The effects of magnesium treatment on short-term changes in heart rate variability, cardiac ventricular function and lipid profile in streptozotocin-induced diabetic rats.

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ABSTRACT

INTRODUCTION

Diabetes mellitus is a major and rapidly growing worldwide health problem, causing mortality largely in developing countries such as South Africa. Diabetes induces life threatening cardiovascular complications including cardiac autonomic neuropathy, ventricular dysfunction and dyslipidaemia, which are dependent on the duration and severity of the diabetes. Most complications are identified at a late, irreversible stage following long-standing diabetes; therefore, early detection and treatment of cardiovascular complications may reverse impairments and improve outcomes. The early treatment of diabetic complications remains ineffective, as the associated underlying features, such as electrolyte disturbances, are poorly understood. A key electrolyte disturbance in diabetes is hypomagnesaemia, which is also an independent cardiovascular risk factor. However, the effects of magnesium (Mg^{2+}) supplementation are unclear. Therefore, this study investigated the effects of Mg^{2+} treatment on the early manifestations of streptozotocin (STZ)-induced diabetic cardiac complications.

METHODS

Adult male Wistar rats were treated once with STZ (50 mg/kg, i.p.) or vehicle (citrate), and daily for seven days with MgSO4 (270 mg/kg, i.p.) or saline. Blood glucose and body weight were monitored daily. On the eighth day, in vivo tail-pulse plethysmography was recorded for analysis of heart rate variability (HRV), a marker of cardiac autonomic function. Ex vivo, Langendorff-based left ventricular (LV) pressure-volume parameters were measured using an intraventricular balloon. Other hearts were stained with Masson's trichrome and haematoxylin and eosin for histological analysis. Cardiac tissue Mg^{2+} concentration as well as plasma lipid- and Mg^{2+} levels were measured by colorimetric assays.

RESULTS

Diabetes reduced heart rate and increased the low-frequency (LF)/high-frequency (HF) power ratio. Mg^{2+} treatment prevented theses diabetes-induced changes in heart rate and in the low-frequency (LF)/high-frequency (HF) power ratio ($p < 0.05$, n = 9/group). In addition, Mg^{2+} restored orthostatic stress induced changes in heart rate, and LF/HF ratio in diabetic rats ($p < 0.05$, n = 9/group). In isolated hearts, Mg^{2+} reversed the diabetes-induced decrease in LV end-diastolic elastance ($p < 0.05$, n = 6/group) and the right shift of end diastolic equilibrium volume intercept from 49 ± 6 µL to 25 ± 5 µL ($p < 0.05$, n = 6/group), without altering LV developed pressure or end systolic elastance. Diabetes significantly increased plasma triglyceride, total cholesterol and blood glucose ($p < 0.05$, n = 7/group), and significantly decreased body weight ($p < 0.05$, n ≥ 16/group) compared to control, but these changes were not prevented by Mg^{2+} treatment. Neither diabetes nor Mg^{2+} treatment altered plasma- and tissue Mg^{2+} levels. Histologically, diabetes and Mg^{2+} treatment also did not alter cardiomyocyte size or the amount of interstitial collagen in myocardial tissue.

CONCLUSION

These results show that Mg^{2+} treatment attenuates diabetes-induced autonomic dysfunction and improves LV diastolic distensibility in short-term diabetes. However, the diabetic metabolic disturbances of hyperglycaemia and dyslipidaemia, the changes in cardiac microstructure or the plasma- and cardiac tissue Mg^{2+} levels were uninfluenced by Mg^{2+} treatment. This suggests that Mg^{2+} exerted its beneficial effects independent of these factors, highlighting the underling mechanisms remain to be clarified. The Mg^{2+} levels not measured in this study by which changes could have been mediated was intracellularly; an aspect that should be further explored in future studies. Furthermore, whether these effects would be translatable to chronic diabetes is an important next question. Thus, the results of this study suggest that Mg^{2+} may have a modulatory role in treating early diabetic cardiovascular complications, but future studies will need to clarify the underlying mechanisms.

CHAPTER 1: INTRODUCTION

1.1 DIABETES MELLITUS: A RAPIDLY GROWING HEALTH PROBLEM

Diabetes mellitus is a complex metabolic disorder characterized by underlying hyperglycaemia and glucose intolerance, due to an insulin deficit, resistance or both (Kumar et al., 2010a; Kerner, Bruckel & German Diabetes Association, 2014). Diabetes is a major and rapidly growing health problem worldwide. According to the International Diabetes Federation (IDF), over 387 million of the world's 7 billion people, consistent with the World Health Organisation (WHO) that estimates the prevalence of diabetes to be 9%; emphasizing that diabetes is a major problem affecting approximately 1 in 10 adults (IDF, 2013; WHO, 2013). Furthermore, the diabetes prevalence has been steadily increasing by over 50% each decade and in 2002 was projected to double by the year 2030 (Mathers & Loncar, 2006).

Approximately 1.6 million people die from diabetes and its complications every year. The majority of diabetes-related deaths, about 70%, are estimated to occur in low- to middle-income countries (Mathers, Boerma & Ma Fat, 2009). In Sub-Saharan Africa, it was estimated that 12 million people were living with diabetes in 2010 and this is projected to double to 23.9 million by 2030 (Hall et al., 2011). South Africa specifically, has the highest prevalence of diabetes in Sub-Saharan Africa (Bradshaw et al., 2003), with prevalence proportions as high as 9% in rural communities (Motala et al., 2008). Non-communicable diseases such as diabetes have become a major health problem, largely attributable to urbanisation and increased lifestyle risk factors such as unhealthy diets, sedentary lifestyles and smoking (Mayosi et al., 2009). WHO projected that the burden of mortality due to diabetes in Africa will account for 3.5% of deaths; overtaking infectious diseases such as tuberculosis and HIV which will account for 0.8% and 2.6% respectively, by 2015 (WHO, 2013).

Diabetes causes various metabolic derangements that induce cellular and tissue injury, resulting in the diabetic complications of end-organ damage. These complications are the main problem in diabetes with measured prevalence of 75% in some African countries (Bos & Agyemang, 2013). Diabetic end-organ damage affects various organ systems, most commonly the heart, eyes and kidneys (Kengne, Amoah & Mbanya, 2005). Moreover, most of the morbidity and mortality in diabetes is due to complications of cardiac- and vascular tissue damage, which leads to terminal cardiovascular disease (CVD) in diabetic patients (Bradshaw et al., 2007; Soedamah-Muthu et al., 2006). A review by Kengne and associates (2005), demonstrated that although CVD complications of diabetes are on the rise with approximately 30% of patients treated for CVD are found to have co-morbid diabetes. Thus, there is a need to understand and develop more effective therapies for diabetes and its cardiovascular complications.

1.2 DIABETES: A CARDIOVASCULAR DISEASE

There has been a change in perception regarding the classification of diabetes, which was widely known as an endocrine/metabolic disease, to now being thought of as a CVD. In 1999, the American Heart Association and the American Diabetes Association published a joint statement concerning the challenge of CVD prevention in diabetes, which highlighted that diabetes must take its place alongside the other major risk factors as important causes of CVD. The statement suggested a re-classification of diabetes, with the rationale that from the point of view of cardiovascular medicine, it is appropriate to say, "*Diabetes is a cardiovascular disease*" (Grundy et al., 1999). This supported the revolutionary thinking of exemplar scientists such as Joseph Kraft, the father of the insulin assay, who had for decades argued that all diabetic patients had CVD and those without were simply undiagnosed due to insensitivity of testing methods (Kraft, 2011).

Diabetes is thus a significant cause of CVD, compounding the problem of CVD, which is the leading cause of morbidity and mortality worldwide, including in South

Africa (Bradshaw et al., 2003; Groenewald et al., 2013). This is because diabetes causes various metabolic derangements that induce cellular and tissue injury, predominantly cardiac and vascular tissue damage, resulting in the diabetic complications of end-organ damage in the heart, brain and nerves, kidneys and eyes (Stanley, Lopaschuk & McCormack, 1997; Soedamah-Muthu et al., 2006). Cardiovascular complications of diabetes account for the highest proportion of morbidity and mortality in diabetes with a prevalence of up to 75% in some African countries and accounting for over 60% of diabetic deaths (Duckworth et al., 2009; Bos & Agyemang, 2013). Furthermore, diabetic patients are highly likely to have CVD co-morbidity. From the Framingham study, Kannel & McGee (1979) found that there was a two- to three-fold increase in cardiovascular mortality in diabetic patients when compared to non-diabetic patients. More recently, according to Wong and collaborators (2012), 60% of diabetics have a pre-existing CVD or will be diagnosed with CVD at the time of diagnosis of diabetes. This makes the diagnosis and treatment of diabetic CVD complicated, as the temporal relationship and underlying mechanisms in early diabetic cardiovascular complications of diabetes is not completely understood.

1.2.1. EARLY DIABETIC CARDIOVASCULAR COMPLICATIONS

Diabetes causes a wide spectrum of cardiovascular complications that are related to the duration of diabetes as well as the severity of hyperglycaemia and insulin resistance (The Diabetes Control and Complications Trial Research Group, 1993; UK Prospective Diabetes Study (UKPDS) Group, 1998). Cardiovascular complications of diabetes are of insidious onset and often masked by compensatory mechanisms, only becoming overt once the compensatory mechanisms fail due to longstanding progression of diabetic complications (Malpas & Maling, 1990). The severity of the diabetes and its complications is also related to the type of diabetes, with type 1 or insulin-dependent diabetes mellitus (IDDM) associated with more severe hyperglycaemia and type 2 or non-insulin-dependent diabetes mellitus (NIDDM) associated with hyperinsulinaemia (Lehto et al., 2000; Snell-Bergeon & Wadwa, 2012). However, both hyperglycaemia and hyperinsulinaemia drive the

onset of diabetic cardiovascular complications in a concordant manner (Kim & Reaven, 2004). Diabetic cardiovascular complications are reversible in the early stage of diabetes, but become irreversible and permanent as diabetes becomes long-standing, characterized by progressive pathological processes leading to terminal deterioration of the cardiovascular system (Aneja et al., 2008).

Reaven (1988; 2005) argues that the nature of diabetic testing, which relies on the findings of impaired glucose tolerance and hyperglycaemia to diagnose diabetes lead to the failure to detect early diabetes and thus early complications of diabetic CVD leading to the problem of established CVD at first diagnosis of diabetes. Early complications such as atherosclerosis and coronary vasculopathy, which drive a majority of cardiovascular complications, may precede the overt symptom of hyperglycaemia, and diabetes; reflecting early metabolic derangements of lipid signalling regulatory mechanisms related to insulin resistance. However, without invasive testing, this complication is difficult to assess. Moreover, large clinical trials have found that undiagnosed early diabetes and the pre-diabetic state of impaired glucose tolerance are a common finding in patients with newly diagnosed myocardial infarction (MI) (Norhammar et al., 2002). This is supported by the converse findings that diagnosed diabetic patients have a five-fold greater risk of compared to non-diabetic patients (Haffner et al., 1998; Juutilainen et al., 2005).

1.2.2. ISCHAEMIC HEART DISEASE

Ischaemic heart disease (IHD) refers to states of reduced or hypo-perfusion of the myocardium relative to its energy demand causing ischaemia and in severe cases infarction. IHD is synonymously used with coronary artery disease (CAD), which is the predominant cause of myocardial ischaemic states, and is due to pathology of coronary arteries in which there is narrowing of the vessel lumen that results in the hypo-perfusion (Kumar et al., 2010b). IHD is the leading pathology underscoring the epidemic of CVD in developing countries and accounting for the largest cardiovascular age-adjusted mortality even in developing countries (Ahern et al., 2011). There is a strong association between IHD and diabetes: over a third of patients with IHD have diabetes, as highlighted by the EUROASPIRE (Norhammar et

al., 2002; Gyberg et al., 2015). Moreover, diabetic patients with IHD were found to be at a significantly higher risk of morbidity and mortality with mortality rates of almost double that of non-diabetic patients in both short- and long-term (Norhammar et al., 2007). Conversely, diabetes has also been well established as a cause and independent risk factor for IHD since the Framingham study in which the prevalence of atherosclerotic (ischaemic) CVD was observed to be twice that of non-diabetic men and thrice that of non-diabetic women (Kannel & McGee, 1979). Further large studies produced similar findings, demonstrating that CAD is 2-4 times more prevalent in diabetics, and observing that this risk due to diabetes is independent of dyslipidaemia and atherosclerosis (Koskinen et al., 1992), as well as severity or diffuse nature of vessels involved (Dortimer et al., 1978; Fallow & Singh, 2004) or the infarct size (Timmis, 2001; Laing et al., 2003).

Not only is the risk and severity of CAD disease increased, diabetes also portends a substantially worse prognosis post-MI and leads to poorer outcomes complicated by increased incidences of complications such as angina and re-infarction, arrhythmias, ventricular dysfunction and heart failure. Large clinical trials such as the Global Use of Strategies To Open Occluded Coronary Arteries in Acute Coronary Syndrome (GUSTO-IIb, McGuire et al. 2000) and the Organisation to Assess Strategies for Ischaemic Syndromes (OASIS, Malmberg et al. 2000) found that diabetic patients had poorer outcomes including death and re-infarction (13% *vs*. 8% in non-diabetics) with increased incidences of complications including stroke, heart failure, atrioventricular block, arrhythmias and major bleeding. Furthermore, Kahn et al. (2012) found that in diabetics, the mortality rate post percutaneous coronary intervention (PCI) remains substantially higher than in patients without diabetes, which remains closer to non-intervention mortality rates seen in other studies.

