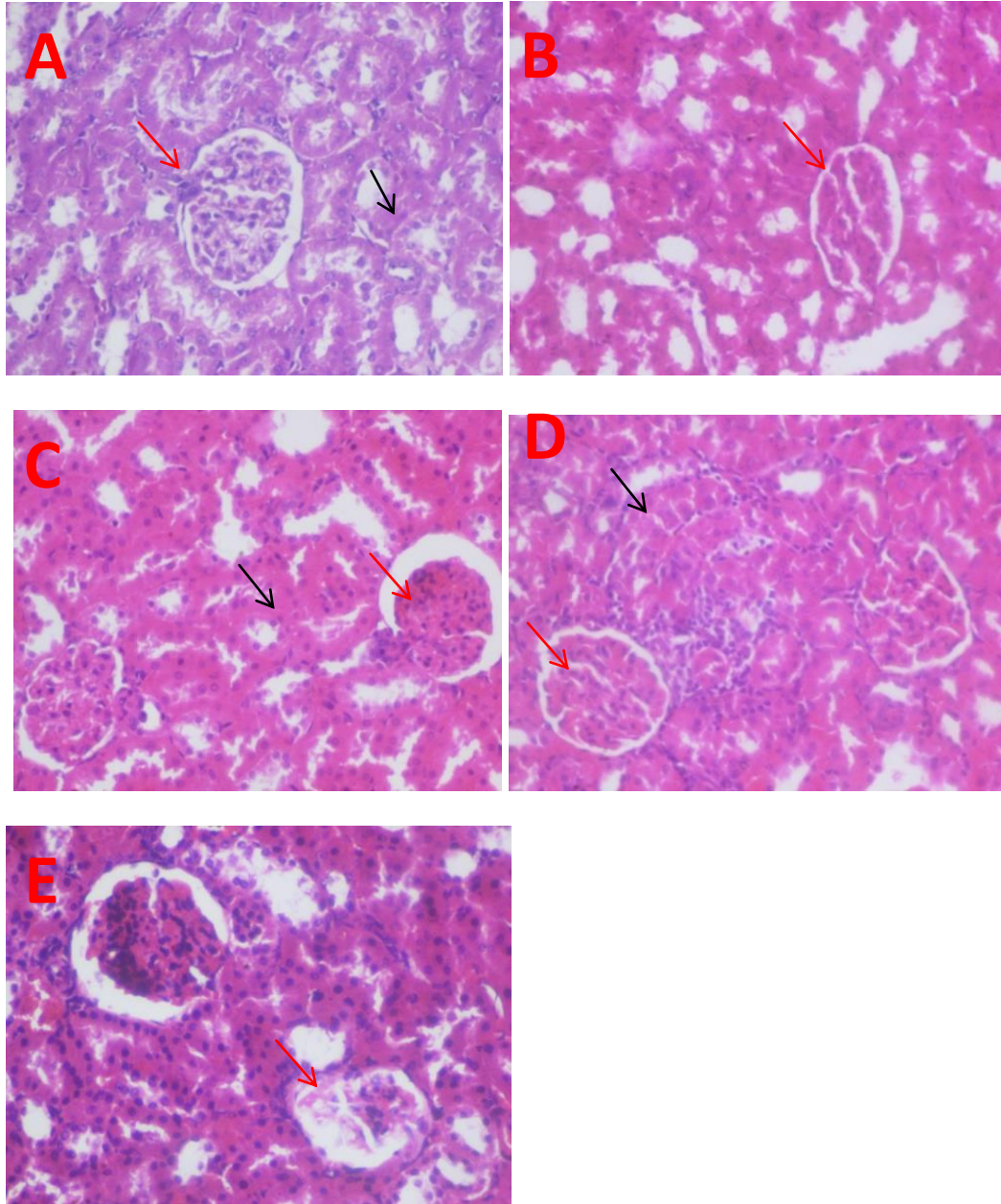


Figure 4.30: Photomicrographs of sections of the rat kidney stained with hematoxylin and eosin. The slides show normal renal morphology in the non-diabetic control group and varying degree of renal injury in the DCAN subgroups.

Key: A= Control specimen showing normal renal parenchymal outline

B= DCAN 2B specimen showing loss of Bowman's space

C=DCAN 2C specimen showing dilated glomeruli

D=DCAN 2A specimen showing dilated proximal and distal tubules

E= DCAN 2D specimen showing crumpling glomeruli, and dilated tubules and Bowman's space

Red arrows= glomerulus; Black arrows=tubules

DCAN, diabetic cardiac autonomic neuropathy.

4.8. Effect of DCAN and carvedilol on the heart

4.8.1. Cardiac histology

Histological sections of the atrial/SA region of the heart using H&E stain showed a normal cardiac muscle fibres arrangement with deeply staining basophilic nuclei in the non-diabetic control group. However, sections from the DCAN group showed varying patterns ranging from swelling of cardiac tissue, increased cellularity, graded to complete loss of normal cardiac morphology, to outright disorganisation of cardiac muscle architecture with degenerating myocyte nuclei and increased cellularity, as shown in Figures 4.31 and 4.32.

Beilchowsky histochemical stain of specimens from the non-diabetic control group showed normal cardiac tissue, with the axons innervating the intercalated discs staining black. Cardiac connective tissue stained various shades of grey; nuclei were round- to oval-shaped and stained grey to black. Sections from the DCAN group showed varying degrees of axonal dystrophy characterized by excessive vascular tissue reaction, disorganization of the cardiac tissue, inflammatory cells, clumping together of nerve fibres, and thickening of the nerves and axons that supplied the intercalated discs, as shown in Figures 4.33 and 4.34.

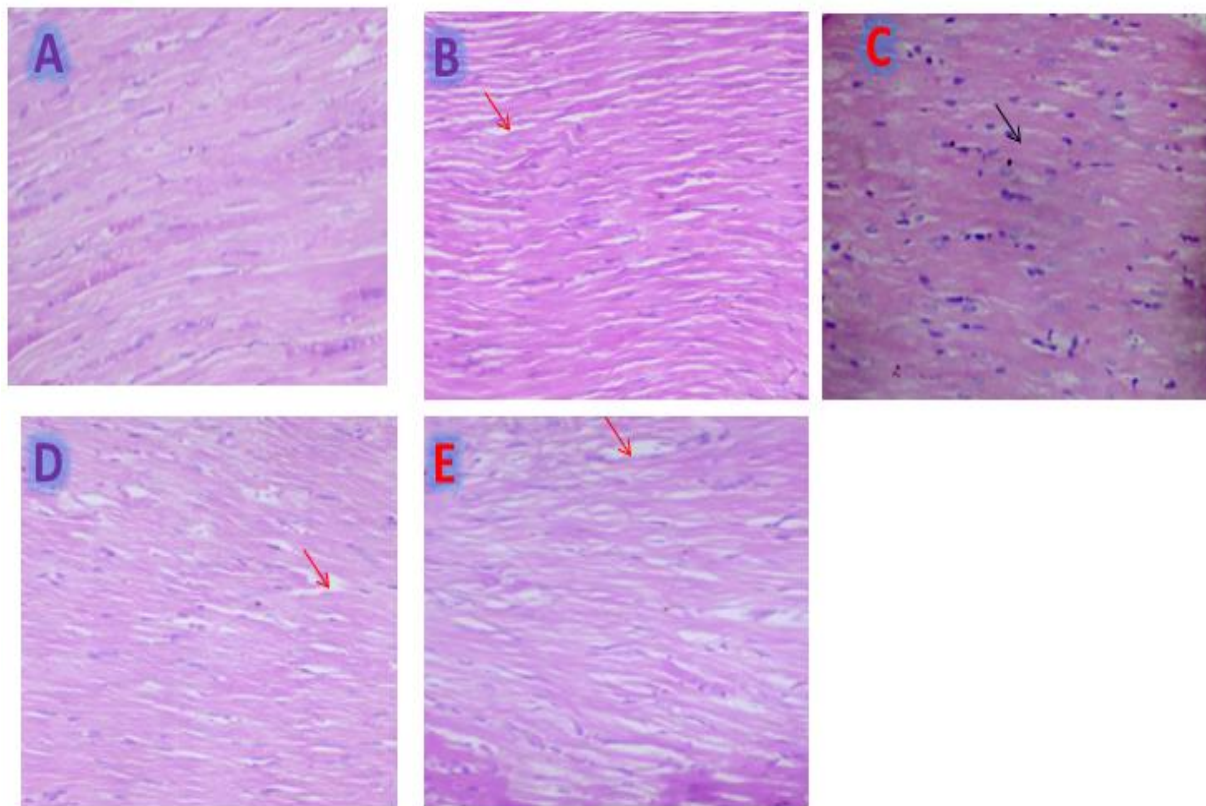


Figure 4.31: Photomicrographs representative of slides of the atrial/sinoatrial region of the heart stained with hematoxylin and eosin in the DCAN 1 rat model. The non-diabetic control group showed normal cardiac architecture, while the DCAN group had varying degree of cardiac damage as highlighted below.

Key:

A= Control specimen showing normal cardiac muscle fibres and basophilic nuclei

B= DCAN 1A specimen showing waxy pattern with minimal swelling

C=DCAN 1B specimen showing increased cellularity

D=DCAN 1C specimen showing swelling with disorganised muscle architecture

E= DCAN 1D specimen showing degenerating myocyte nuclei, swelling, and disorganization

Red arrows= edema

Black arrow=nuclei

DCAN, diabetic cardiac autonomic neuropathy.

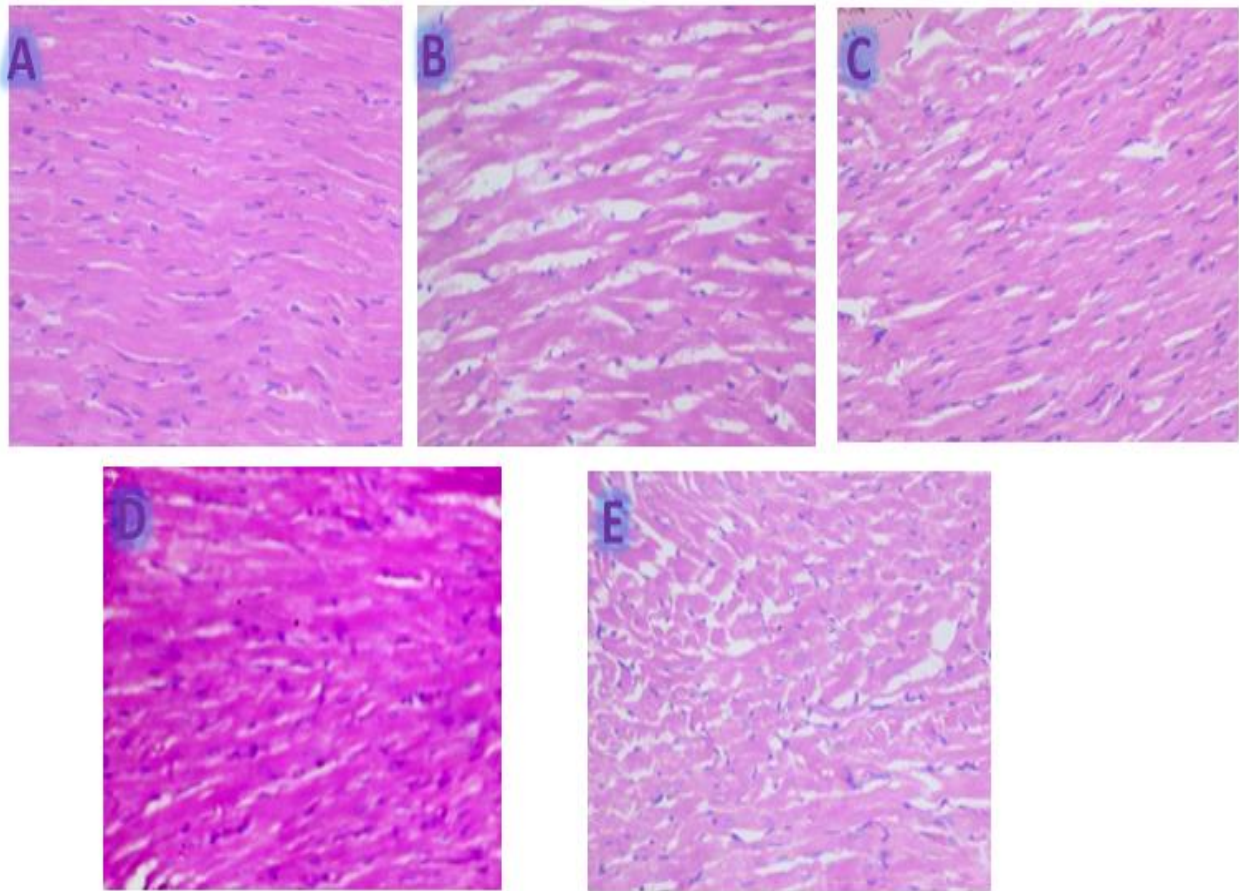


Figure 4.32: Photomicrographs representative of slides of the atrial/sinoatrial region of the heart stained with hematoxylin and eosin in the DCAN 2 rat model. The non-diabetic control group had normal cardiac architecture while the DCAN group had varying degree of cardiac damage as highlighted below.

Key:

A= Control specimen showing normal cardiac muscle fibres and basophilic nuclei

B= DCAN 2A specimen showing waxy pattern with minimal swelling

C=DCAN 2B specimen showing increased cellularity

D=DCAN 2C specimen showing swelling with disorganised muscle architecture

E= DCAN 2D specimen showing degenerating myocyte nuclei, swelling, and disorganization

Red arrows= oedema; Black arrow=nuclei

DCAN, diabetic cardiac autonomic neuropathy.

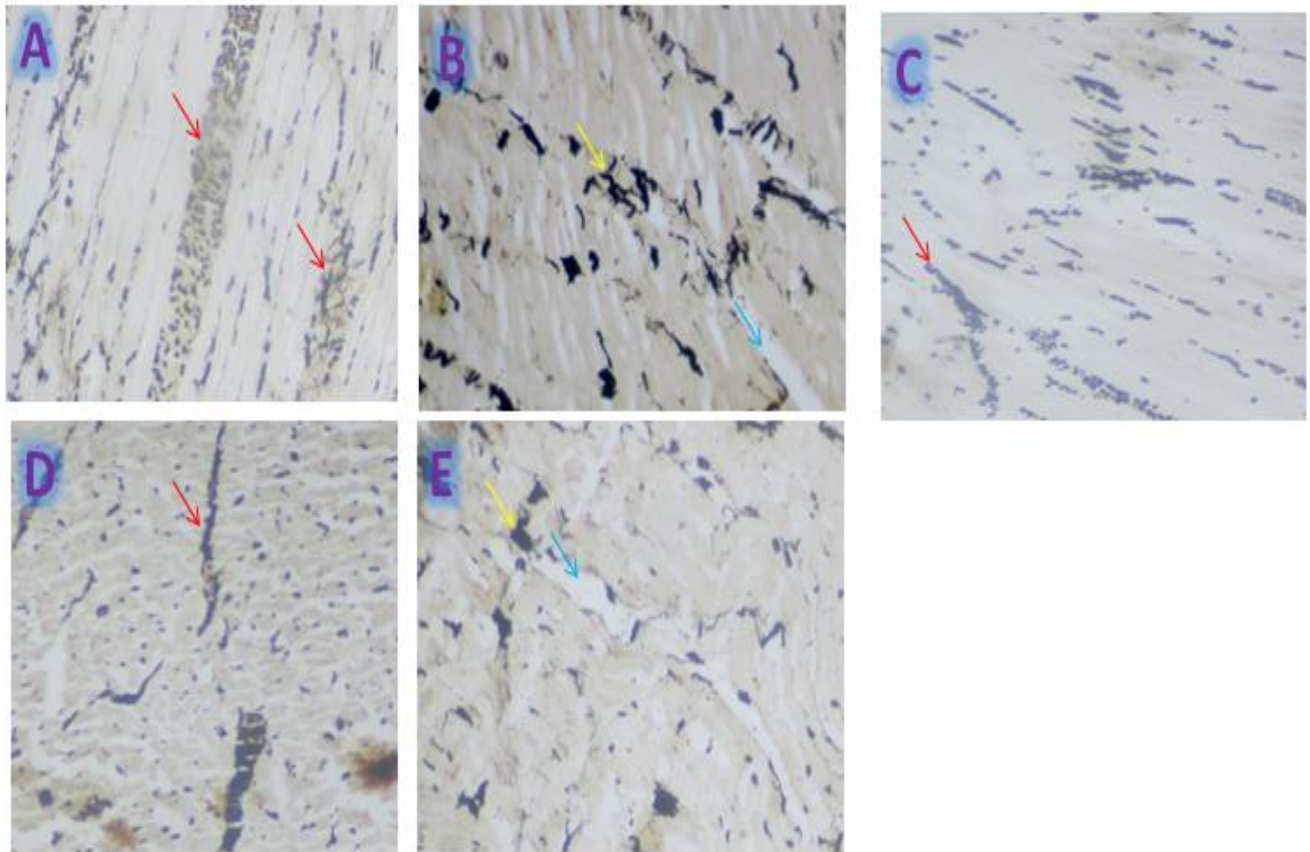


Figure 4.33: Photomicrographs representative of slides of the atrial/sinoatrial region of the heart stained with Beilchowsky histochemical silver in the DCAN 1 rat model. The non-diabetic control group had normal cardiac architecture, while the DCAN group had varying degree of cardiac damage as highlighted below.

Key:

A= Control specimen showing normal cardiac muscle fibres and basophilic nuclei

B= DCAN 1A specimen showing waxy pattern with minimal swelling

C=DCAN 1B specimen showing increased cellularity

D=DCAN 1C specimen showing swelling with disorganised muscle architecture

E= DCAN 1D specimen showing degenerating myocyte nuclei, swelling, and disorganization

Red arrows= nerve sheaths; Yellow arrows=axonal dystrophy; Blue arrows=oedema

DCAN, diabetic cardiac autonomic neuropathy.