In early diabetes, metabolic- and vascular derangements induce dysfunction and damage in coronary vasculature leading to vasculopathy and CAD. The main pathological process implicated in CAD is atherosclerosis, which is accelerated/aggravated by diabetic derangements (Airaksinen, 2001; Kalogeris et al., 2012). Accelerated atherogenesis is the product of endothelial dysfunction, prothrombosis and vascular inflammation, which is driven by the early diabetic derangements of insulin resistance. Another factor that plays an important role in the

pathogenesis and complications of early manifestations of CAD is autonomic dysfunction. Autonomic dysfunction has been implicated to play a pathogenic role in the development of coronary artery disease in diabetes (Sayer et al., 2000). Coronary vascular tone, among other factors including endothelial function is regulated by the cardiac autonomic nervous system, the failure of which has been implicated in driving myocardial ischaemia and infarction (Ewing, Campbell & Clarke, 1980). Autonomic dysfunction is a negative independent prognostic factor that predicts poor outcomes and sudden death in CAD (La Rovere et al., 1998); which is exaggerated in diabetes (Spallone et al., 2014). Moreover, in risk stratification, the combination of assessing autonomic function indices and structural indices such as the left ventricular function has been proposed as a high-standard method for risk assessment in patients with CVD, particularly CAD and heart failure (Priori et al., 2001; Goldberger et al., 2008). Therefore, the focus of this dissertation will be on these aspects of diabetic cardiovascular complications as early markers that can be used in risk stratification and prevention of diabetic CVD.

1.2.3. CARDIAC AUTONOMIC DYSFUNCTION

The cardiac autonomic nervous system is an essential regulatory mechanism that modulates chronotropy and inotropy in response to physiological and psychological changes. Regulatory centres in the midbrain, hypothalamus, pons, and medulla form the central autonomic nervous system (ANS) and provide efferent innervation to various organs including the heart. Both divisions of the ANS – the sympathetic- and parasympathetic, innervate the heart. Sympathetic innervation to cardiac and vascular tissue is derived from the sympathetic chain that is para-vertebra from about the 1st thoracic vertebra to the 5th lumbar vertebra; while the parasympathetic innervation is derived from the vagus nerve (Carrio, 2001).

Diabetes induces damage and dysfunction in the autonomic nerves including the sympathetic and parasympathetic innervation of the heart. Diabetic autonomic neuropathy (DAN) is a common early complication of diabetes that causes morbidity and is a poor prognostic factor in mortality due to diabetes, particularly in CVD (Vinik et al., 2003). The pathogenesis pattern of DAN leads to dysfunction of long nerves in

early stages, including the large vagus nerve (Spallone et al., 2014). DAN is an under-diagnosed and undertreated complication of diabetes. The prevalence varies greatly by population studied and method of measurement with conservative large study estimates of 50% and smaller studies estimating the prevalence at 70%; highlighting that it is a major problem requiring urgent attention (Young, Ewing & Clarke, 1983; Maser et al., 2003).

Findings of abnormalities in autonomic function in type 1 diabetic patients that could be consistent with early onset of autonomic neuropathy has led to the hypothesis that poor glycaemic and metabolic control of early derangements is a major determinant of nerve dysfunction and damage (Pfeifer et al., 1984). Moreover, optimising metabolic control and preventing severe diabetic metabolic derangements in patients can reverse early autonomic dysfunction (Hreidarsson, 1981). Early autonomic dysfunction, characterized by impairment in normal heart rate variability (HRV), progresses to irreversible autonomic neuropathy that contributes to stress and abnormalities of neurohormonal over-activation leading to remodelling culminating in terminal heart failure (Howarth et al., 2006). Another characteristic impairment of the cardiac ANS in response to integrated physiological stimuli from the brain and peripheral sensory apparatus is the response to orthostatic stress and changes in blood pressure.

Baroreceptor reflex impairment and autonomic dysfunction

At a sensory level, physiologic stimuli are detected by receptors such as those of the aortic- and carotid body baroreceptors as well as the ventricular pressure sensors that detect changes in blood pressure and ventricular filling respectively. These in turn stimulate the cardiovascular ANS to respond by increasing or reducing vascular tone, chronotropism and inotropism, forming the baroreceptor reflex (La Rovere, Pinna & Raczak, 2008). The parasympathetic and sympathetic divisions of the ANS have different speeds of effect activation, with the parasympathetic division inducing immediate change within about 600 ms and the sympathetic division inducing a slower activation within 2-3 s; a characteristic that is used in analysis to independently assess these arms (Pickering & Davies, 1973). The role of the baroreceptor reflex is to prevent wide fluctuations in blood pressure as well as

ensure that the cardiovascular system responds adequately to any changes in the physiological homeostasis state to maintain organ perfusion. This is illustrated by animal models of arterial baroreceptor denervation, which leads to increased variability and fluctuation in blood pressure, which leads to poor blood flow and organ perfusion (Cowley, Liard & Guyton, 1973). Autonomic dysfunction and neuropathy in diabetes affects the nerves involved in the baroreceptor reflex arc. Impaired baroreceptor reflex is a serious complication of diabetes related to morbidity and mortality and is a negative prognostic factor in patients with CVD, in particular post-myocardial infarction (Clarke & Ewing, 1982; Pitzalis et al., 1998). Preliminary evidence suggests that this derangement in blood pressure and heart rate regulation in the early stage of diabetes-induced baroreceptor reflex impairment may be reversed by preventing metabolic derangements driven by hyperglycaemia, and by better understanding and targeting the underlying mechanisms (Ferreira et al., 1998). The exact nature of baroreceptor reflex failure in diabetes is controversial: in early diabetes, reflex tachycardia in response to falling arterial pressure was observed in experimental diabetes (Maeda et al., 1995); however in chronic experimental diabetes, this reflex tachycardia appears to be preserved (McDowell et al., 1994). Thus, there is a need to assess the baroreceptor reflex in autonomic dysfunction in early diabetes.

One source of contention that has received growing interest is the assessment of assessing autonomic function and baroreceptor sensitivity in experimental animals by invasive methods requiring anaesthesia. Anaesthetics alter central sympathetic output and cardiac autonomic activity, thereby influencing autonomic function and confounding experimental results (Shimokawa et al., 1998). Methods of recording and assessing cardiac autonomic function in conscious animals are thus gaining favour. One such method is HRV analysis. Functions of the cardiac ANS effector control as well as responses to baroreceptor stimuli can be sensitively assessed as changes in HRV (Mesangeau, Laude & Elghozi, 2000). HRV refers to the changes or variability in the heart rate or the interval between successive beats. This reflects intact autonomic function that is constantly changing and responding effectively to physiological changes. With sensitive methods of determining early autonomic dysfunction, the question of investigating early ventricular dysfunction arises as co-

occurrence of these complications is common and a temporal link has been suggested by animal models studies (Marangoni et al., 2014).

1.2.4. VENTRICULAR REMODELLING

The detrimental effects of diabetes on ventricular function have been studied since Rubler et al. (1972) described a cardiomyopathy syndrome in patients without any other common risk factors for this condition, except diabetes. Although end-stage diabetic cardiomyopathy is an established clinical entity, temporality and drivers of its progression are poorly understood and remain controversial in early diabetes, particularly in terms of the timing of detectable dysfunction. In early diabetes, ventricular function, comprising of systolic (contraction) and diastolic (relaxation), is compromised in a pattern of diastolic dysfunction with preserved systolic function; and progresses to systolic and diastolic function failure leading to a dilated cardiomyopathy and heart failure in end-stages (Aneja et al., 2008).

In early diabetes, diastolic dysfunction precedes systolic impairment in type 1 diabetic patients and animal models of diabetes (Litwin et al., 1990; Schannwell et al., 2002), and occurs even in short-term conditions like gestational diabetes (Oliveira et al., 2015). This early diastolic dysfunction is reversible by ameliorating the metabolic derangements induced by diabetes and hyperglycaemia, which can be achieved by optimising glycaemic control or improving glucose tolerance in the postpartum period (von Bibra et al., 2004; Freire et al., 2006). The nature of diastolic pathology in early diabetes is not well described. While the nature of the diastolic pathology reported in most chronic diabetes studies is ventricular stiffness, which has been attributed to hyperglycaemia-induced deposition of advanced glycation end-products (AGEs) and myocardial fibrosis (Van Heerebeek et al., 2008), there remains controversy on the role of these factors in early diabetes. While in-vivo non-invasive investigations such as echocardiography are popular and can provide accurate information on ventricular function, the humoral and other multifactorial contributors to cardiac function, such as the anaesthesia necessary to perform these investigations, confound the results of such investigation. Perhaps, studying these aspects of ventricular function and structure in isolation can derive some clarity. The

factors driving the progression from the reversible complications seen in early diabetes to permanent CVDs seen in chronic diabetes are unclear. Oral anti-diabetic drugs and insulin are effective but do not always correct the associated metabolic derangements and gluco-regulatory dysfunctions (Campbell, 2003). This has raised questions as to whether glycaemic control is beneficial at all in diabetes given the results of large randomized trials, which showed little or no benefit.

According to the ADVANCE trial (ADVANCE Collaborative Group et al., 2008), tight glycaemic control in type 2 diabetic patients by a single agent, gliclazide, improved nephropathy but not the incidence of retinopathy or macrovascular complications. The ACCORD trial (The Action to Control Cardiovascular Risk in Diabetes Study Group et al., 2008) was prematurely stopped after the finding that tight glycaemic control increased mortality in high-risk type 2 diabetic patients. Hope was found when although the United Kingdom Prospective Diabetes Study did not find benefit in strict glucose control for myocardial infarction (UK Prospective Diabetes Study (UKPDS) Group, 1998), a follow-up study observed a benefit of preventing CVD in type 2 diabetes derived from hyperglycaemic control in the long-term (Holman et al., 2008). In scrutinizing the reasons for failure of these trials, emerged the concept and understanding of metabolic memory in diabetes. This concept postulated that certain factors are activated in early diabetes, which then drive the progression of diabetic cardiovascular complications despite normalization and effective treatment of the primary symptom of hyperglycaemia by tight glycaemic control (Ceriello, Ihnat & Thorpe, 2009). Closer analysis revealed that there was a substantial increase in hypoglycaemic events due to the tight glycaemic control regimen, which are known to confer cardiovascular risk that maybe comparable to hyperglycaemia (Turnbull et al., 2009; Snell-Bergeon & Wadwa, 2012). This evidence highlights the question of the insufficiency of glycaemic control in treating and preventing the onset and progression of diabetic cardiovascular complications, and the need to better understand the underlying factors to guide the search for an appropriate therapy.

Diabetes also reduces the effectiveness of a majority of interventions and therapeutics used to manage CVD and cardiovascular complications of diabetes. Studies have observed a mixed effectiveness of anti-ischaemic and anti-failure medication, in particular the loss of survival benefits of nitrates and calcium (Ca^{2+})

antagonists in diabetics compared to non-diabetics (Held, Yusuf & Furberg, 1989; Rydén et al., 2007). Antithrombotic and antiplatelet therapy also have reduced effectiveness in diabetic patients and the use of combination or higher dosages is prescribed to achieve therapeutic outcomes (Mak et al., 1997; Antithrombotic Trialists' Collaboration, 2002; Rydén et al., 2013). Therefore, these observations of more severe CAD and complications with poor prognosis and poor response to effective therapy despite comprehensive management, compared to matched non-diabetics, points to differences in the nature and underlying factors of CAD (Arnold et al., 2015). Changes in cardiovascular function and metabolism in diabetes have been suggested to be the underlying mechanisms behind the differences in CVD onset and progression in diabetics (Goyal & Mehta, 2013).

1.3 MECHANISMS OF CARDIOVASCULAR COMPLICATIONS IN DIABETES

The various metabolic, structural and functional derangements induced by diabetes cause stress and injury to the cardiovascular system. These factors cause cellular dysfunction and death, and lead to abnormal remodelling processes that lead to contraction-relaxation insufficiency/abnormalities and overall myocardial insufficiency. The importance of these factors is that they are potential therapeutic targets for prevention or modulation of the course of these conditions, but the underlying mechanisms need to be clarified.

1.3.1 METABOLIC DERANGEMENTS AND TOXIC METABOLITES

Lipid Derangements in Diabetes

Diabetes induces significant derangements in lipid metabolism and plasma lipid composition known as diabetic dyslipidaemia, which predispose to atherogenesis and CVD (Taskinen, 2003). The core components of established diabetic lipid abnormalities are metabolically linked abnormalities comprised of increased triglycerides, very- low-density cholesterol (VLDL-C) and low-density cholesterol

(LDL-C), and reduced high-density lipoprotein cholesterol (HDL-C) (Adiels et al., 2006). Our understanding of the pathophysiology underlying these abnormalities remains incomplete, particularly in early diabetes. It is postulated that in early diabetes abnormalities of VLDL-C drive the inception of diabetic dyslipidaemia. Insulin resistance modulates the metabolism and production of apo B lipoproteins including VLDL-C and LDL-C through increased hepatic production and reduced degradation, increased expression of regulatory microsomal transfer protein (MTP) and reduced HDL production (Chirieac et al., 2000).