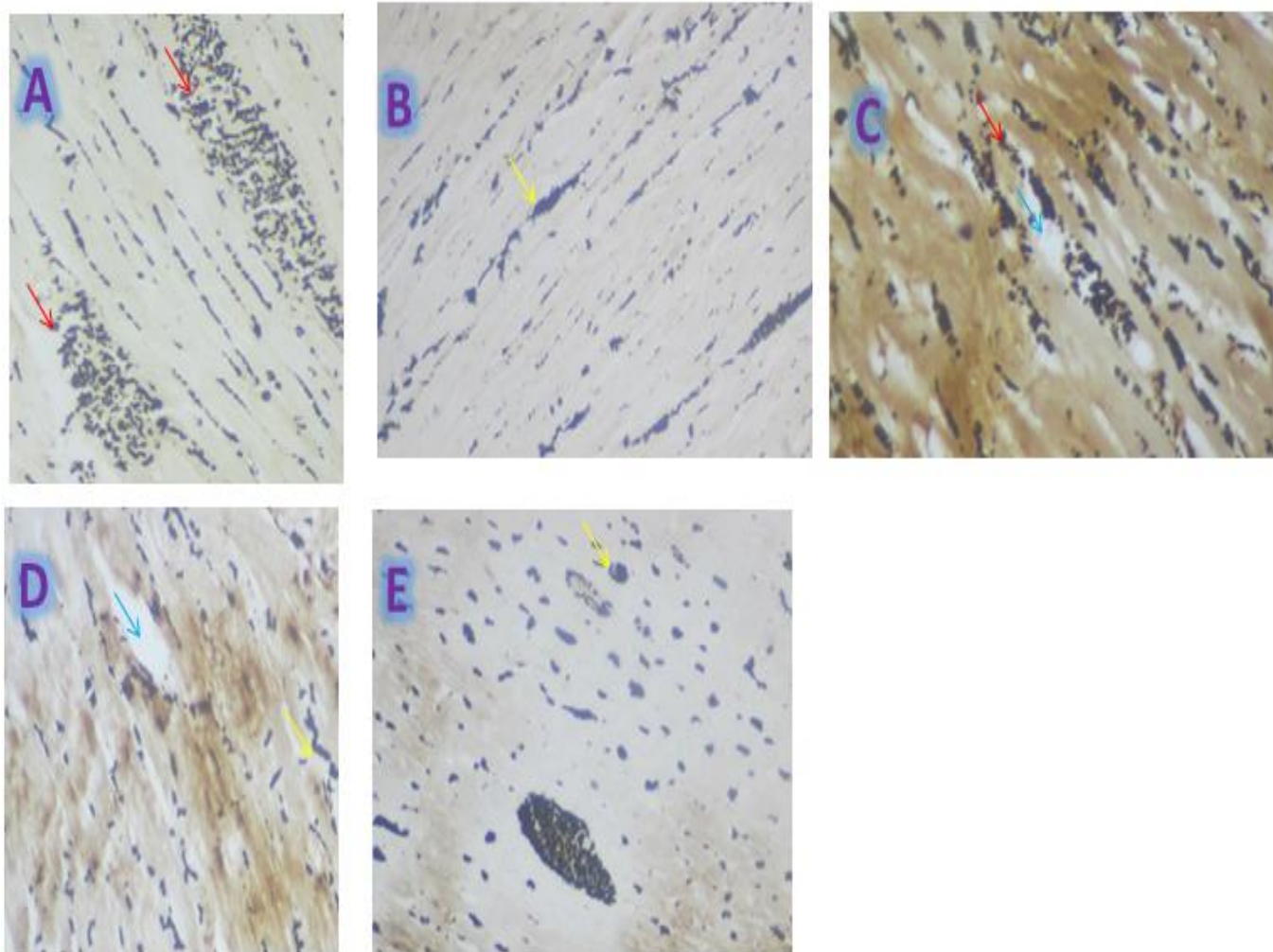


Figure 4.34: Photomicrographs representative of slides of the atrial/sinoatrial region of the heart stained with Beilchowsky histochemical silver in the DCAN 2 rat model. The non-diabetic control group had normal cardiac architecture, while the DCAN group had varying degree of cardiac damage as highlighted below.

Key:

A= Control specimen showing normal cardiac muscle fibres and basophilic nuclei

B= DCAN 2A specimen showing waxy pattern with minimal swelling

C=DCAN 2B specimen showing increased cellularity

D=DCAN 2C specimen showing swelling with disorganised muscle architecture

E= DCAN 2D specimen showing degenerating myocyte nuclei, swelling and disorganization

Red arrows= nerve sheaths; Yellow arrows=axonal dystrophy;Blue arrows=oedema

DCAN, diabetic cardiac autonomic neuropathy.

4.8.2. Nerve density estimates and correlation with HRV parameters and laboratory parameters

Morphometric nerve estimates using image J profiling showed significantly lower nerve density in the DCAN group compared to the non-diabetic controls in both T1DM and T2DM models. Carvedilol showed a trend towards improved nerve densities in the various interventional subgroups, but this did not achieve statistical significance, as shown in Figure 4.35.

There were significant correlations between the cardiac nerve density and both biochemical laboratory with time varying HRV parameters. Positive correlation was found with SDH ($p=0.048$), NGF ($p=0.018$), glutathione ($p=0.025$), and choactase ($p=0.048$). However, there was a significant negative correlation with AGEs ($p=0.026$) and noradrenaline ($p=0.049$). The time spectral-domain showed a significant positive correlation with nerve density, unlike the frequency domain that did not correlate significantly with the cardiac nerve density (Table 4.2).

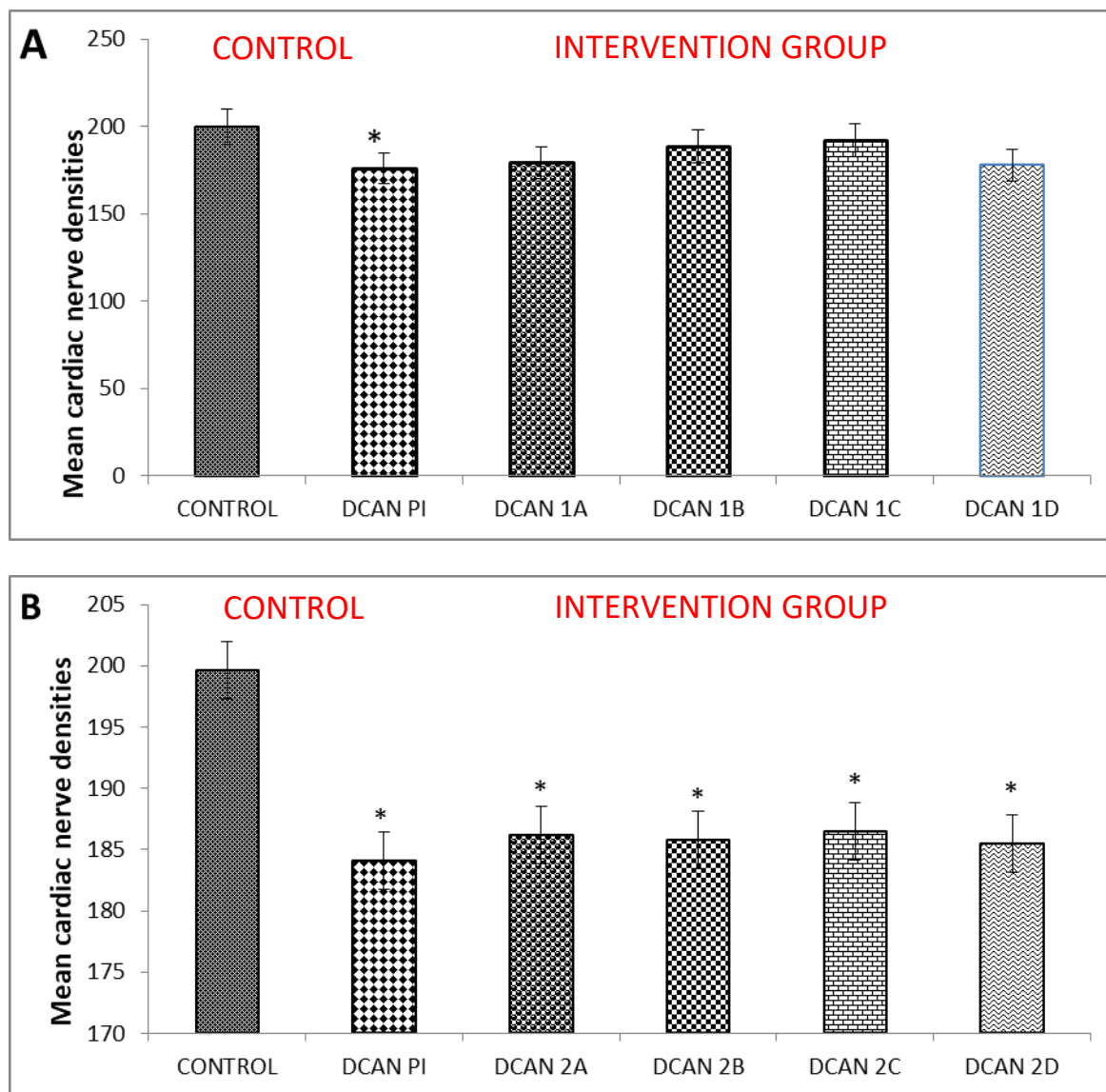


Figure 4.35: Effect of carvedilol on morphometric estimates of the cardiac nerve densities.

* $p < 0.05$ compared with control

Key

Control: Control (+10 ml/kg normal saline), DCAN 1A: DM 1 (+0.1 mg/kg carvedilol)
 DCAN 1B: DM 1(+1 mg/kg carvedilol), DCAN 1C: DM 1 (+10 mg/kg carvedilol)
 DCAN 1D: DM 1 (+10 ml/kg normal saline), DCAN 2A: DM 2 (+0.1 mg/kg carvedilol)
 DCAN 2B: DM 2(+1 mg/kg carvedilol), DCAN 2C: DM 2 (+10 mg/kg carvedilol)
 DCAN 2D: DM 2 (+10 ml/kg normal saline)
 DM, diabetes mellitus; DCAN, diabetic cardiac autonomic neuropathy

Table 4.2: Correlation of morphometric nerve densities with laboratory parameters and heart rate variability indices

Parameters	r^*	p - value
TG	-406	0.133
AGEs	-0.571	0.026*
SDH	0.469	0.048*
NGF	0.363	0.018*
NA	-191	0.049*
Choactase	0.203	0.048*
GSH	0.575	0.025*
SDNN	0.351	0.019*
RMSSD	0.231	0.048*
PNN50	0.235	0.039*
HRV index	0.311	0.259
LF	-0.236	0.398
HF	-0.343	0.211

KEY;

TG, triglyceride; AGEs, advanced glycated end product; SDH, sorbitol dehydrogenase;

NGF, nerve growth factor; NA, Noradrenaline; Choactase, acetyl choline transferase;

GSH, glutathione;

SDNN index (SDNNI), mean of the standard deviations of all the NN intervals for each 5 min segment of a 24 h HRV recording; RMSSD, root mean of squared successive differences;

PNN50, Percentage of successive RR intervals that differ by more than 50ms;

HRV, heart rate variability; LF, Low frequency; HF, High frequency.

* $p < 0.05$ compared with normal control.

Dose effect of carvedilol

Carvedilol at 1 mg/kg and 10 mg/kg had optimal dose effect on both the biochemical and HRV indices; however, more of the animals died when 10 mg/kg was administered, compared to when 1 mg/kg was administered. The mortality rates were 12.5%, 25%, 37.5%, and 37.5% respectively in DCAN 2B (1 mg/kg carvedilol), DCAN 2A (0.1 mg/kg carvedilol), DCAN 2C (10 mg/kg carvedilol), and DCAN 2D (no carvedilol) subgroups. Therefore from the findings of the index study, the recommended dose of carvedilol for optimal effect and least mortality is 1 mg/kg.

CHAPTER FIVE

5.0. DISCUSSION

5.1. Diabetic induction in T1DM and T2DM animal models

The present study showed induction of DM in a rat model using STZ alone (T1DM) and using STZ in combination with a high-fat diet (T2DM). High-fat feeding has been proposed to lead to obesity, hyperinsulinemia, and altered glucose homeostasis due to insufficient compensation by the beta cells of the pancreatic islets (Vatandoust *et al.*, 2018). The T2DM model in this study typifies human T2DM progression with associated dyslipidaemia, insulin resistance, pre-diabetes, and DM.

DM is a complicated, heterogenic, and polygenic disease. While T1DM is associated with the destruction of the pancreatic acini cells commonly via an autoimmune mechanism, T2DM is associated with the development of insulin resistance by target organs and decreased insulin production from pancreatic beta cells (Paton *et al.*, 2002; Srinivasan *et al.*, 2005; Algoblan *et al.*, 2014; Skovsø, 2014). In this study, a high single dose of STZ was associated with sudden and significant destruction of the pancreatic cells. This may explain the insulin deficiency seen in patients with T1DM and therefore other attendant clinical features.

Progressive multiple doses of STZ after HFD causes less rapid destruction of the pancreas. Accordingly, the animal model treated in this study portrays the same characteristics and mimics the pathogenesis and clinical features of human T2DM. Some researchers earlier showed that HFD for 2–7 weeks would induce stable insulin resistance, and HFD in combination with low doses of STZ induces DM in rats (Zygmunt and Stanczyk, 2010; Vatandoust *et al.*, 2018). The index study observed a significant increase in the blood glucose and total cholesterol of the animals, although some other studies showed significantly elevated triglyceride levels

(Kusunoki *et al.*, 2000; Reed *et al.*, 2000; Skovsø, 2014). This disparity may be in part due to the differences in the composition of the high-fat diets used.

Since there were different doses and numbers of STZ injections reported for the development of the T2DM rat model, this study tried to use a protocol that will be a similar model to the human disease. While Reed *et al.* used 50 mg/kg STZ for inducing T2DM (Reed *et al.*, 2000), Srinivasan *et al.* used 35 mg/kg intraperitoneal injection (Srinivasan *et al.*, 2005). The period of HFD usage was also variable across studies: some protocols administered HFD for 2–4 weeks before STZ injection, but Zhang *et al.* administered HFD for two months (Zhang *et al.*, 2008). This present study showed that HFD administration for eight weeks decreases insulin secretion by pancreatic cells and induces insulin resistance in target tissues.

5.1.1. Effect of Carvedilol on glucose and insulin sensitivity after DM induction.

Nguyen *et al.* using the HFD-induced obese mouse model, observed persistent elevation of basal plasma noradrenaline reflecting an over-activation of the sympathetic nervous system, which had been shown to cause profoundly adverse effects on glucose metabolism (Nguyen *et al.*, 2019). Persistent adrenergic overdrive caused by excess catecholamine can impair insulin sensitivity and glucose metabolism by causing complex interactions in several metabolic organs, with associated increased lipolysis, decreased muscular glucose uptake, and increased hepatic glucose output (Nguyen *et al.*, 2019).

Earlier studies had shown that traditional beta-blockers showed adverse effects on body metabolism, possibly due to their incomplete adrenoceptor blockade properties (Ripley and Saseen, 2014; Wiysonge *et al.*, 2007). The current study uses carvedilol, a third-generation beta-blocker agent with vasodilating action, which can completely block the effects of excessive

catecholamine, thereby leading to metabolic benefits on glucose metabolism. Carvedilol therapy in HFD-induced DM enhanced glucose tolerance and insulin sensitivity, according to recent research, likely by suppressing hepatic glucose overproduction and improving the muscular insulin signaling pathway (Bakris, 2004; Sica, 2005; Kveiborg *et al.*, 2006; Ahmad, 2017; Sirenko, 2017).

Although a report from a previous study showed that carvedilol had a hyperglycemic effect (Suresha *et al.*, 2013), the present study showed that carvedilol treatment had no adverse effect neither on blood glucose levels nor bodyweight of the HFD-fed models. The findings of carvedilol's effects on blood glucose levels and glucose tolerance were consistent with the metabolic parameters of HFD-fed mice treated with carvedilol in a study by Wang *et al.* that was used to investigate diabetes-associated cardiac dysfunction (Wang *et al.*, 2017). The inconsistent findings on blood glucose of carvedilol treatment might be due to differences in the treatment periods and the experimental rodent models since Wang *et al.* and Nguyen *et al.* used HFD-induced rodents and treated carvedilol for four weeks, unlike Suresha *et al.* that treated carvedilol in normal albino rats for only five days (Suresha *et al.*, 2013; Wang *et al.*, 2017; Nguyen *et al.*, 2019). Nonetheless, the present findings and others indicated that the use of carvedilol in DM and obesity might provide long-term benefits on glucose metabolism.

Earlier studies have shown that carvedilol improves lipid profile and insulin activity in DM and hypertensive subjects (Sharp *et al.*, 2008; Gokhan *et al.*, 2017). It was proposed as a possible replacement for insulin in the management of DM patients (Ahmad, 2017). The findings in the index study did show a trend towards improved insulin sensitivity and lipid profile in T2DM following carvedilol administration.

5.1.2. Oral and Intra-peritoneal glucose tolerance test

The index study showed differences in the response of plasma glucose levels to IPGTT and OGTT in the STZ-induced animal DM model. The peak blood glucose level in both T1DM and T2DM models following bolus glucose challenge was higher in IPGTT than in OGTT. This was similar to findings by Watada *et al.* and Andrikopoulos *et al.* and possibly implies that a significant amount of glucose administered through the gastric route is taken up by the splanchnic bed during its first pass through the organs (Andrikopoulos *et al.*, 2008; Watada *et al.*, 2013). Moreover, earlier researchers have reported that glucose ingestion during OGTT stimulated more GLP-1 production than during intravenous glucose tolerance test (IVGTT) in patients with T2DM. Thus intragastrically administered glucose may be more efficient in stimulating insulin release from pancreatic beta cells for lowering blood sugar level (Færch *et al.*, 2008).

Carvedilol improved glycaemic control and glucose tolerance in this study, which agrees with earlier studies that showed significant benefit of carvedilol for maintaining glycaemic control (George, 2004, Nguyen *et al.*, 2019).

5.2. Effect of DCAN and carvedilol on biochemical parameters.

5.2.1. The Sorbitol Pathway and oxidative stress in the Pathogenesis of Experimental Diabetic Autonomic Neuropathy

Numerous metabolic derangements associated with increased sorbitol pathway activity have been implicated in the pathogenic mechanism of diabetic complications. Earlier studies by Schmidt *et al.* showed that inhibition of SDH exacerbated autonomic neuropathy in rats with STZ-induced diabetes (Schmidt *et al.*, 1998, 2001). This further supports the fact that sorbitol

may play a significant role in mediating nerve damage in diabetic autonomic neuropathy (Bril *et al.*, 2004; Yagihashi *et al.*, 2011). The index study observed that while STZ-induced DCAN was associated with reduced SDH activity, an improvement in SDH activity on exposure to carvedilol occurred, which was further corroborated by the HRV indices. A possible mechanism of production of reactive oxygen radicals through the sorbitol pathway is the transfer of electrons and protons from sorbitol to NAD⁺ faster than they can be utilized for the synthesis of ATP and fatty acids (Wolin, 1996; Obrosova *et al.*, 1999).

Several mechanisms have been postulated to be involved in the pathogenesis of DM neuropathy in the STZ-induced DM rat. However, hyperglycaemia with the formation of AGEs and the toxic effect of sorbitol on myelin sheaths play a profound role. Peripheral nerves have been shown to have structural changes that mimic those of human DM neuropathy, and these changes are preceded by hyperglycaemia-induced biochemical abnormalities. Recent findings have emphasized the significance of vascular dysfunction, driven by metabolic impairments in the nerves, as aetiology of DM neuropathy. Non-enzymatic glycosylation of myelin components, deprivation of NGF, reduced endoneural blood flow, and increased free oxygen radical activity and production have also been hypothesized (Lee *et al.*, 2001). Improved SDH activity appears protective against toxic neural effect in DCAN.

5.2.2. Effect of DCAN and carvedilol on advanced glycated end products

This study showed significantly increased AGEs after DM induction in the rat model. Previous studies have shown that accumulations of AGEs are associated with neuronal fibre loss in the human diabetic peripheral nerve. AGEs interfere with axonal transport, thus contributing to the development of atrophy and degeneration of nerve fibres (Singh *et al.*, 2014; Vlassara and

Uribarri, 2014; Rhee and Kim, 2018). AGEs contribute to diabetic complications through (i) formation of cross-links between key molecules in the basement membrane of the ECM, thus permanently altering cellular structure and architecture, and (ii) interaction of AGEs with cell surfaces, thus altering the signalling cascades and cellular function (Singh *et al.*, 2014; Rhee and Kim, 2018).

AGEs are heterogeneous molecules produced from non-enzymatic reactions between free amino groups of lipids, proteins and nucleic acids and reducing sugars. A series of reactions, including oxidation-reduction reactions, successions of dehydration and other arrangements, lead to the formation of AGEs. Some reactive or precursor AGEs, such as cellular matrix, basement membranes, and vessel-wall components, form covalent crosslinks between proteins, altering their structure and function. Other significant characteristics of AGEs are their interactions with a range of cell-surface AGE-binding receptors, which result in either endocytosis or degradation (Ott *et al.*, 2014; Fishman *et al.*, 2018).

Several studies suggest that AGEs play a vital pathogenetic role in all the complications of DM, both microvascular and macrovascular. AGEs, for example, are present in diabetic patients' retinal vessels, and their levels correspond with those in the blood and the magnitude of retinopathy (Stitt *et al.*, 2002; Saxena *et al.*, 2012). Aminoguanidine, an inhibitor of AGE formation, has been shown to prevent retinopathy in diabetic animals (Mishra and Newman, 2012). It is also understood that AGEs accumulate in diabetic patients' peripheral nerves and that anti-AGE therapy enhances nerve conduction velocities and neuronal blood flow abnormalities (Yagihashi *et al.*, 2011).

The thickened glomerular basement membrane and mesangial expansion that characterize diabetic nephropathy are associated with AGE accumulation, which leads to glomerulosclerosis and interstitial fibrosis. Non-diabetic rats given AGEs for a long time developed similar morphological changes as well as severe proteinuria. AGE inhibitors (aminoguanidine) were observed to prevent DM nephropathy in animal models (Forbes *et al.*, 2003).