Since the Framingham study, growing evidence has established an inverse relationship between HDL-C and CVD (Superko et al., 2012). In diabetes, HDL-C is protective to the cardiovascular system in preventing cardiovascular events and atherosclerosis by exerting endothelial protective effects mediated by nitric oxide (NO) and progenitor cells (Sorrentino et al., 2010). However, HDL-C is a heterogeneous group of molecules, which differ from each other in size, composition and interaction with the cardiovascular system; particularly playing unique roles in diabetes and atherogenesis (Bakogianni et al., 2001). With the advent of analytical ultracentrifugation, unique HDL-C molecules are being identified; two major subfractions of HDL-C have been identified as HDL_2 and HDL_3 (De Lalla & Gofman, 1954). The larger HDL_2 has been shown to be a valuable risk indicator for- and is inversely correlated to myocardial infarction, but the role of the smaller more dense HDL_3 remains unclear while others suggest it to be anti-atherogenic (Brugger et al., 1986; Kontush, Chantepie & Chapman, 2003). Later studies showed HDL_2 and HDL_3 to have up to 3 further subfractions each: HDL_2 can be separated into HDL_2a and HDL_2b, and HDL_3 can be resolved into HDL_3a, -b and -c (Blanche et al., 1981). More recent studies have shown up to 10 subfractions, which are still unclassified (Filippatos et al., 2008). Moreover, differences in these HDL subfractions are associated with coronary disease and have been detected even in patients without differences in total HDL-C concentration (Ballantyne et al., 1982), suggesting that these subfractions play different roles that significantly impact health and disease. The function and roles of these subfractions in health and disease are still being clarified, but preliminary evidence suggests large HDL-C subfractions including HDL_2 are associated with cardioprotection (Barter & Rye, 1996; Frias et al., 2012).

Therefore, further investigation into the changes in these HDL-C subfractions in early diabetes as well as the role they play in diabetic dyslipidaemia and the development of diabetic cardiovascular complications in imperative.

Aberrant Glucose Metabolism and Oxidative Stress

A key mechanism implicated in tissue damage in diabetes is the overproduction of reactive oxygen species (ROS) causing oxidative stress (Brownlee, 2005). Particularly in atherosclerosis and neuropathy where diabetes causes an imbalance in the oxidative and anti-oxidative species. For example, endothelial function involves a balance of the production of antithrombotic NO production and pro-thrombotic endothelin-1 (ET-1), both regulated by insulin signalling via the phosphatidylinositol-3 kinase (PI3-K)/Akt transduction pathway and the ERK/MAPK pathway respectively (Cardillo et al., 1999). In diabetes, the antithrombotic NO signalling is impaired leading to dominance of ET-1 and prothrombotic oxidative stress (Pitocco et al., 2013).

During periods of increased cardiac workload, stress, or ischaemia, the heart relies on glycolysis and pyruvate oxidation as main energy sources. Hyperglycaemia induces a primary cardiac metabolic derangement in glycolysis and pyruvate oxidation with reduced supply of substrates and increased toxic metabolites (Mokuda et al., 1990). These toxic intermediates include polyol pathways, hexosamines, AGEs and their receptors (RAGEs). Hyperglycaemia also induces mitochondrial damage and activation of protein kinase C (PKC), which results in increased production of reactive oxygen species (ROS) (Davidoff et al., 2004). These ROS activate signalling cascades that damage cardiovascular cells (Joseph et al., 2014).

The inhibition of metabolism driving toxic metabolite production also occurs via the direct inhibitory effect of free fatty acids (whose concentration is significantly increase in diabetic dyslipidaemia) on pyruvate dehydrogenase, a rate-limiting step in the glycolytic cycle (Stanley et al. 1997). There is also a restriction of glucose supply to cardiomyocytes that occurs due to reduction in glucose transporters (GLUT) 1 and 4, whose transcription and translocation is regulated by insulin (Russell et al., 1998). Furthermore, oxidative stress, hyperglycaemia and elevated levels of circulating

lipids, inhibit endothelial NO synthetase resulting in reduced NO and endothelial dysfunction and vascular structural remodelling (Goyal & Mehta, 2013).

1.3.2 STRUCTURAL AND FUNCTIONAL REMODELLING

The role of structural and functional remodelling in early diabetes is not well understood. Reduced insulin sensitivity and hyperglycaemia lead to myocardial structural abnormalities and functional impairment. The mechanisms underlying this are predominantly mediated by Ca^{2+} influx abnormality as well as ROS, AGE/RAGE signalling and hexosamine fluxes (Clark et al. 2003). In early diabetes, ventricular functional alterations are observed due to bradykinin-induced noradrenaline release and sympathetic nervous system glucose toxicity, which have also been implicated in the raised neurohormonal levels of noradrenaline observed despite reduced systemic levels (Pietrzyk et al., 2000).

Functional alterations precede structural disturbances in early diabetes (Goyal & Mehta, 2013); nonetheless, provision is made for the argument that structural derangements can be observed in conjunction with, and possibly underlying the functional derangements observed (Litwin et al., 1990; Riva et al., 1998). Diabetic effects on cellular expression of adrenergic receptors are altered leading to abnormalities in adrenergic receptor density (Uekita, Tobise & Onodera, 1997). Stimulation of the β-adrenergic system induces cytotoxic effects (stress response) on the myocardium as well as intrinsic apoptotic signalling cascades via oxidative stress and mitochondrial Ca^{2+} influx. β-adrenergic hyperstimulation also leads to pathological changes in structural protein gene expression pathological changes, which drive the processes of cardiac remodelling involving hypertrophy, interstitial fibrosis and contractile dysfunction (Krenek et al., 2009; Takaki, 2012).

Diabetes also induces an electrical remodelling and is known to be an independent risk factor for arrhythmias. Remodelling of the diabetic atrium has been shown to predispose to atrial tachyarrhythmia and action potential duration abnormalities (Watanabe et al. 2012). This is compounded by cardiac autonomic dysfunction, which can induce fatal ventricular arrhythmias (Wang et al. 2013). At a cellular level, diabetes is known to induce electrical current flux abnormalities including K^+ and

Ca^{2+} current abnormalities by altering gene expression of these ion's transporter proteins e.g. ion channels (Qin et al. 2001; Nishiyama et al. 2001; Abe et al. 2002). These ionic aberrations are associated with electrolyte homeostasis imbalances.

1.3.3 ELECTROLYTE DISTURBANCES

Diabetes causes various imbalances in electrolyte and acid-base homeostasis (Sotirakopoulos et al., 2012). Ca^{2+} disturbance is of particular importance in the heart as Ca^{2+} is an important signalling and intracellular ion involved in various processes and functions. A proposed mechanism of this disturbance in cardiac cells is by ion channel dysregulation in the sarcolemma and sarcoplasmic reticulum, in addition to mitochondrial dysfunction (Heyliger, Prakash & McNeill, 1987; Takeda et al., 1996; Qin et al., 2001). Diabetes is also extensively associated with magnesium (Mg^{2+}) deficiency (Sales & Pedrosa, 2006). The underlying mechanism of this remains unclear although increased renal excretion, reduced absorption and red blood cell sequestration of Mg^{2+} have been implicated. Furthermore, hypomagnesaemia is known to be responsible for cardiac arrhythmias and ventricular contractile dysfunction. Thus, an area of growing interest and research is the role of Mg^{2+} in the pathogenesis and treatment of diabetes and CVDs.

1.4 THE ROLE OF MAGNESIUM IN DIABETES AND CARDIOVASCULAR DISEASE

Mg^{2+} deficiency is implicated in the pathophysiology of various endocrine and metabolic disorders, and is most commonly observed in diabetic patients (Sales & Pedrosa, 2006; Wells, 2008). Significant negative correlations have been demonstrated between plasma Mg^{2+} levels and fasting plasma glucose levels, glycated haemoglobin (a marker of poor glucose control) and the homeostatic model of assessment for insulin resistance (HOMA-IR) (Kao et al., 1999). At a molecular level, serum hypomagnesaemia has been associated with increased production of pro-inflammatory and fibrogenic species, which result in reduction of antioxidative

enzymes and increased apoptosis (Rodriguez-Morán & Guerrero-Romero, 2004). Intracellular Mg^{2+} plays an important role in regulating the production and release of insulin; Mg^{2+} also regulates cellular sensitivity to insulin and acts as a cofactor for insulin-mediated including glucose uptake and utilisation as well as other non-insulin related functions such as maintaining cell polarity and vascular tone (Lee et al., 2009; Shechter, 2010). Furthermore, cellular Mg^{2+} deficiency results in abnormalities of insulin secretion, defective tyrosine-kinase activity, post-receptor impairment of insulin action, glucose transport and oxidation impairment and vascular dysfunction, which are hallmarks of diabetes and its complications (Chetan, Chaudhary & Bansal, 2003; Takaya, Higashino & Kobayashi, 2004)

Mg^{2+} supplementation has been shown to improve insulin sensitivity and glucose control and prevent the development of diabetic complications including CAD (Rodriguez-Morán & Guerrero-Romero, 2004; Nagai & Ito, 2013). Plasma Mg^{2+} imbalances are also a common feature of hospitalised patients with CVD as well as present in the pathogenesis of the cardio-metabolic syndrome (Jee et al., 2002; Mubagwa et al., 2007). Experimentally, Mg^{2+} is known to be cardioprotective (Christensen et al. 1995; Sameshima et al. 1999). We recently showed Mg^{2+} supplementation to be protective to the hypertrophied heart in limiting ischaemia-reperfusion injury, reducing arrhythmogenesis and preserving hemodynamic function (Amoni et al. 2015). However, the effects of- and role of Mg^{2+}, including its potential cardioprotective role and role in the development of dysfunction in the diabetic heart remain unknown.

MAGNESIUM HOMEOSTASIS

Mg^{2+} homeostasis is maintained by regulating its intake, distribution and excretion. Mg^{2+} transverses biologically membranes during the processes of absorption, distribution, cellular uptake and excretion via transport through specialized proteins called ion channels. The function of some of these ion channels, and downstream signalling and metabolic pathways are implicated in diabetic cardiovascular complications and can be modulated by Mg^{2+} (Gomez, 1998; Delva, 2003; Gwanyanya et al., 2004).

Some of the channels involved in Mg^{2+} cellular transport belong to group of channels called transient receptor potential (TRP) channels, which are non-selective channels involved in transport of various ions including Mg^{2+}, Ca^{2+} and zinc depending on various regulatory conditions such as pH, temperature and ionic concentrations including Mg^{2+} concentration (Montell & Rubin, 1989; Nilius & Owsianik, 2011). TRPs have received increasing attention as to their role in accounting for ionic abnormalities not explained by the well-known ion channels (Inoue et al., 2006). In diabetes, TRPs have been pivotal in understanding insulin homeostasis and glucose sensing. The abundant expression of TRPs in pancreatic β-cells has been implicated in the cells' high Ca^{2+} selectivity and the role of Ca^{2+} in insulin release (Watanabe et al., 2009; Cao et al., 2012). McCoy and colleagues (2013) demonstrated TRPM8-mediated neuronal control of insulin secretion that is regulated by a liver insulin-degrading enzyme. TRPV1 is a hepatic sensor for hypoglycaemia and mediates pancreatic insulin resistance (Razavi et al., 2006; Fujita et al., 2007). Thus, TRPs are a growing area of interest in diabetes pathophysiology.

Among TRP channels, TRPM7 is Mg-sensitive and is also a pathway for Mg^{2+} and Ca^{2+} entry into cells (Macianskiene et al., 2008). TRPM7 is a member of the melastatin-related subfamily of TRP channels. TRPM7 is involved in various signalling pathways including regulation of proliferation, survival and remodelling. TRPM7 is abundantly expressed in the myocardium and is critical to electrical function and development of ventricular automaticity and prevention of arrhythmias (Sah et al., 2013). Du and collaborators (2010) observed that TRPM7 is the underlying Ca^{2+} permeable channel involved in atrial fibrillation in cardiac enlargement, stiffening and remodelling as seen in diabetes. Sun and co-workers (2013) showed that TRPM7 expression is increased by hyperglycaemia, implicating TRPM7 in hyperglycaemia induced-endothelial injury. However, the effects of diabetes on magnesium homeostasis and the transporters involved in maintaining homeostasis, such as the cardiac TRPM7 expression and function remain unclear.

The role of Mg^{2+}, as well as the mechanisms underlying the development of cardiovascular complications in early diabetes needs to be clarified. The proposed study will seek to understand the role of Mg^{2+} in diabetic cardiovascular

complications and in the possible mechanisms of cardiac and neural dysfunction in diabetes.

HYPOTHESIS

The null hypothesis in this study is that Mg^{2+} treatment does not attenuate early diabetic cardiac complications of cardiac autonomic dysfunction or left ventricular structural and functional remodelling. Furthermore, Mg^{2+} treatment does not modulate factors implicated as underlying mechanisms in these complications, including hyperglycaemia, dyslipidaemia and hypomagnesaemia.

AIM

To investigate the effects of Mg^{2+} treatment on early diabetic cardiac complications and elucidate possible underlying mechanisms.

SPECIFIC OBJECTIVES

1. To investigate the effects of Mg^{2+} treatment on early diabetic cardiac complications of autonomic dysfunction *in vivo,* and on left ventricular pressure responses to volume in the isolated perfused heart.

2. To determine whether abnormalities of lipid metabolism and magneisum homeostasis are possible underlying mechanisms of diabetic cardiovascular complications, by assessing effects on Mg^{2+} treatment on lipid profile, plasma- and cardiac tissue Mg^{2+}, and ventricular structure.

CHAPTER 2: METHODS

All experimental protocols and procedures were approved by the University of Cape Town Animal Ethics Committee (AEC) (project number FHSAEC 014/014, appendix 1). All animal husbandry and studies were performed in compliance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health, 2011), following training and accreditation by the South African Veterinary Council.