Diabetic patients have a faster rate of atherosclerosis, which is linked to a higher risk of cardiovascular and cerebrovascular mortality. AGEs are implicated in the development and progression of atherosclerotic lesions in both animal and human studies (Fishman *et al.*, 2018). Changes in endothelial cell, smooth muscle cell and macrophage function have been linked to increased AGE accumulation in diabetic vascular tissues.

The positive effect of carvedilol in T2DM model in the index study may show a promising effect of reducing the progression and subsequent development of complications in DM patients.

5.2.3. Effect of DCAN and carvedilol on noradrenaline

Noradrenaline has been proposed as a surrogate marker of muscle sympathetic activity. Its significant elevation in the DCAN group in this study further supports the development of autonomic neuropathy as recommended by the Toronto Consensus Committee on the diagnosis of DCAN (Wallin, 1984; DeBeck *et al.*, 2010; Zygmunt and Stanczyk, 2010; Spallone *et al.*, 2011, Thorp and Schlaich, 2015; Spallone, 2019).

Carvedilol administration ameliorated the noradrenaline effect, which may be due to the primary function of carvedilol as an antagonist at adrenergic receptors. The same property makes it an essential drug in patients with heart failure as it inhibits the sympatho-adrenergic drive, thus conferring survival benefits to such patients.

5.2.4. Effect of DCAN and carvedilol on antioxidants and nerve growth factor

Structural changes that occurred in peripheral nerves in this study resembled those of human DM neuropathy and were preceded by hyperglycaemia-induced biochemical abnormalities. Recent findings have emphasized the importance of vascular dysfunction, driven by metabolic insults to the nerves, as aetiology of DM neuropathy. Non-enzymatic glycosylation of myelin components, reduction of endoneurial blood flow, increased oxygen free radical activity or production, and deprivation of NGF are also involved (Lee *et al.*, 2001). The findings in the index study strengthens the earlier findings that showed impaired antioxidants activity and reduced NGF, which also correlate with the HRV spectral events shown. The antioxidant effect of carvedilol was seen in the significant improvement in TAC activity and GSH, which agreed with earlier studies (Dandona *et al.*, 2007; Ayashi *et al.*, 2016).

The finding that carvedilol improves antioxidant activity, as observed in this study, corroborates earlier research that showed its role in reducing oxidative stress in diabetic patients (Huang *et al.*, 2007; Diogo *et al.*, 2017). Fabiyi-Edebor recently showed that vitamin C, due to its antioxidant activity, ameliorated cardiac autonomic neuropathy (Fabiyi-Edebor, 2020). This may explain one of the mechanistic roles of carvedilol in ameliorating DCAN through its antioxidant effect.

5.2.5. Effect of DCAN and carvedilol on Choline acetyltransferase

Choactase, a protein marker of parasympathetic nerve activity, has been shown to correlate with autonomic nerve densities. Yang found a decreased concentration of choactase in the Akita diabetic rat model, similar to what was observed in this study. It was further shown that the reduced choactase correlated with lower Western blot-detected choactase antibodies and

autonomic nerve rarefaction (Yang, 2011). The finding of a significantly lower level of choactase further supports the development of cardiac autonomic neuropathy in these animal models. The effect of carvedilol on choactase may show that aside from ameliorating sympathetic derangements in diabetic models, it may additionally improve parasympathetic function following cardiac autonomic neuropathy in these animal models.

5.3. Effect of DCAN and carvedilol on heart rate variability indices

The findings in this study showed that STZ-induced DM rats have changes in some of the HRV indices, especially the frequency domain spectral activity, early in the course of diabetes. However, autonomic functions evaluated by time-domain indices of HRV were more pronounced about 28 days after STZ injection. Interestingly, all of these changes negatively correlated with plasma glucose and AGEs, while they were positively correlated with SDH and antioxidant markers.

Earlier studies have suggested that DCAN includes both parasympathetic and sympathetic dysfunction with early defective parasympathetic control, represented by persistent resting tachycardia and loss of beat-to-beat variation during deep respiration (Fazan *et al.*, 1997; DeBeck *et al.*, 2010; Ernst, 2017; Young and Benton, 2018). Similar to what Faran *et al.* documented, this study observed early changes in PNN50 immediately after DCAN induction, unlike SDNN that took a longer time to show significant changes in the DCAN model (Ernst, 2017). SDNN has been shown to provide an estimate of overall HRV and sympathetic balance, while PNN50 correlates with overall parasympathetic events. The findings in this study thus show that parasympathetic dysfunction occurs early in DCAN. Carvedilol had less effect on SDNN relative to its effect on improving PNN50. This may be due to the fact that it takes a

longer time for sympathetic dysfunction to manifest in abnormality of HRV. Since blood parameters showed a significant increase in noradrenaline levels early after DCAN induction, it may then mean that HRV indices lag behind plasma markers of sympathetic dysfunction. SDNN has important correlates with sympathetic function. It has been proposed as a prognostic marker for myocardial infarction and sudden death (Tsuji *et al.*, 1996; Mozaffarian *et al.*, 2008; Anichkov and Platonova, 2009; Poirier, 2014; Soares-Miranda *et al.*, 2014; Fyfe-Johnson *et al.*, 2016; Young and Benton, 2018). Studies have earlier shown significant derangement in PNN50 in heart failure subjects with autonomic dysfunction (Goldberger *et al.*, 2001; Guzzetti *et al.*, 2005; Sacha, 2014).

The RR interval variations present during resting conditions represent fine-tuning of the beat-to-beat control mechanisms. An understanding of the modulatory effects of neural mechanisms on the sinus node has been enhanced by spectral analysis of HRV. Some researchers have proposed that the efferent vagal activity is a major contributor to the HF component, PNN50, and RMSSD, as seen in clinical and experimental observations of autonomic manoeuvres. However, there seem to be conflicting views with regards to the LF component.

The effect of carvedilol on both SDNN and PNN50 may also corroborate the fact that it may regulate both parasympathetic and sympathetic output, thus providing a balance in the autonomic interplay. SDNN and RMSSD have been proposed as a surrogate for LF/HF, which represents the sympathovagal balance of the autonomic nervous system (Wang and Huang, 2012). The HRV indices vary with heart rate, age, and sex, among others; thus, ranges are used to express expected norms. Studies have proposed various ranges, means, and standard deviations for both time and frequency domain spectra activity (Sammito and Böckelmann, 2016; Shaffer and Ginsberg, 2017). RMSSD and SDNN have also been integrated into smart watches for

estimation of HRV in daily life, thus sensitizing individuals to the need for early health care visits in the case of deviation from the expected reference values (McCraty and Shaffer, 2015).

5.4. Effect of DCAN on pancreas histology

The index study showed a similar trend to other earlier findings with regards to the effect of STZ on pancreatic tissues (Mythili *et al.*, 2004; Nurdiana *et al.*, 2017). For instance, Mythili *et al.* showed that 30 mg/kg body weight STZ produced mild changes in pancreatic tissues, while 50 mg/kg proved to be fatal (Mythili *et al.*, 2004). The doses used were similar to those used in this study; 25 mg/kg and 50 mg/kg for T2DM and T1DM models, respectively. Findings in this study also corroborate the nearly complete destruction of the beta cells in T1DM patients. In contrast, T2DM patients still have preserved residual beta cells till very late in the disease spectrum.

5.5. Effect of DCAN on cardiac histology and cardiac nerve density estimates

Diabetic cardiomyopathy is characterized by myocellular hypertrophy and myocardial fibrosis, which lead to diastolic dysfunction and a high incidence of heart failure in diabetes. Previous studies have shown that AGEs can affect the physiological properties of proteins in the ECM by inducing the formation of cross-links, thereby causing intracellular changes in vascular and myocardial tissue (Kralova *et al.*, 2013). Carvedilol has been shown to improve left ventricular function, diabetic cardiomyopathy, and overall cardiac function, although the effect was not significantly seen in DCAN rats that had carvedilol intervention in the index study, probably due to the duration of administration (Bristow *et al.*, 1996; Feuerstein and Ruffolo, 1996; Huang *et al.*, 2005; Kralova *et al.*, 2013; Li *et al.*, 2014; Zheng *et al.*, 2018).

These results corroborate the fact that cardiac autonomic neuropathy and axonal dystrophy seem to be closely associated with biochemical derangements in the STZ-induced diabetic model. These biochemical derangements include increased AGEs, glucose, and noradrenaline, with reduced SDH activity, antioxidants, and NGF, which all correlate with HR autonomic indices derangements. Moreover, contrary to researchers who showed that autonomic nerve structural lesions do not appear as early as these functional changes, this index study shows that neuronal structural dystrophy may be seen early in the course of autonomic neuropathy (Yagihashi and Sima, 1986; Vinik *et al.*, 2013). However, the finding that carvedilol ameliorated both the biochemical and HRV indices but had no significant effect on structural changes and nerve densities may reflect the fact that early treatment of DCAN before permanent structural damage occurs has the potential for reversing neurological dysfunction. The correlation observed between HRV dysfunction and plasma biomarkers are in accordance with these observations.

5.6. Dose effect of carvedilol

This study also shows the varying dose-dependent effect of carvedilol. The high mortality rate observed in the group that received 10 mg/kg of carvedilol may be attributed to the higher volume load for dilution and subsequent volume overload. In contrast, the high mortality rate in the group without carvedilol intervention may be attributed to diabetic toxicity and progression of complications.

CHAPTER SIX

6.0. SUMMARY, CONCLUSION AND RECOMMENDATIONS

6.1 Summary

This study shows that HFD with multiple low doses of STZ administered intra-peritoneally and single high dose of STZ in adult animals were effective for inducing T2DM and T1DM, respectively, in rats. These models are proposed for studying cardiac autonomic neuropathy in rodents in resource-constrained settings where transgenic models may be technically challenging to develop.

In resource-constrained research laboratories, the development of transgenic T2DM animal models remains technically complicated, time-consuming, and costly; therefore, diet manipulation may be a promising alternative. Moreover, since the protocols presented in the literature for diet-induced T2DM animal models are heterogeneous, with no consensus existing for a standard procedure, conducting studies in this field to reach a common procedure for T2DM animal model production is of intensive value. Accession to an easy, cost-effective, and accurate animal model will facilitate more studies to unravel the pathogenic mechanism of the disease and to develop new therapeutic interventions in diabetes.