2.1 EXPERIMENTAL LOGISTICS AND DESIGN

2.1.1 HUSBANDRY OF LABORATORY ANIMALS

In this study, adult male rats (*Rattus norvegicus* species - *Wistar* strain) of weight between 250-300g were used. Only male animals were used, to avoid the confounding effects of the female hormonal cycle, which has an effect on mechanisms underlying susceptibility to cardiac remodelling (Wong et al., 2013), abnormalities of lipid profiles (Bittner, 2005; Kleiblová, Springer & Haluzík, 2006) and insulin insensitivity/impaired glycaemic control (Adolfsson et al., 2012), which are essential aspects of this study. The Wistar strain, which I have used previously (Amoni et al. 2015), is a commonly used laboratory rat due to its passive nature and ease of husbandry and was used for continuity. The selected weight is also widely used as it is comparable to young-middle aged humans (Dorsett-Martin, 2004; Horn et al., 2012). The animals included in this study were obtained from the University of Stellenbosch Animal Unit, while test animals (used for experimental procedure optimisation, and not included in the study) were obtained from University of Cape Town Animal Unit. All animals were given two days to acclimatise to the Department of Human Biology satellite animal facility environment after transportation from the Animal Unit. Animals were housed in conventional open top cages in a temperature-controlled room and had access to standard rat chow (SafMed, SA) and water *ad libitium*. The temperature was kept between 22-26 °C and a 12-hour light/dark cycle

with optimal light intensity of 250-350 lux was maintained (Bellhorn, 1980; Clough, 1982). The animal facility's temperature and environment were monitored daily as these can affect the animals' sensitivity and responses to drugs (Horn et al., 2012; Ferguson et al., 2014).

2.1.2 THE DIABETES MELLITUS DISEASE MODEL

We studied an insulin dependent diabetes mellitus (IDDM) model in which diabetes was induced by pharmacological 'pancreatectomy' with streptozotocin (2-deoxy-2-(3-(methyl-3-nitrosoureido)-D-glucopyranose). Streptozotocin (STZ) is an antibiotic synthesized by *Streptomycetes achromogenes* that induces pancreatic β-cell specific cytotoxicity and death by DNA alkylation and damage (Elsner et al., 2000; Delaney et al., 2015). STZ is taken up by insulin-producing β-cells in the pancreas via glucose transporter 2 (GLUT2), that is primarily specific to pancreatic cells; this makes it a pancreas-specific toxin sparing other tissues including the cardiovascular system from toxicity (Fein, Malhotra & Strobeck, 1981; Sanyal et al., 2012). The STZ model is widely used to study early cardiovascular complications of diabetes due to the rapid onset of diabetes and negligible cardiotoxicity (Deeds et al., 2011).

A single intraperitoneal injection of STZ (50 mg/kg) was administered to induce diabetes. Usage of a such a relatively low dosage has the advantage of low incidences of STZ-toxicity and mortality without the need for insulin therapy (Young et al., 2005; Choi et al., 2013). Furthermore, the intraperitoneal route is as effective as the intravenous route and less invasive (Katsumata & Katsumata, 1992; Szkudelski, 2001). The STZ was prepared freshly before administration in a 0.1M citrate buffer at pH 4.5 (Appendix 2) (Deeds et al., 2011). Control animals were injected with equivalent volumes of the vehicle, citrate (figure 2-1). Daily tail blood glucose measurements were conducted with a standard glucometer (Acu-Check, SA). Animals that had a glucose level above 15 mmol/L after 3 days were considered diabetic (Cagalinec et al., 2013; Choi et al., 2013). Daily monitoring involved weighing the animals and observing them for signs of dehydration or distress.

2.1.3 THERAPEUTIC INTERVENTION

The therapeutic intervention studied in this protocol was Mg^{2+} treatment. Therefore, animals were treated with therapeutic doses of Mg^{2+} (270mg/kg i.p.), once daily for 7 days. This optimal Mg^{2+} dose has been shown to be neuroprotective and is therapeutically effective as a membrane stabiliser in clinical treatment of eclampsia, epilepsy and arrhythmias (Gomez, 1998; Sameshima, Ota & Ikenoue, 1999; Dubé & Granry, 2003). Furthermore, our lab has recently shown this dose of Mg^{2+} to be cardioprotective and antiarrhythmic in isoprenaline-induced cardiac hypertrophy (Amoni et al., 2015). Mg^{2+} was given as magnesium sulphate ($MgSO_4$) dissolved in isotonic saline. The treatment control groups received the equivalent volume of vehicle (saline) injections (figure 2-1).

2.1.4 EXPERIMENTAL DESIGN

During the 7-day treatment phase, all animals underwent non-invasive *in vivo* studies, including daily body weight and blood glucose monitoring. Animals that died during the treatment stage were excluded and replaced. On day 8, *in vivo* assessment of HRV, lipid profile and CVD risk was performed. Thereafter, all animals underwent terminal *ex vivo* experiments, which required harvesting of the heart for either Langendorff perfusion studies or ventricular tissue analysis (histological analysis and tissue Mg^{2+} assay). The animals in both sets of terminal experiments were further divided into 4 groups of 17-20 animals per group. These were then further sub-divided into three groups assigned to terminal experiments: 1) In-vivo HRV & ex-vivo isolated heart experiments (n = 8-10/group); 2) Histological studies (n = 5-7); and Plasma lipid- and Mg^{2+} assay, and Cardiac tissue Mg quantification (n = 8-10/group). Estimated numbers in each sub-group were derived by power calculations using effect sizes obtained from literature (figure 2-1).

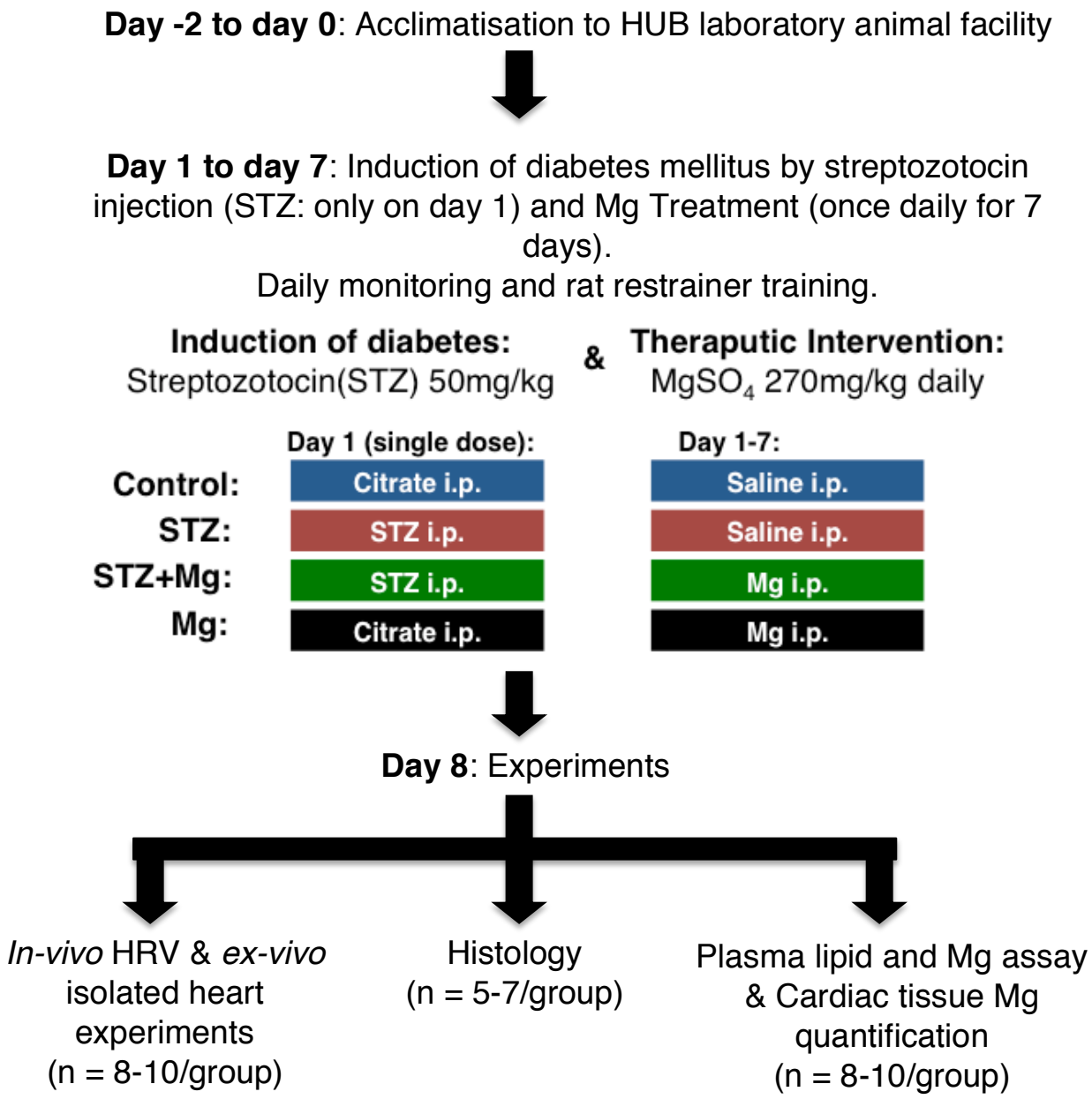

Figure 2-1: Experimental design and treatment protocols.

Animals were divided in to 4 groups: control, diabetes disease model, diabetes and Mg^{2+} treatment, and Mg^{2+} treatment; treated for 7 days and then some animals underwent *in*-vivo HRV testing and then were sacrificed for Langendorff experiments while others were sacrificed for cardiac tissue- and plasma studies. STZ – Streptozotocin; Mg^{2+} – Magnesium; i.p. – Intraperitoneal.

2.2 EXPERIMENTAL PROTOCOLS

2.2.1 *IN VIVO* HRV EXPERIMENTS

Classically, HRV is assessed from electrocardiogram (ECG) recordings in anaesthetized rats (de Moura, dos Santos & Fontes, 2005). Although this method is technically effective and accurate, the results are confounded by anaesthesia which alters the sympathetic output and modulates cardiac automaticity (Shimokawa et al., 1998). Anaesthesia also stimulates humoral factors, such as the renin-angiotensin system, which modulate cardiovascular function via Angiotensin I and II receptor signalling (Colson, Ryckwaert & Coriat, 1999). Therefore, to assess physiological HRV and cardiac autonomic integrity, on the 8th day, prior to terminal experiments, I performed recordings of heart rate in the absence of anaesthesia by restraining the animals in a standard rat restrainer. Resting heart rate of restrained, conscious animals was recorded by non-invasive tail pulse plethysmography, as described by Bedette and associates (2008). This is a validated method of recording heart rate and assessing variability, working on the premise that the peak of the pulse wave corresponds with the R wave peak of the cardiac cycle on an ECG (Rodríguez et al., 2012).

(a) Tail pulse recordings

To facilitate the recording of resting heart rate by reducing the stress of being restrained, all animals were acclimatized to the rat restrainer for at least five days (during treatment phase) before recording, which occurred separately from the restraining for blood glucose level monitoring (Rodríguez et al., 2012; DeAndrade et al., 2014). On the day of recording (Day 8), each animal was placed in the restrainer with the tail passed through the open end of the restrainer (figure 2-2), and the pulse transducer (Lasec, SA) attached to the base of the tail on the left inferior-lateral side next to the ventral tail artery (in a manner to avoid total compression to prevent ischaemia). The transducer was connected to a Power Lab data acquisition system (ADInstruments, Aus, figure 2-2). The animal was then given 5-10 min to acclimatize, prior to recordings. At least 3 recordings were taken for at least 5 min duration for each animal.

(b) Heart rate recording protocol

The protocol for recording heart rate consisted of three phases: (1) a clean pulse recording for 5 min when the animal was lying flat (0° to the ground); (2) The animal (rat-head side of the restrainer) was then elevated to a 70° angle head-up-tilt (HUT) to induce orthostatic stress followed by recording for 15 min, as shown in figure 2-2. The orthostatic stress of HUT induces cardiovascular reactivity which gives an indication of baroreceptor reflex to changes in pressure due to pooling of blood under gravity in the lower parts of the body and accentuates differences in cardiac sympathetic *vs.* parasympathetic innervation (Bedette, Santos & Fontes, 2008; DeAndrade et al., 2014). Finally (3) the animal was returned to the resting flat position and heart rate was recorded for 5 min. All recordings were visualised using LabChart Pro software (ADInstruments, Aus), see appendix 3 for more details.

(c) HRV analysis in LabChart Pro

Analysis of the heart rate was conducted using LabChart Pro software (ADInstruments, Aus) using the inbuilt rat pulse settings that were modified to fit the transducer threshold (appendix 3). The tracing analysis was also manually reviewed to ensure all beats were detected and included in the analysis, and that all artefacts were excluded. HRV analysis was performed on both time domain and frequency domain parameters (power spectral density analysis), and was used to assess the sympathetic and parasympathetic functional components of the cardiac autonomic innervation. The frequency limits within the power spectra were classified according to the Task Force of The European Society of Cardiology and The North American Society of Pacing and Electrophysiology (1996) guidelines into: very low frequencies (VLF) < 0.2Hz; low frequencies (LF) 0.2-0.8Hz; and high frequencies (HF) 0.8-2.8Hz. HF analysis (as well as time domain parameter: root mean square of successive NN differences, RMSSD) was used to assess vagal tone, while the ratio of LF/HF was used as an indication of the sympathetic balance.