The results in this study emphasize some well-known cardiac alterations in the STZ-induced diabetic rat model, such as increased AGEs, glucose and noradrenaline, and reduced SDH activity, antioxidants and NGF, which all correlate with HRV autonomic indices derangements. In agreement with previous researchers who showed that autonomic nerve structural abnormalities might not become apparent before functional changes, this study showed that these changes could be due to the early development of autonomic neuropathy in this model. The

correlation observed between cardiovascular HRV dysfunction and plasma biomarkers are in support of the possible presence of reversible neurological dysregulation.

Carvedilol may have a promising role in the early management of DCAN before permanent, irreversible structural nerve changes occur. In addition to its use in the management of patients with chronic heart failure, it may show some promising trends in diabetic patients if commenced early in the course of autonomic neuropathy, thus ameliorating the progression of autonomic derangement. Early detection of DCAN should be encouraged using routine ECG Holter in conjunction with clinical features so that early intervention with strict glycaemic control and possible use of carvedilol may retard the progression of the disease.

6.2 Conclusion

The present study shows that STZ alone and in combination with a high-fat diet induced DCAN in T1DM and T2DM rat models, respectively. Carvedilol ameliorated the cardiac autonomic effects caused by diabetes in both T1DM and T2DM animal models. It had more effect on the HRV indices and plasma biomarkers, with less effect on structural parameters. The effective doses of carvedilol were 1 mg/kg and 10 mg/kg. However, carvedilol at 10 mg/kg was associated with higher mortality relative to 1 mg/kg, thus making the recommended dose to be 1 mg/kg.

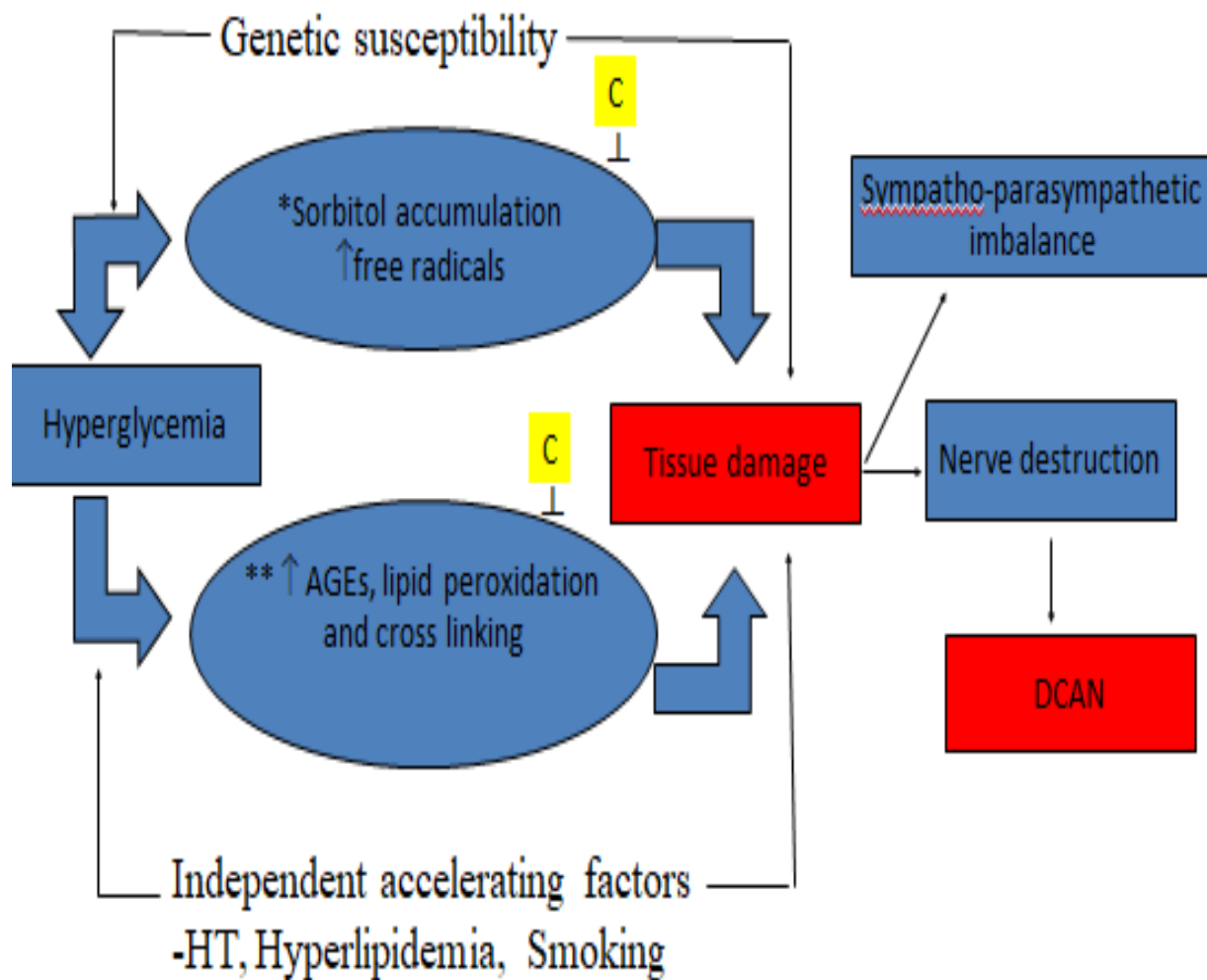


Figure 6.1: Diagrammatic illustration of the mechanism of diabetic cardiac autonomic neuropathy and the possible site of action of carvedilol

Key; AGEs, advanced glycated end products; C, carvedilol; DCAN, diabetic autonomic neuropathy; HT, hypertension

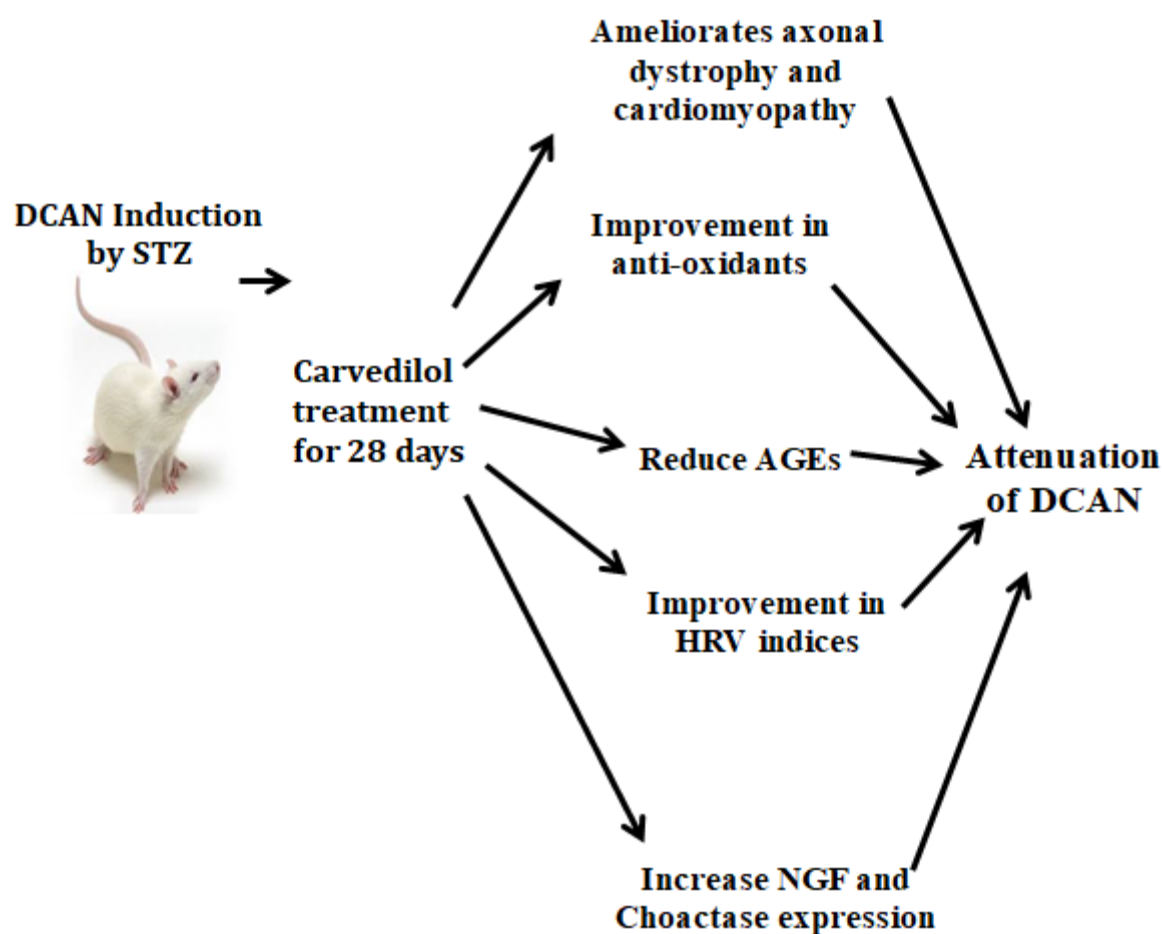


Figure 6.2: Diagrammatic illustration of the role of carvedilol in ameliorating diabetic cardiac autonomic neuropathy

Key; AGEs, advanced glycated end products; choactase, choline acetyl transferase; DCAN, diabetic autonomic neuropathy; HRV, heart rate variability; NGF, nerve growth factor; STZ, streptozotocin

6.3. Contribution to knowledge

- (i) High-dose streptozotocin (STZ) alone and low-dose STZ combined with high-fat diet can be used to induce diabetic cardiac autonomic neuropathy (DCAN).
- (ii) DCAN can be mediated through activation and production of advanced glycated end products and sorbitol activity.
- (iii) Heart rate variability indices and plasma biochemical derangements occur early in the setting of DCAN.
- (iv) Cardiac axonal dystrophy and reduced cardiac nerve densities are possibly late features of DCAN.
- (v) Carvedilol ameliorates DCAN in experimental rat model effectively at a dose of 1 mg/kg with least complications.