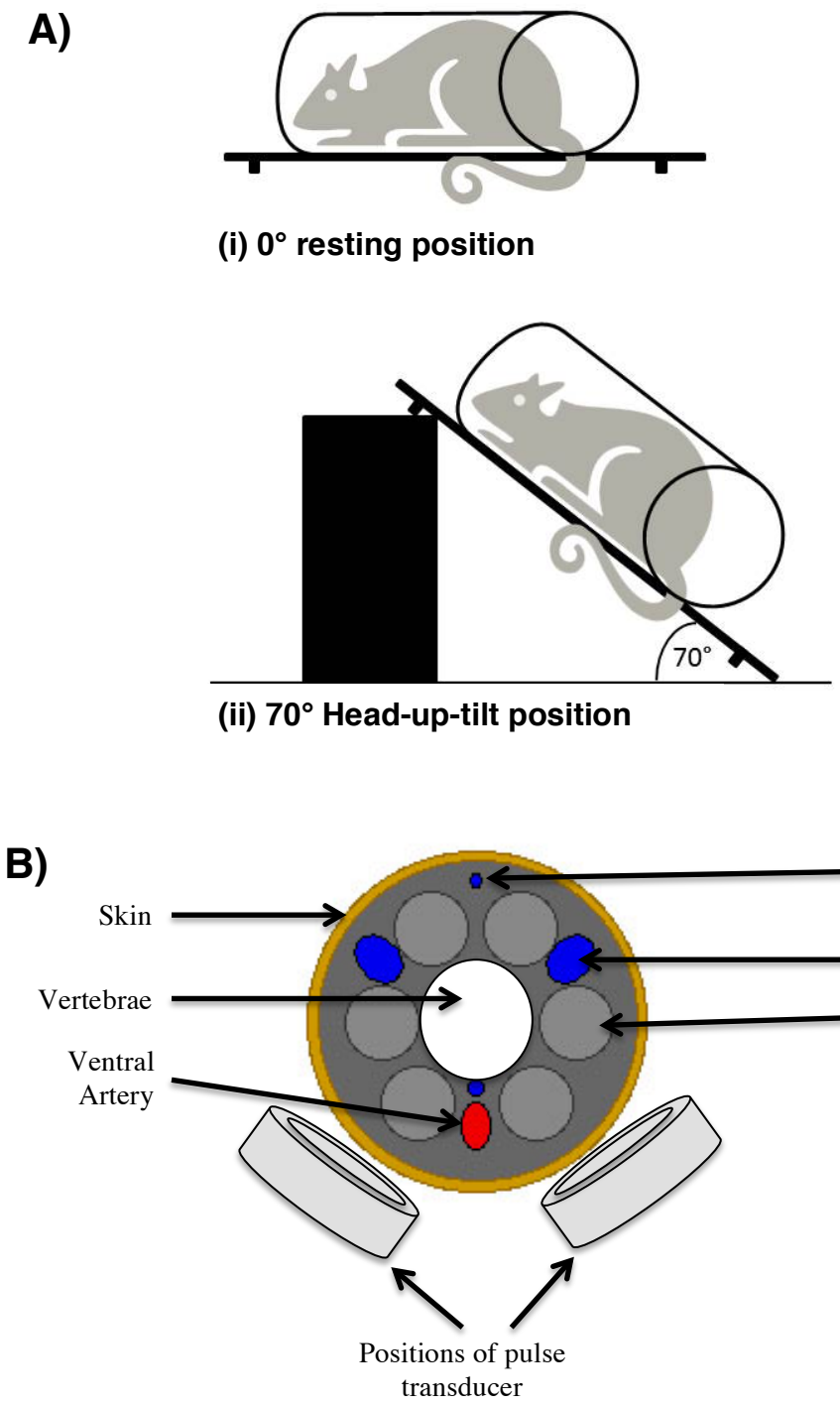

Figure 2-2: Heart rate variability recording technique.

(A) The recording positions for heart rate variability analysis: (i) the resting baseline position; (ii) the 70° Head-Up-Tilt position. (B) Transverse section of a rat-tail anatomy showing the positioning of the pulse transducer for heart rate plethysmography recordings.

2.2.2 PREPARATION OF *EX VIVO* EXPERIMENTS

For *ex vivo* experiments to study the effects of diabetes and Mg^{2+} treatment on, plasma lipids and Mg^{2+} concentration, cardiac structure, function and tissue Mg^{2+} concentration, the treated rats were sacrificed and their hearts isolated for perfusion according to the preparation described by Oscar Langendorff (Skrzypiec-Spring et al., 2007). At the end of perfusion, hearts were fixed for histological studies or stored for tissue Mg^{2+} concentration assays.

Anaesthesia and surgical cardiac harvesting

Prior to the surgical extraction of the heart, the animals were anticoagulated with heparin (500 I.U./kg i.p.) and after waiting 15 min for the heparin to take effect; the animals were anaesthetized with sodium pentobarbital (70 mg/kg i.p.). Once appropriate depth of anaesthesia was achieved (assessed by loss of a positive pedal withdrawal reflex), a transabdominal-thoracotomy was performed; exposing the heart that was to be excised at the level of the great vessels. The heart was then quickly transferred into ice-cold filtered (7µm pore filter paper, GE Healthcare, Germany) Krebs-Henseleit (K-H) buffer solution (0-4°C), containing 118.5 mM NaCl, 4.7 mM KCl, 25 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 1.2 mM Mg_2SO_4, 1.8 mM CaCl and 11 mM Glucose at pH 7.4 that was oxygenated with Carbogen of 95% oxygen and 5% Carbon dioxide (Air Liquid, SA). The heart was rapidly mounted on the Langendorff perfusion system by aortic cannulation and perfused as described below.

2.2.3 BLOOD PLASMA ANALYSIS

Immediately following removal of the heart, approximately 3-4 ml of blood was collected from the chest cavity and centrifuged at 1500 rpm (C2500R Labnet, USA) for 15 min at room temperature. The plasma supernatant was decanted into 2 tubes and stored at -80°C to be batch analysed at a later stage once all samples were collected. An aliquot (0.5ml) of the plasma was used for plasma Mg^{2+} determination and the rest, 2-3ml used for lipid profile analysis (figure 2-3).

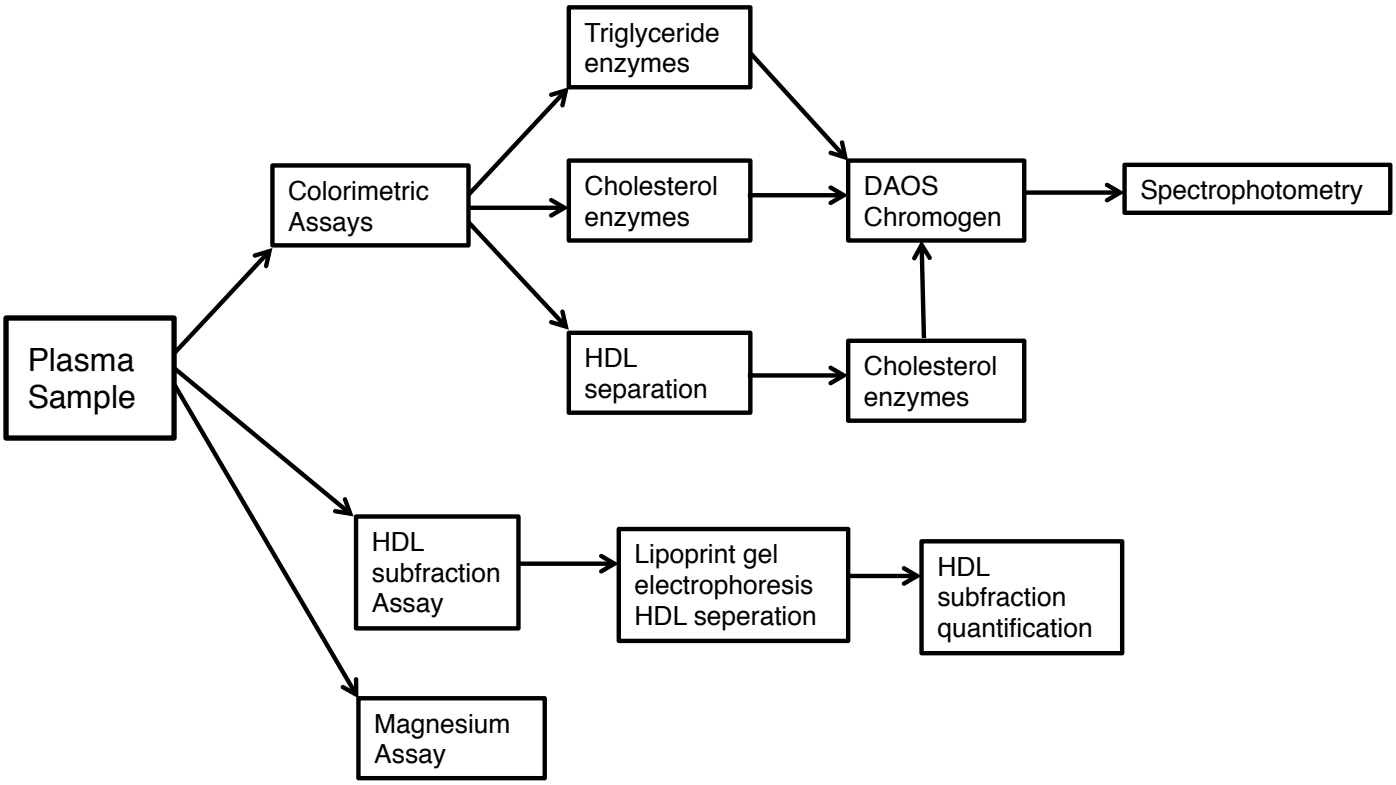

Figure 2-3: Flow diagram of plasma analysis protocol. HDL- High-density lipoprotein.

(a) Lipid Profile Studies

The lipid profile determination consisted of the quantification of the triglyceride (TG), total cholesterol (TC), and high-density lipoprotein cholesterol (HDL-C) concentration by colorimetric assay and spectrophotometry; the HDL-C subfractions were also separated and quantified by HDL-C fractionation. The colorimetric assays utilised specific enzymatic reactions for TGs and cholesterol to generate hydrogen peroxide, which was then quantified by its reaction with a chromogen, dimethoxyaniline sodium salt (DAOS), while the separation of HDL-C into its subfractions used a specialized electrophoresis apparatus.

i) Determination of triglyceride concentration

TGs form major components of very low-density lipoprotein cholesterol (VLDL-C) and chylomicrons, and play an essential role in energy metabolism, transport and signalling pathways. TG derangements are associated with diabetes and have been implicated in the pathogenesis of cardiac dysfunction and CVD including

atherosclerosis. Therefore, plasma TG concentrations were determined by the glycerol-3-phosphate oxidase (GPO)-DAOS enzymatic reaction colorimetric assay method (Wako pure chemicals, USA) as described by Spayd et al. (1978). The assay is based on hydrolysis of TG to glycerol by lipoprotein lipase. Glycerol was then converted to glycerol-3-phosphate (G3P) by glycerolkinase. G3P was then oxidized by GPO producing hydrogen peroxide, which was quantified by spectrophotometry.

ii) **Determination of total cholesterol**

Hypercholesterolemia is a risk factor for CVD and is associated with diabetes. Therefore, plasma TC, was determined by the cholesterol oxidase-DAOS enzymatic reaction colorimetric assay method (Wako pure chemicals, USA) as described by Allain et al. (1974). The assay principle was: cholesterol esters in the sample were decomposed by cholesterol esterase to free cholesterol; free cholesterol was then oxidized by cholesterol oxidase producing hydrogen peroxide, which was then quantified by the chromogen condensation using spectrophotometry.

iii) **Determination of high-density lipoprotein cholesterol**

Cholesterol can be classified by lipoprotein density into high-, low- or very low-density lipoprotein cholesterol. Low-density lipoprotein cholesterol (LDL-C) is associated with oxidative stress and increased risk of CVD, while high-density cholesterol (HDL-C) is cardioprotective and reduces the risk of CVD. The concentration of HDL-C was quantified, and then used to calculate the concentration of LDL-C using the Friedewald formula (equation 1). To quantify HDL-C, the LDL-C and VLDL-C was removed from the plasma sample by precipitation as described by Gidez et al., 1982, using a method adapted from Warnick & Albers, 1978. The principle of this assay is that LDL-C and VLDL-C are apolipoprotein-B (apo-B) containing-cholesterols, while HDL-C is the only non-apolipoprotein-B (apo-B) containing-cholesterol. Apolipoprotein-B (apo-B) containing-cholesterols form insoluble complexes with sulphated polysaccharides in the presence of divalent cations. Therefore, by adding sodium heparin sulphate-manganese chloride to the plasma sample, only LDL-C and VLDL-C are specifically precipitated out of solution,

leaving only HDL-C in the plasma (appendix 4). This HDL-C was then quantified using the enzymatic cholesterol assay (2.2.3(a) (ii)), by spectrophotometry.

***iv)* Colorimetric reaction and spectrophotometry**

The hydrogen peroxide produced by the specific enzymatic reactions with either TGs or cholesterol was quantified as a surrogate marker of the actual concentration. This was done by allowing it to react with a chromogen, DAOS, producing a blue pigment (McGowan et al., 1983). The reaction is dependent on hydrogen peroxide and thus, the amount of blue pigment produced is proportional to the TG, TC or HDL-C concentration. The amount of pigment produced was determined by measuring the absorbance of the solution by spectrophotometry (SpectraMax, Labotec, SA) at 600 nm for both triglyceride and cholesterol assays. The corresponding concentration was determined using a standard curve of known concentrations of TG, TC or HDL-C (detailed protocols in appendix 4).

***v)* Low-density lipoprotein cholesterol and CVD risk estimation**

LDL-C was estimated using the Friedewald formula (Friedewald, Levy & Fredrickson, 1972):

LDL-C = [Total cholesterol] – [HDL-C] – ([TG]/5) ……Equation 1

The plasma atherogenic risk index (or coronary risk index), a predictor of CVD was quantified by the formula (Ishiguro et al., 1998; Dobiásová, 2006):

Plasma atherogenic index = Log_{10}(TC/HDL) ……..…Equation 2

***vi)* Determination of HDL-C subfractions**

HDL-C was separated into subfractions grouped into large, intermediate and small sub-fractions by electrophoresis and quantified by the Quantimetrix Lipoprint System HDL subfractions kit (Quantimetrix, California, USA). This is a pre-packed kit of polyacrylamide gel electrophoresis tests that separates and measures HDL-C subfractions in plasma.

A 40 µL aliquot of the plasma sample was mixed with 60 µL loading dye and loaded into the top of a Lipoprint HDL precast gel tube. The dye binds to the cholesterol in the lipoproteins as the gel sets aided by photopolymerization. These stained lipoproteins are then subjected to an electrophoresis current. The stacking gel

concentrates the stained lipoprotein particles into a sharp narrow band. Then as they migrate through the separating gel, they are resolved by the sieving action of the gel, into lipoprotein bands according to their size (figure 2-4). Albumin separates farthest from the lipoproteins, ending up near the bottom of the tube; this is used as a running control.

After this electrophoretic separation, the stained HDL-C subfractions are then identified by their mobility (Rf) using the VLDL/LDL as the lagging reference (VLDL/LDL = 0) point and albumin as the leading reference (albumin = 1).

The relative area for each HDL-C subfraction is then determined and multiplied by the total HDL-C concentration of the sample to give the concentration of the subfractions in mMl/L. The HDL-C concentration was also compared to the concentration obtained from the colorimetric analysis (details in appendix 4).

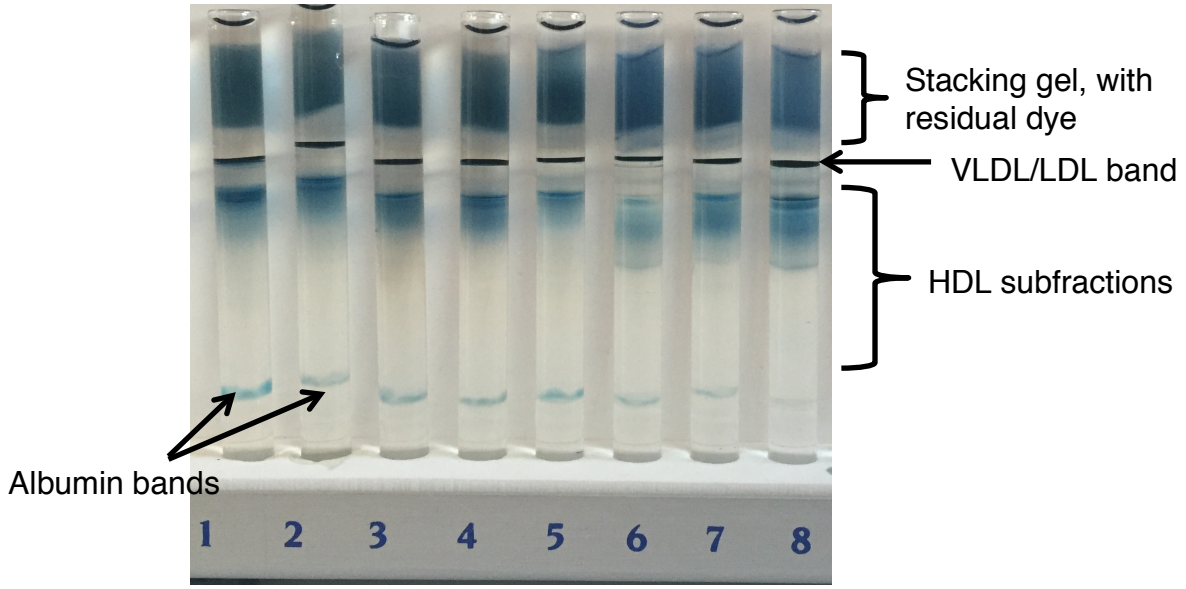

Figure 2-4: Quantimetrix Lipoprotien high-density cholesterol (HDL-C) subfraction separation. Note the HDL-C being resolved into bands of subfractions, the VLDL/LDL and albumin as the reference points.

(b) Plasma magnesium assay

Diabetes is known to induce Mg^{2+} deficiency. Moreover, Mg^{2+} treatment can alter blood or tissue Mg^{2+} concentration. Therefore, I performed blood plasma and cardiac tissue Mg^{2+} concentration studies to investigate any changes.

A 0.5mL aliquot of the plasma samples was used for plasma Mg^{2+} determination. Unfortunately, our lab did not have the equipment or reagents to perform the Mg^{2+} assay. Therefore, this section of tests was performed at PathCare (SA), a veterinary pathology laboratory. The Mg^{2+} plasma assay was performed by automated photometric assay (Beckman Coulter AU600 Analyser, USA). The principle of the assay involves the reaction of Mg^{2+} with a chromogen xylidyl blue in a basic solution of pH 11.4 to produce a purple pigment (Chromý, Svoboda & Stěpánová, 1973). Ca^{2+} is chelated by glycolethendiamine-N,N,N,N-tetracetic acid (GEDTA) to prevent cross-reaction. The absorbance is read at 520 nm and is proportional to the Mg^{2+} concentration in the plasma or solution.

2.2.4 PRESSURE-VOLUME RESPONSE STUDIES

To study ventricular functional properties, isolated heart perfusion experiments were performed on a constant pressure (73 mmHg / 100 mmH_2O) Langendorff perfusion system (Litwin et al. 1990; Riva et al. 1998, figure 2-5). Prior to use, the Langendorff was cleaned with boiling water and then distilled water. It was then primed with the K-H solution that was used to perfuse the heart. The K-H solution was freshly prepared daily. The K-H buffer was maintained at a temperature of 38°C by a water bath surrounding the Langendorff ‘s reservoir and heat exchange coil; and the pH was maintained between 7.2-7.4.

(a) Preparation of Isolated Hearts for Functional Measurements

Once the heart was harvested and mounted on the primed Langendorff perfusion system (as described above); it was secured on the cannula by tying a 3-0 silk suture around the aorta in a groove at the bottom end of the cannula. A left ventricular (LV) balloon was then inserted into the left ventricle via the left atrial appendage and connected to a pressure transducer (Lasec, SA) to measure the left ventricular pressure changes. ECG electrodes were attached as follows: a superficial needle (negative electrode) was threaded superficially into the apex of the heart and hung in such a way as not to impede the contraction of the heart; a clip (positive electrode) was placed around the free end of the aorta just above the ligature securing it to the cannula, and a neutral electrode clip was placed around the free

metal part of the cannula to serve as a ground electrode. The heart was then lowered into an air bath chamber maintained between 36-38°C, which was then covered to minimize heat loss, (figure 2-6). Once the Langendorff preparation had successfully been established, either a pressure-volume relationship protocol (2.2.3) was carried out, or the hearts were frozen and stored for tissue Mg^{2+} concentration assays (2.2.5).

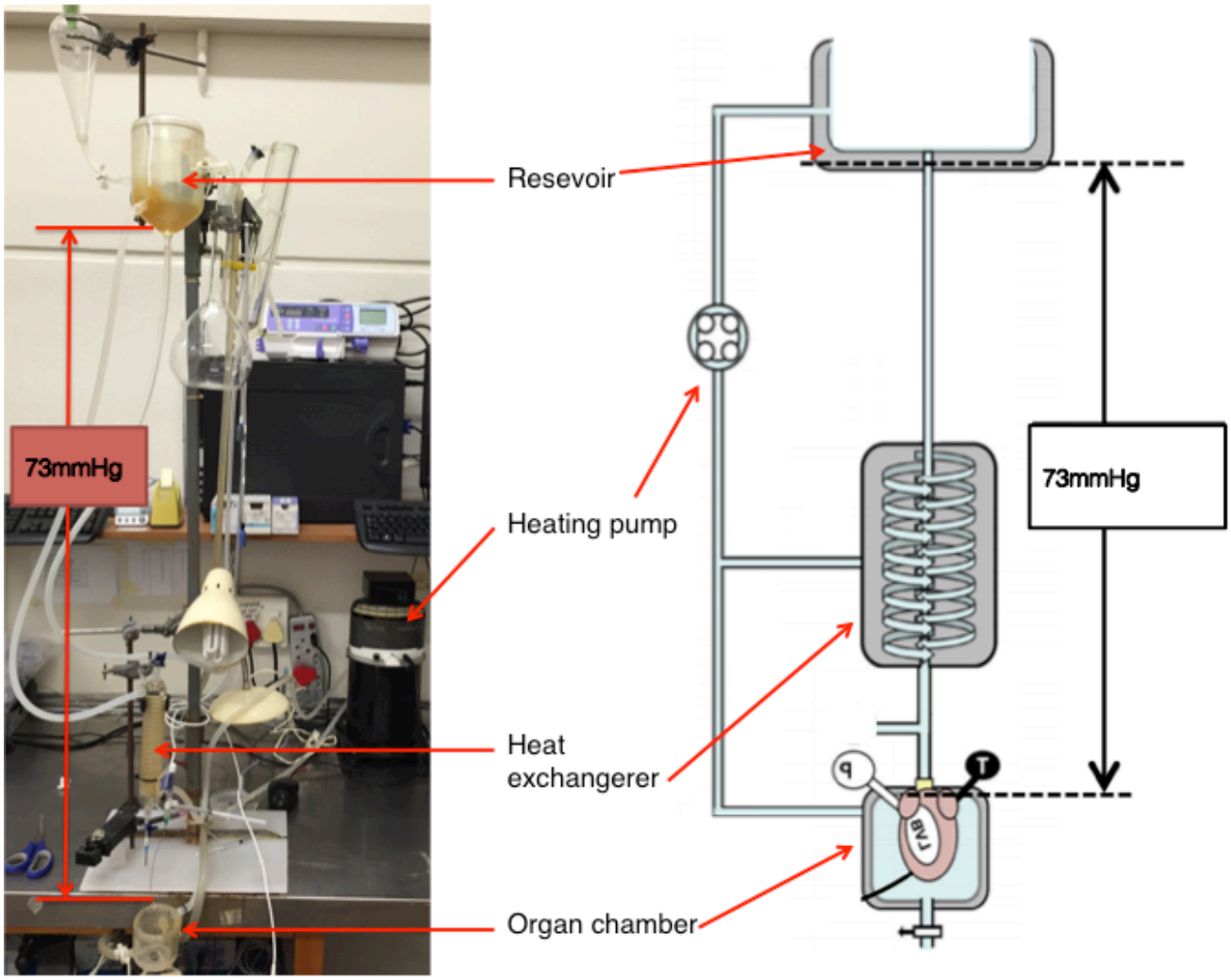

Figure 2-5: The Langendorff perfusion system.
Schematic diagram adapted from Bell et al. (2011).

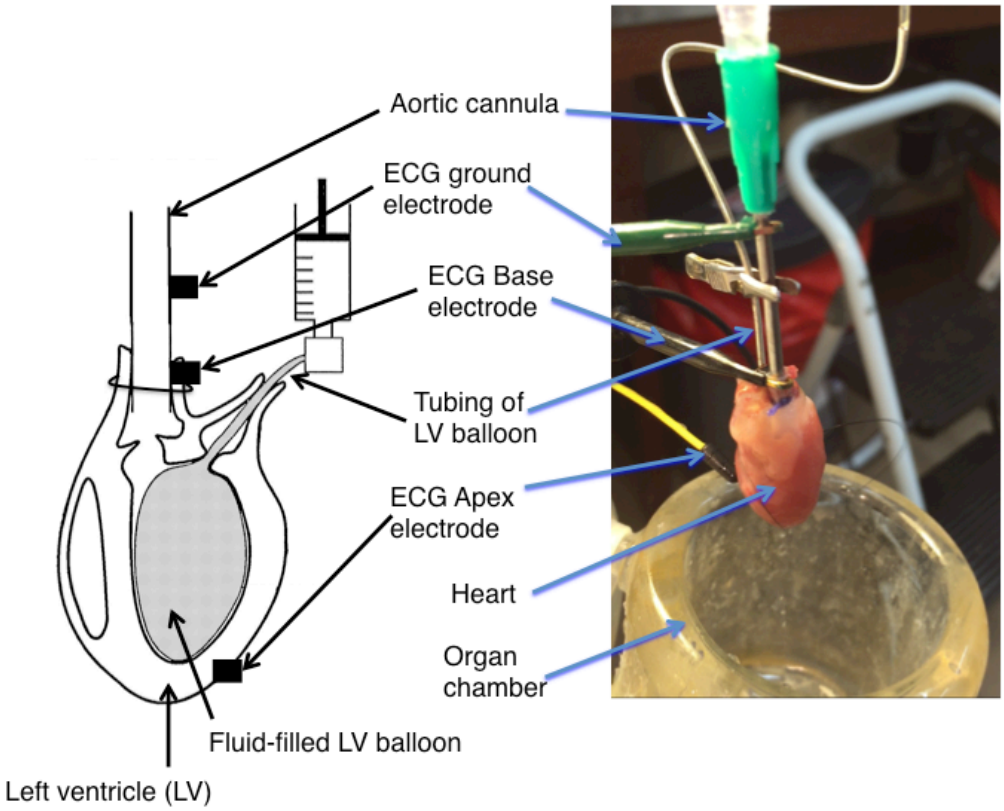

Figure 2-6: The isolated rat heart mounted on a cannula for Langendorff perfusion. (Right) Actual image from experiments showing the organisation of a heart cannulated on the Langendorff rig with ECG leads attached and a LV balloon *in situ*. (Left) Schematic of cannulated heart correlating internal positioning of the LV balloon.
ECG – Electrocardiogram; LV – Left ventricle.
Schematic diagram adapted from Dick & Lab (1998)

(b) Pressure-Volume Relationship Protocol

The P-V protocol involved stabilization for 10 min, during which the LV balloon was deflated completely, and the heart perfused without intervention. Thereafter, the LV balloon was periodically filled with 10 µl increments of bubble-free water using a Hamilton syringe (Sigma-Aldrich, SA). After every increment, the heart was allowed 1-3 min to adjust and come to a stable rhythm. At this point, the end-diastolic pressure (EDP) and end-systolic pressure (ESP) for the given volume were recorded. The LV diastolic volume intercept at zero pressure (V_0) described as the volume at which the end diastolic pressure was 0 mmHg, was recorded. This gives an indication of LV dimensions and a measure of LV function that could be

modulated by remodelling: dilatation or hypertrophy (Osadchii et al., 2007; Hodson et al., 2014). After the cut off EDP of 20 mmHg was reached, the experiment was terminated. The hearts were taken off the Langendorff apparatus, weighed and fixed in formalin for histological assessment (section 2.2.5).