6.4. Recommendations

Based on the findings from this study, the following key recommendations can be made:

- (i) Early and routine check-up for features of diabetic cardiac autonomic neuropathy (DCAN) should be advocated among patients with diabetes.
- (ii) Holter electrocardiography using time-varying domain and spectral frequency evaluation will detect DCAN early and should be advised among diabetic patients, in addition to clinical parameters and reflex testing.
- (iii) Carvedilol should be recommended for diabetic patients with or without hypertension to help retard and improve DCAN development and progression. This should be in addition to standard treatment protocols and glycaemic control.

6.5. Suggestions for future research

Further studies are needed to investigate the following:

- (i) The effect of other beta blockers other than carvedilol on diabetic cardiac autonomic neuropathy
- (ii) The effect of long-term duration of diabetes on cardiac autonomic functions and the effect of carvedilol on already damaged nerves in DCAN rat models, since a lot of patients present late to the hospital after significant damage have been done to the nerves
- (iii) The effect of DCAN induction on the autonomic ganglia and peripheral nerves to assess which one occurs first (since many clinicians check routinely only for peripheral neuropathy during clinic visits), thus predicting possible time for development of autonomic neuropathy

PUBLICATIONS

Published Journal Articles

- i. **Akinlade, O.M.,** Owoyele, B.V. and Soladoye, A.O. (2020). Carvedilol improves sorbitol dehydrogenase and heart rate variability indices thereby ameliorating autonomic neuropathy in T1DM rats with streptozotocin-induced diabetes. *American Journal of Biomedical Science and Research*, 7(5), 434-440.
- ii. **Akinlade, O.M.,** Owoyele, B.V. and Soladoye, A.O. (2020). Heart rate variability indices, biomarkers, and cardiac nerve density: Independent surrogate markers for diagnosis of diabetic cardiac autonomic neuropathy in type 2 diabetes mellitus animal model. *International Journal of Health Sciences*, 14(6), 24-31.
- iii. **Akinlade, O.M.,** Owoyele, B.V. and Soladoye, A.O. (2021). Carvedilol improves heart rate variability indices, biomarkers but not cardiac nerve density in streptozotocin induced T2DM model of diabetic cardiac autonomic neuropathy. *Journal of Basic Clinical Physiology and Pharmacology*, 21(18), 282-296.
- iv. **Akinlade, O.M.,** Owoyele, B.V. and Soladoye, A.O. (2021). Streptozotocin-induced type 1 and 2 diabetes in rodents: models for studying diabetic cardiac autonomic neuropathy. *African Health Sciences*, (accepted for publication: WKR0-2020-05-0837).

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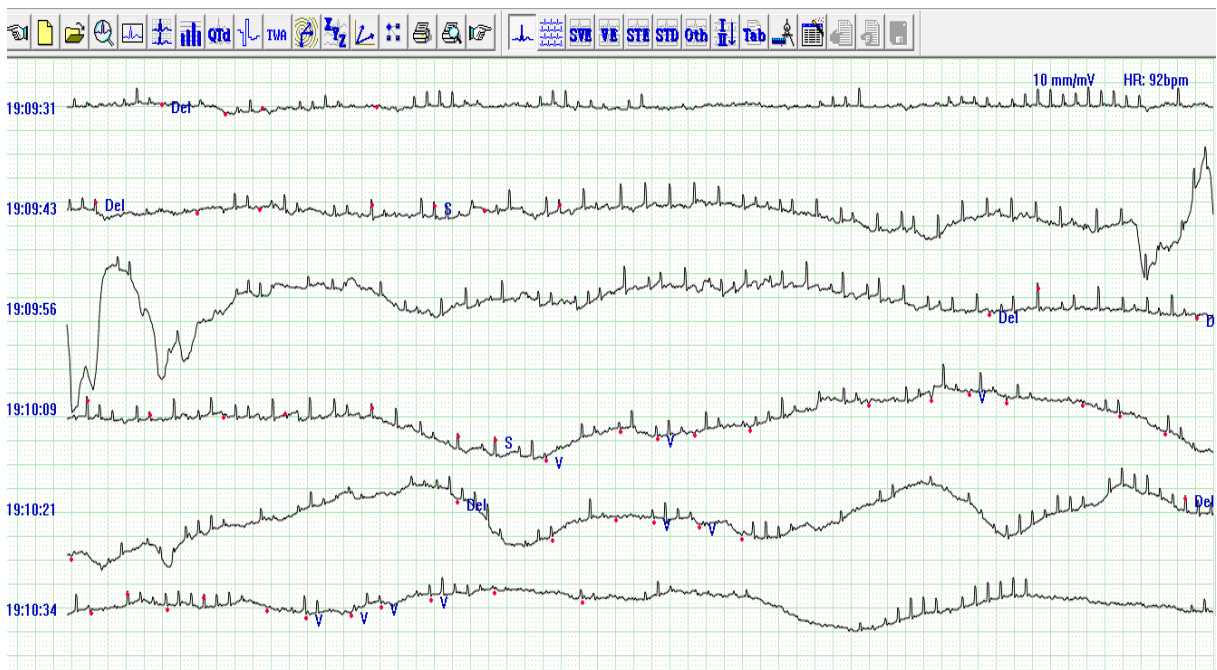
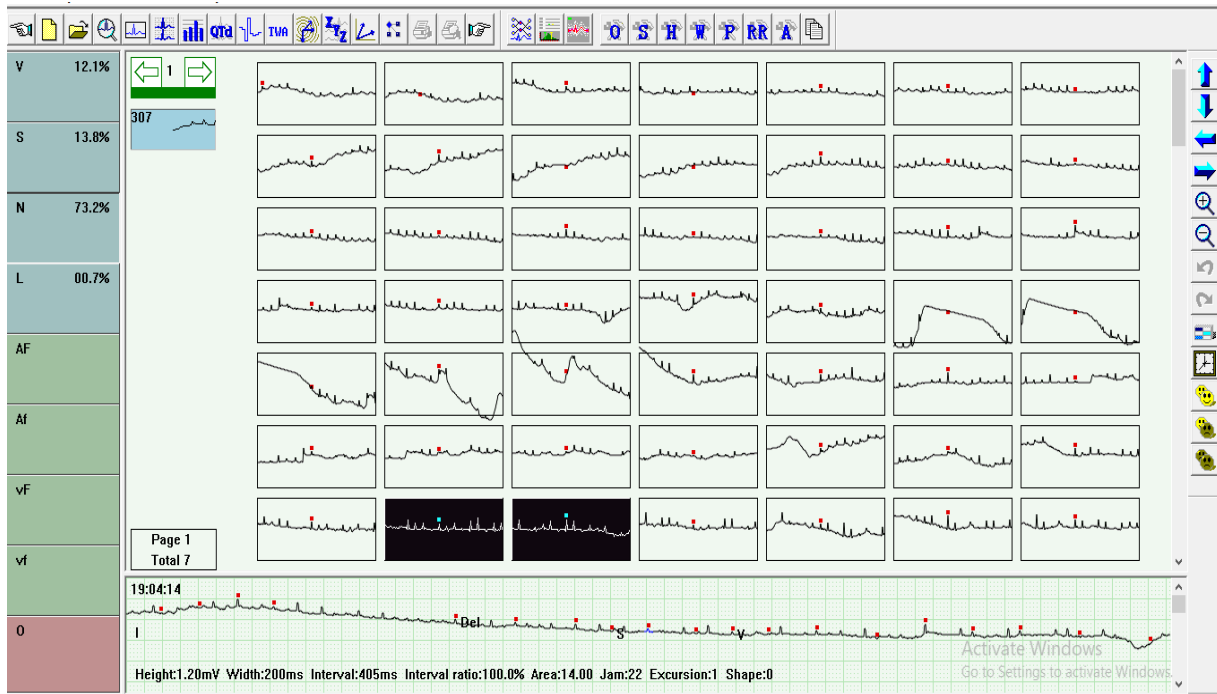
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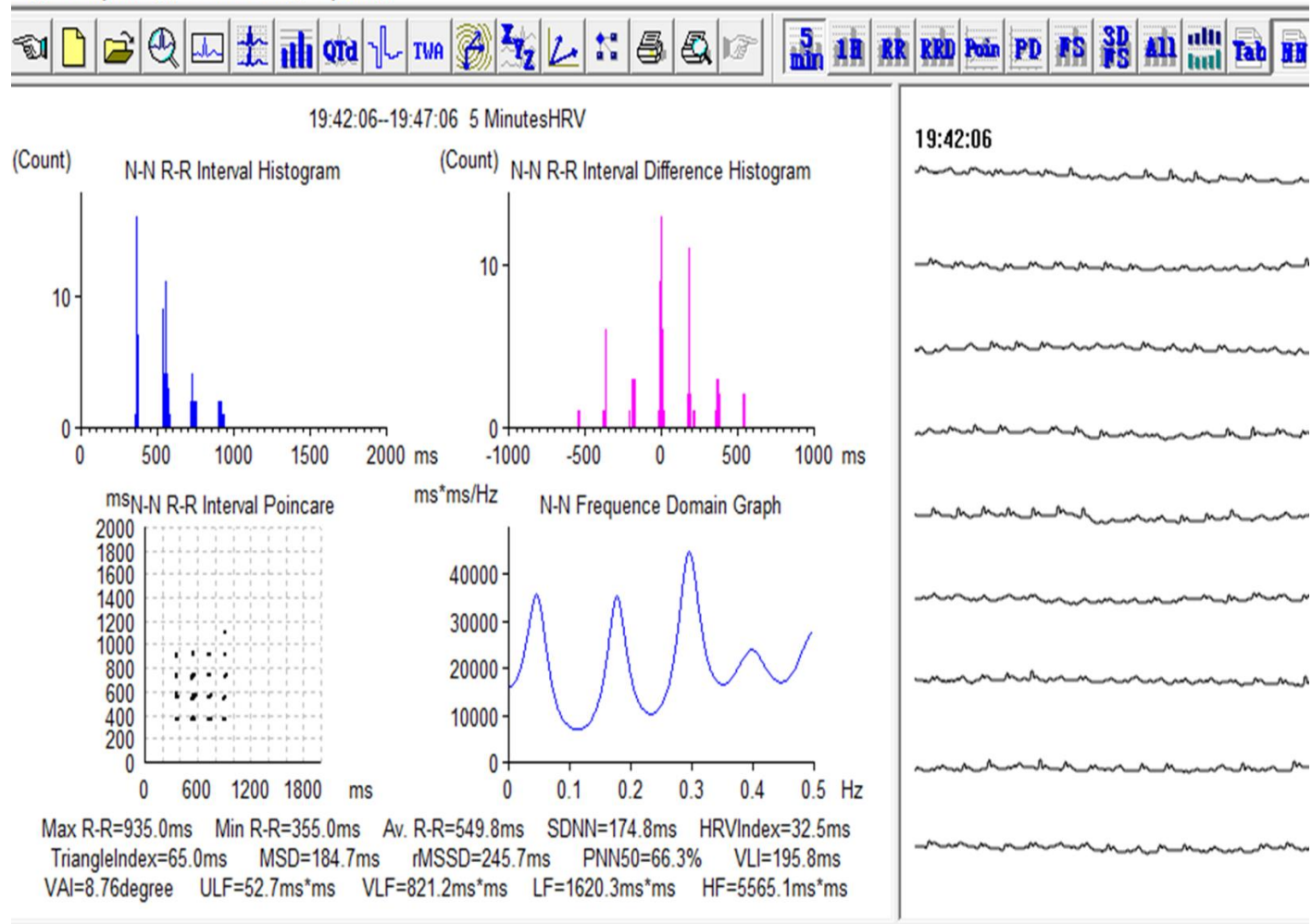
APPENDIX I: Holter ECG heart rate variability tracing.