The pressure-volume relationship was used to derive the end-diastolic and end-systolic elastance, which is the gradient of the pressure-volume relationship graph. The gradient was calculated between 50 and 70 µl, being the most stale and central portion of the graph, using the following formulae:

$$\Delta Pressure/\Delta Volume = EDP2 - EDP1 / Volume2 - Volume1 \quad \text{Equation 4}$$

$$\Delta Pressure/\Delta Volume = ESP2 - ESP1 / Volume2 - Volume1 \quad \text{Equation 5}$$

(c) LabChart Analysis

EDP and ESP were captured and analysed using LabChart v8 software (ADInstruments, Aus). The following exclusion criteria were imposed on all experiments: hearts that took longer than 3 min to be mounted on the Langendorff system; hearts that did not beat after 5 min post-excision; hearts that were bradycardic (< 150 bpm), hypotensive (< 50 mmHg) or had a coronary flow rate less than 4 ml/min were excluded from the protocol and analysis.

2.2.5 CARDIAC TISSUE STUDIES

Animals used in these experiments were anticoagulated and anaesthetized, then their hearts surgically harvested and mounted on the Langendorff perfusion system as described previously (2.2.2). Once the heart was mounted on the primed Langendorff system, it was briefly perfused for approximately 1-3 min to wash out any residual blood from coronary circulation.

(a) Cardiac Tissue Magnesium Concentration Determination

The hearts were then cut into 4 pieces separating the chambers and stored in liquid nitrogen until batch processing could be done. On the day of batch processing, the LV tissue of each of the hearts was retrieved from liquid nitrogen storage and placed in an icebox to maintain the cold chain. An approximately 0.1 g piece of the LV tissue was weighed out. This was then minced in 1 ml millipore-purified distilled water using

surgical scissors. The finely minced sample was then sonicated (Soniprep, MSE, UK) twice for 15 s, and cooled on ice for 5 min between each round. The homogenized samples were then centrifuged at 10000 RCF for 10 min. The supernatant was collected and stored at -20°C. The Mg^{2+} concentration assay was then performed by PathCare (RSA) described above (2.2.3(b)).

(b) Histological Assessment of Ventricular Structure

Following the brief perfusion, the hearts were quickly weighed and sliced into four 2 mm thick slices from the apex to the base of the heart, and fixed in 10% phosphate-buffered formaldehyde for 24-48 hours. The hearts were stored in 70% Ethanol until all hearts could be processed simultaneously. Tissue processing involved dehydration in serial increasing concentration of ethanol: 70%, 96%, 100%; clearing in xylene and finally impregnation with paraffin wax using a standard tissue processing system (Leica TP1020, SA). The heart slices were then embedded in wax blocks to facilitate sectioning. Each of the four heart slices was then sectioned on a microtome (Leica RM2125RT, SA) into 4µm thick sections. The sections were then stained using standard haematoxylin and eosin (H&E), and Masson's trichrome stains (Petersen, 2014).

After staining, the slices were viewed using an upright widefield microscope (AxioSkop 200, Zeiss, DEU) with transmitted light using 5x/10x/40x objective lenses. Images were captured with a colour, digital, charged-coupled device camera (AxioCam HBO, Zeiss, DEU) using AxioVision 4.7 software (Zeiss, DEU). The resolution of the microscope was approximately 0.2 µm and scale bars were used to measure and indicate final magnification size.

After microscopy, the micrographs were analyzed digitally according to the method described by Ruifrok & Johnston 2001. This method was previously optimised in our lab (Garson et al., 2012) using ImageJ software (NHI, USA) and a colour deconvolution plugin. Briefly, deconvolution of the stain vector with the plugin produced red, blue and green colour channels. The red colour channel represents the stained areas of fibrosis/collagen. This was then quantified by: (whole area – red channel area) / whole area.

2.3 DRUGS AND CHEMICALS

All chemicals and drugs used in this protocol were procured from Sigma-Aldrich (SA), unless otherwise stated.

2.4 STATISTICAL ANALYSIS

Data presented in this study is expressed as mean ± SEM. Data analysis was performed with GraphPad Prism 6 (California, USA) software package. Data was subjected to Shapiro-Wilk normality tests. Student's t-test was used to compare two groups of data. Parametric data from more than 2 groups was analyzed using one-way or two-way analysis of variance (ANOVA) for single and multiple variables respectively, with Tukey post-hoc testing. A repeated measure ANOVA was performed on repeatedly sampled data in the same group. Non-Parametric data was analyzed by Kruskal-Wallis test and Mann-Whitney post hoc tests. Differences were considered to be statistically significant if P values were less than 5% ($P < 0.05$). n - represents the number of animals per group.

CHAPTER 3: RESULTS

3.1 DAILY MAGNESIUM TREATMENT DOES NOT ALTER BLOOD GLUCOSE, LIPID PROFILE, PLASMA- OR CARDIAC TISSUE Mg^{2+}

3.1.1 Characteristics of the Streptozotocin-induced model of diabetes

Diabetes was successfully induced in 90% of animals treated with STZ, while in 10%, the blood glucose concentration remained unchanged. There was no significant difference in the success rate of diabetes induction due to Mg^{2+} co-treatment (88% for STZ and 91% for STZ+Mg groups respectively, $P > 0.05$, $n = 24$-27; Table 1). None of the citrate-treated animals, Control- and Mg groups, developed hyperglycaemia.

The overall mortality in the STZ treated animals was 10% (11% for the STZ group and 8% for the STZ+Mg group, $P > 0.05$, $n = 24$-26; Table 1). None of the animals from the Control or Mg groups died during treatment.

Table 1: Success rates for induction of diabetes according to treatment groups and mortality during treatment protocol by treatment groups.

	Number treated	Number Hyperglycaemia	Number of deaths	Diabetes induction success rate (%)	Mortality (%)
Citrate-treated	**34**	**0**	**0**	**0%**	**0%**
Citrate-Saline	17	0	0	0%	0%
Citrate-Mg	17	0	0	0%	0%
STZ-treated	**50**	**45**	**5**	**90%**	**10%**
STZ-Saline	26	23	3	88%	11%
STZ-Mg	24	22	2	91%	8%

3.1.2. The Effects of Magnesium on Body Weight In Streptozotocin-Induced Diabetes

Animals were weighed daily as changes in daily body weight were used as an indicator of severity and progression of disease. The daily changes in body weight are presented as a percentage of the initial weight, i.e. weight on Day 1 (figure 3-1). The non-diabetic animals, Control and Mg groups, showed a similar trend of normal weight gain of about 1% per day over the 7 days of treatment. There was a significant loss of weight in the diabetic animals, STZ and STZ+Mg, compared to control (STZ: 96 ± 0.8%, $P < 0.05$; STZ+Mg 97 ± 0.9%, $P < 0.05$ *vs.* control).

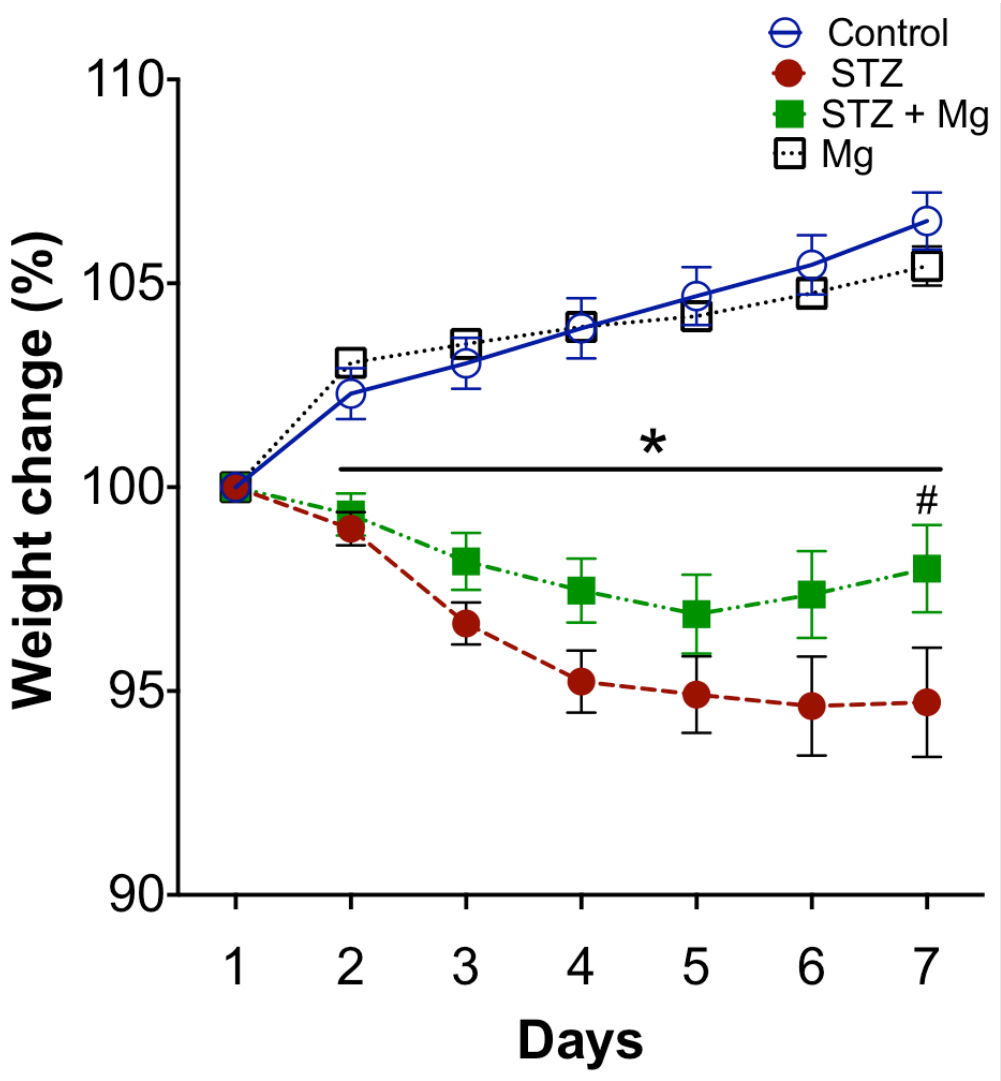

Figure 3-1: Daily body weight changes for the various treatment groups.
Control animals had normal weight gain while STZ treated animals lost weight until day 5-6; Magnesium treatment had no significant effect on body weight changes.
** $P < 0.01$ *vs.* Control; # $P < 0.05$ *vs.* STZ; *n = 17-20.*

3.1.3 The Effects of Magnesium on Streptozotocin -Induced Hyperglycaemia

After 7 days of treatment, non-diabetic animals remained at euglycemic baseline without differences due to Mg^{2+} treatment (Mg: 6.5 ± 0.2 mmol/l, $P > 0.05$ *vs.* control: 6.7 ± 0.1 mmol/l; figure 3-2). STZ-treated animals showed significant hyperglycaemia (STZ: 31 ± 0.4 mmol/l, n = 20/group, STZ+Mg: 29.5 ± 0.7 mmol/l, n = 22/group, $P < 0.001$ *vs.* control). The degree of hyperglycaemia in STZ-treated animals was not significantly altered by Mg^{2+} treatment (STZ+Mg: $P > 0.05$ *vs.* STZ).

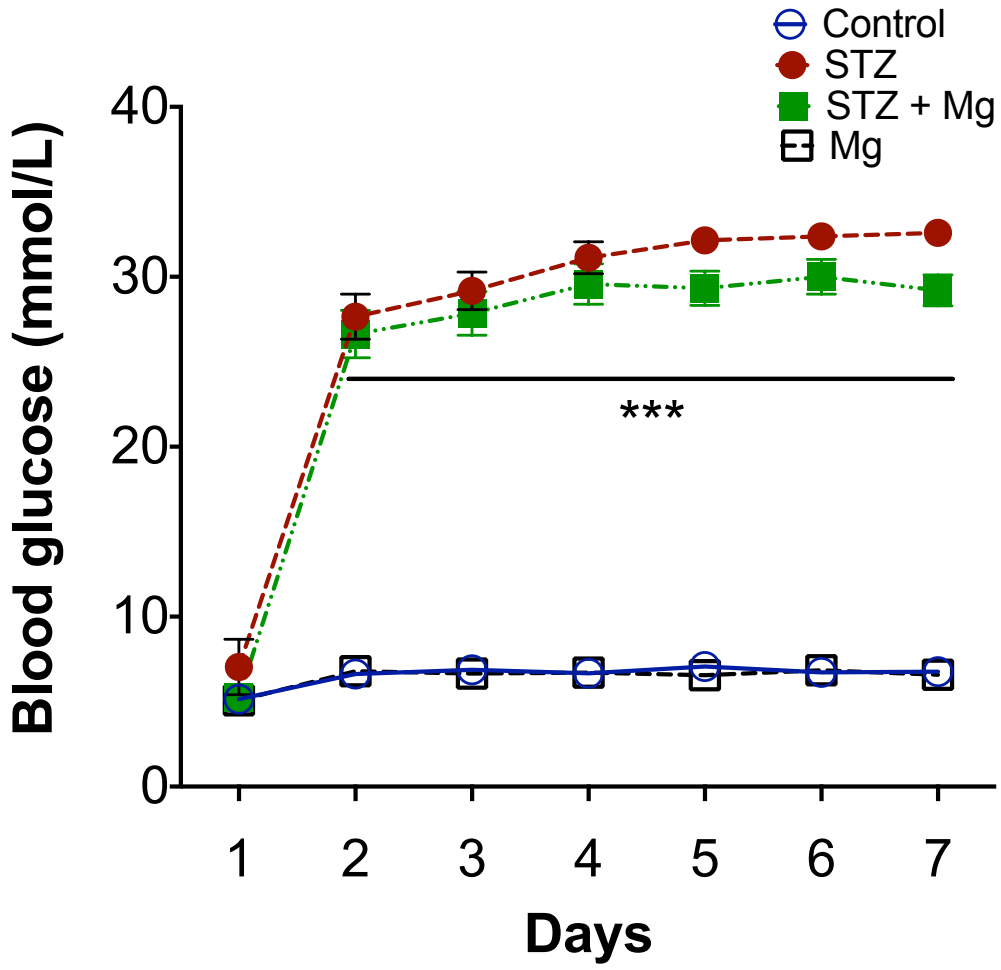

Figure 3-2: Daily blood glucose levels for the various treatment groups.
STZ treated animals developed sustained hyperglycaemia while control animals remained at euglycaemic baseline; Magnesium treatment had no significant effect on blood glucose levels. *** $P < 0.001$ *vs.* Control; *n = 17-20*.