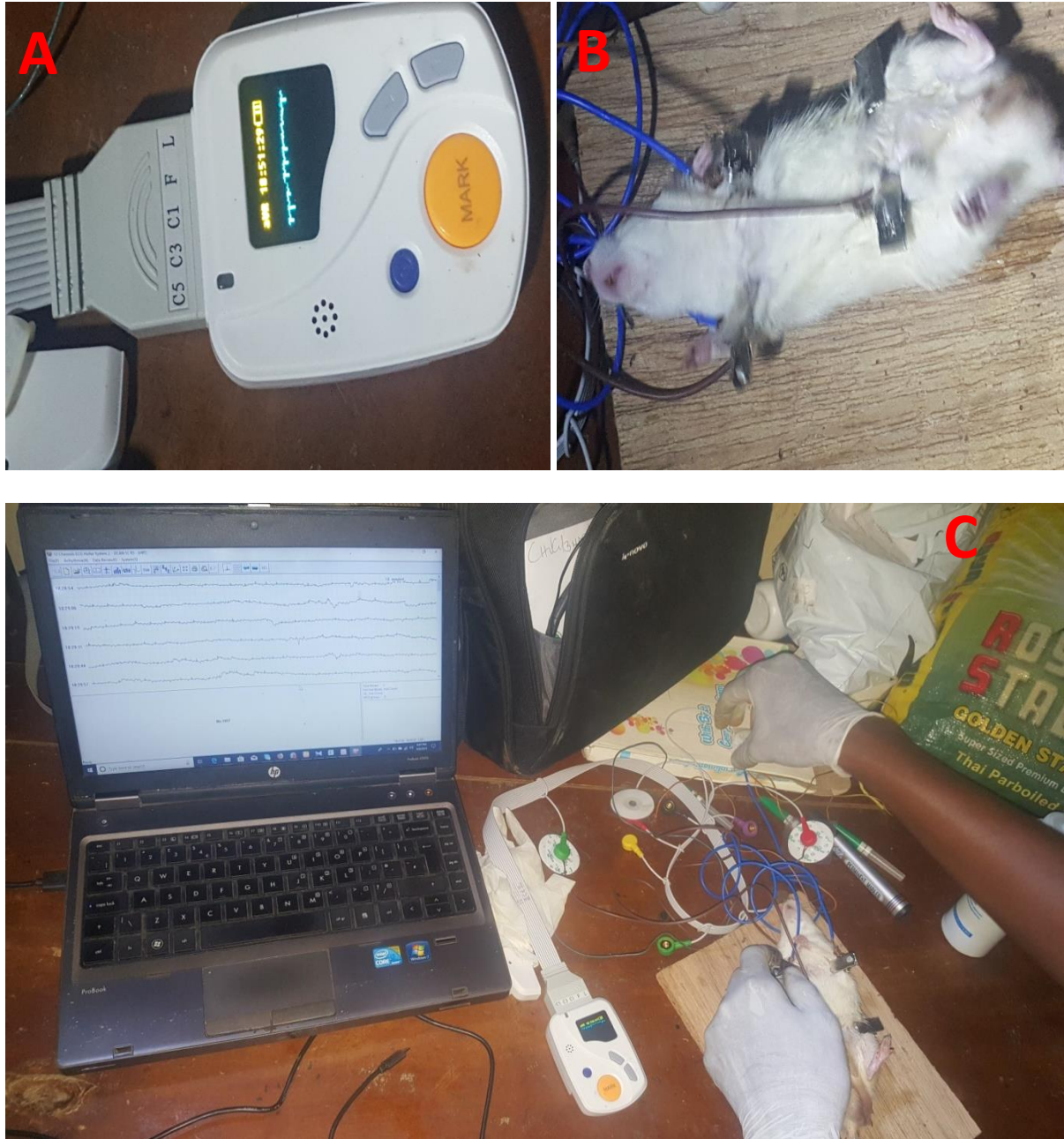
APPENDIX II: Holter ECG heart rate variability spectral tracing of a rat.

12 Channels ECG Holter System_L - 1ST DCAN 2A-8 - [HRV]

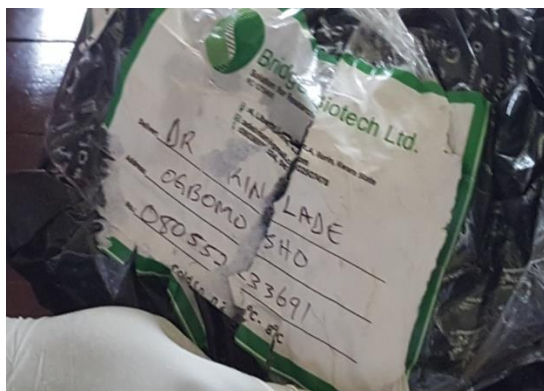
File(F) Arrhythmia(A) Data Review(E) System(S)



Appendix III: Holter electrocardiograph machine (A), attachment of electrodes to the rat (B), and electronic tracing (C)



Appendix IV: Materials, procedure, Inspection



APPENDIX III: Ethical approval certificate.

UNIVERSITY OF ILORIN, ILORIN, NIGERIA
UNIVERSITY ETHICAL REVIEW COMMITTEE

Vice Chancellor: Prof. S.A. Abdulkareem
B.ChE, M.ChE, (Detroit); Ph.D. (Louisville),
MNAICH, MNSE, MACS, R.Eng. (COREN).
Registrar: Dr. (Mrs) F.M. Olowoloni
B.Sc (Zaria); MBA, M.Sc. PGDE (Ilorin)

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ethicalreview.unilorin.edu.ng
Website: www.unilorin.edu.com

UIL/UERC/14/68KJ004
Our Ref:

13th November, 2019
Date:

Protocol Identification Code: UERC/BMS/149
UERC Approval Number: UERC/ASN/2019/1912

INVESTIGATION OF THE EFFECT OF CARVEDILOL ON EXPERIMENTALLY-INDUCED DIABETIC CARDIAC AUTONOMIC NEUROPATHY IN WISTAR RATS

Name of applicant/Principal Investigator: AKINLADE Olawale Mathias
Address of Applicant: Department of Physiology,
Faculty of Basic Medical Sciences,
University of Ilorin, Ilorin.

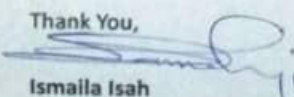
Type of Review: Full Committee Review
Date of Approval: 13/11/2019

Notice of Full Committee Approval
I am pleased to inform you that the research described in the submitted proposal has been reviewed by the University Ethical Review Committee (UERC) and given full Committee approval.

This approval dates from 13/11/2019 to 12/11/2022, and there should be no participant accrual or any activity related to this research to be conducted outside these dates.

You are requested to inform the committee at the commencement of the research to enable it appoints its representative who will ensure compliance with the approved protocol. If there is any delay in starting the research, please inform the UERC so that the dates of approval can be adjusted accordingly.

The UERC requires you to comply with all institutional guidelines and regulations and ensure that all adverse events are reported promptly to the UERC. No charges are allowed in the research without prior approval by the UERC. Please note that the UERC reserves the right to conduct monitoring/oversight visit to your research site without prior notification.

Thank You,

Ismaila Isah
For: University Ethical Review Committee



"...If it's not ethical, it's not scientific, if it's not scientific it's not ethical"

Appendix IV: Approval of Doctoral Research Protocol.

UNIVERSITY OF ILORIN, ILORIN, NIGERIA
POSTGRADUATE SCHOOL

Vice-Chancellor:

Prof. S. A. Abdulkareem
*B.ChE (Detroit); Ph.D. (Louisville);
MNAICH; MNSE; MACS; R.Eng (COREN)*

Dean:

Prof. R. A. Bello
*B.Sc., M.Sc., Ph.D.; Economics (ABU)
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Date: **4th February, 2021**

UIL/PGS/42

AKINLADE, Olawale Mathias
Matric. No: 14/68KJ004
Department of Physiology
Faculty of Basic Medical Sciences
University of Ilorin
Ilorin.

Dear **Akinlade**,

APPROVAL OF DOCTORAL RESEARCH PROTOCOL

I am pleased to inform you that, the Board of Postgraduate School at its 289th Meeting held on Wednesday 20th January, 2021 considered and approved your Doctoral Research Protocol titled:

**Investigation of the Effects of Carvedilol on Experimentally
Induced Diabetic Cardiac Autonomic Neuropathy in Wistar Rats**

You are therefore, to proceed with your Ph.D. programme accordingly, please.

Congratulations.

Yours sincerely,


M.A. Alfania
Secretary, Postgraduate School

MA/AJ