3.1.4 The Effects of Magnesium on Plasma Lipid Profile in Streptozotocin -Induced Diabetes

Non-diabetic animals had normal looking pale-yellow plasma while diabetic animals had milky-white pale plasma, characteristic of hypertriglyceridemia. The appearance of the plasma was graded according to their opacity/cloudiness - adapted from the Frederickson classification (Fredrickson et al. 1967; Belanger et al. 1973, figure 3-3).

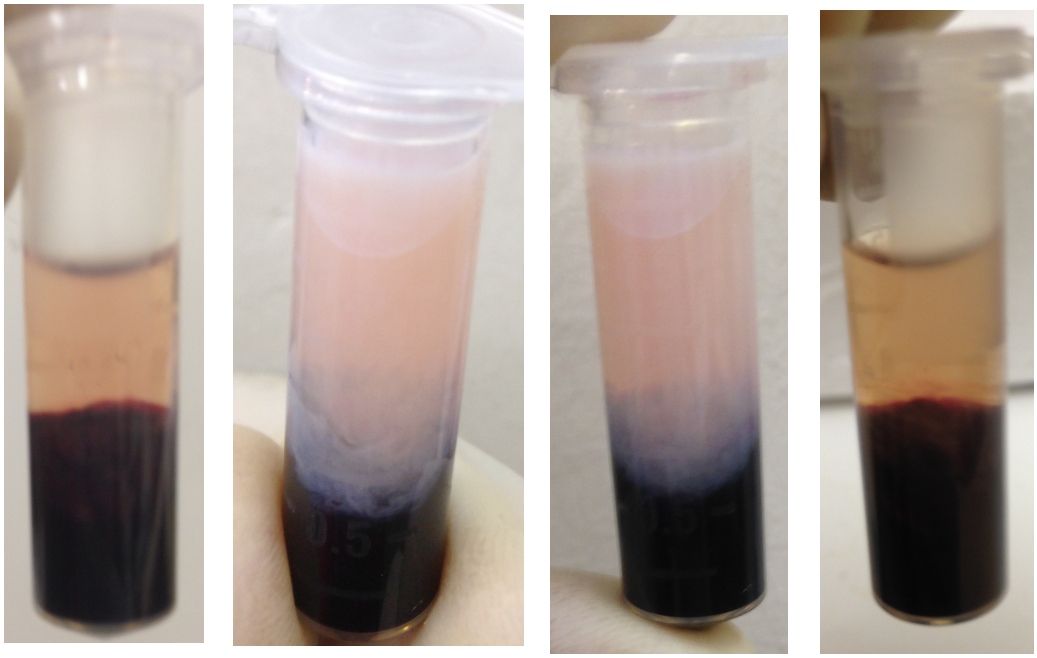

Figure 3-3: Examples of plasma samples from the treatment groups. Note that STZ-treated animals had milky plasma (++ opacity) characteristic of diabetic dyslipidaemia.

(a) Assessment of plasma triglycerides

Having observed the turbid plasma characteristic of lipid abnormalities, the concentration of lipids was quantified (triglycerides and cholesterol) in the plasma samples. Diabetic animals demonstrated severe hypertriglyceridemia that was about 5 times higher than the control (STZ: 5.6 ± 0.15 mmol/l, $P < 0.001$ *vs.* control 0.9 ± 0.05 mmol/l), which was not changed by Mg^{2+} treatment (STZ+Mg: 5.1 ± 0.11 mmol/l, $P > 0.05$ vs. STZ; figure 3-4).

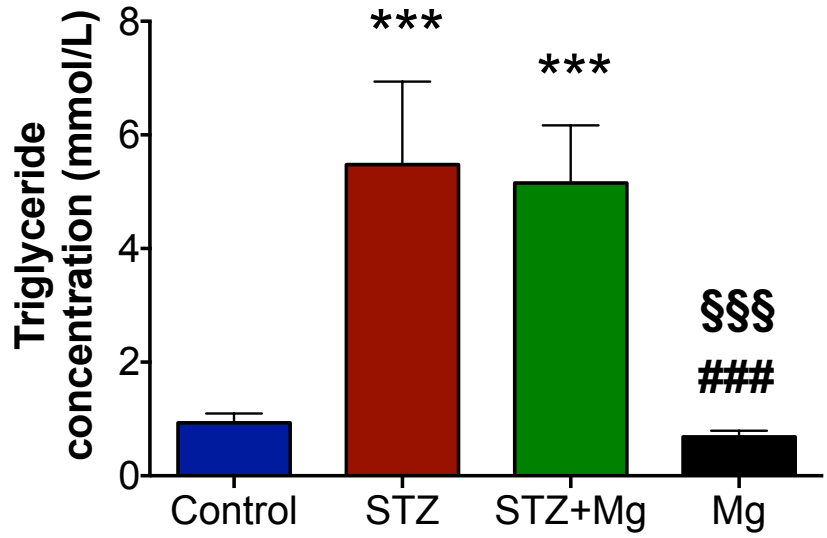

Figure 3-4: Effects of streptozotocin and magnesium on plasma triglycerides after 7 days of treatment. STZ treated animals had severe hypertriglyceridemia, which was not altered by magnesium treatment.

*** $P < 0.001$ *vs.* control, ### $P < 0.001$ *vs.* STZ, §§§ $P < 0.001$ *vs.* STZ+Mg. $n = 8$-10.

(b) Assessment of plasma cholesterol

Diabetic animals had a hypercholesterolemia compared to control animals (STZ: 2.7 ± 0.2 mmol/l, $P < 0.05$, *vs.* control: 1.7 ± 0.06 mmol/l), and this was not changed by Mg^{2+} treatment (STZ+Mg: 2.3 ± 0.1 mmol/l, $P > 0.05$ *vs.* STZ; figure 3-5 (A)).

Further investigation of which subclasses of cholesterol were altered was performed; as the different HDL subfractions are known risk predictors for CVD to varying extents. Diabetic animals showed no significant differences in HDL-C compared to non-diabetic animals (STZ: 1.4 ± 0.09 mmol/l, $P = 0.09$; *vs.* control: 1.8 ± 0.08 mmol/l) and this was not altered by Mg^{2+} treatment (STZ+Mg: 1.5 ± 0.08 mmol/l, $P = 0.72$ *vs.* STZ; figure 3-5 (B))

Although gross HDL-C concentration may be similar, diseased subjects can have differences in HDL-C subfractions that may have implications on health. All treatment groups had minimal amounts of small HDL-C of <1µmol/L that could not be quantified. There was no significant difference in the large HDL-C subfraction between the groups. However, there seemed to be a trend of reduced large HDL-C in the diabetic animals (STZ: 29 ± 5 mg/dl, $P = 0.08$; *vs.* control: 24 ± 3.4 mg/dl;

figure 3-5 (C)). In the intermediate HDL-C, I observed a reversed relationship, with diabetic animals showing a higher level of intermediate HDL-C that control animals. Mg^{2+} treatment did not significantly effect HDL-C.

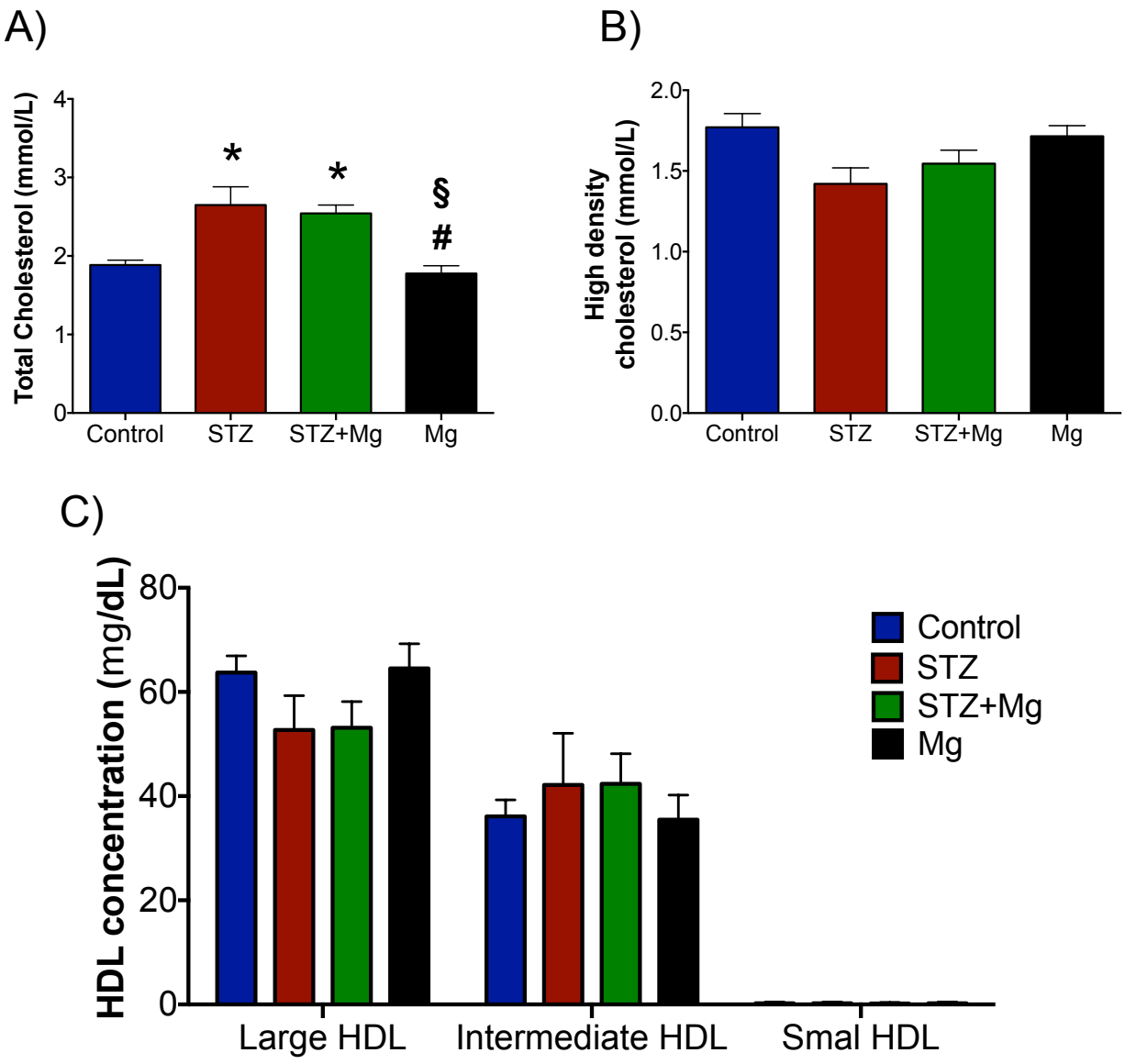

Figure 3-5: The effects of streptozotocin and magnesium on plasma cholesterol.

A) Plasma total cholesterol levels. STZ treated animals had hypercholesterolemia compared to controls, which was not altered by magnesium treatment.

* $P < 0.05$ *vs.* control, # $P < 0.05$ *vs.* STZ, § $P < 0.05$ *vs.* STZ+Mg. $n = 8\text{-}10$.

B) Plasma high-density cholesterol (HDL-C) levels, $P = 0.09$, $n = 8\text{-}10$.

C) Plasma high-density cholesterol (HDL-C) subfraction concentration levels. No significant differences were observed, however, note the trend of a reversed relationship, STZ seems to have less Large- and more Intermediate HDL-C than controls groups. $P = 0.08$, $n = 5\text{-}6$.

(c) Assessment of Atherogenic Index

Congruent with the findings of elevated total cholesterol and the trend of reduced HDL-C in diabetic animals, these animals had a significantly raised atherogenic index compared to control animals (STZ: -0.25 ± 0.06 $P < 0.001$ *vs.* control: 0.026 ± 0.01). This was not changed by Mg^{2+} treatment in diabetic animals (STZ+Mg: -0.19 ± 0.02 $P > 0.05$ *vs.* STZ), and control animals (Mg: 0.022 ± 0.02 $P > 0.05$ *vs.* control, figure 3-6).

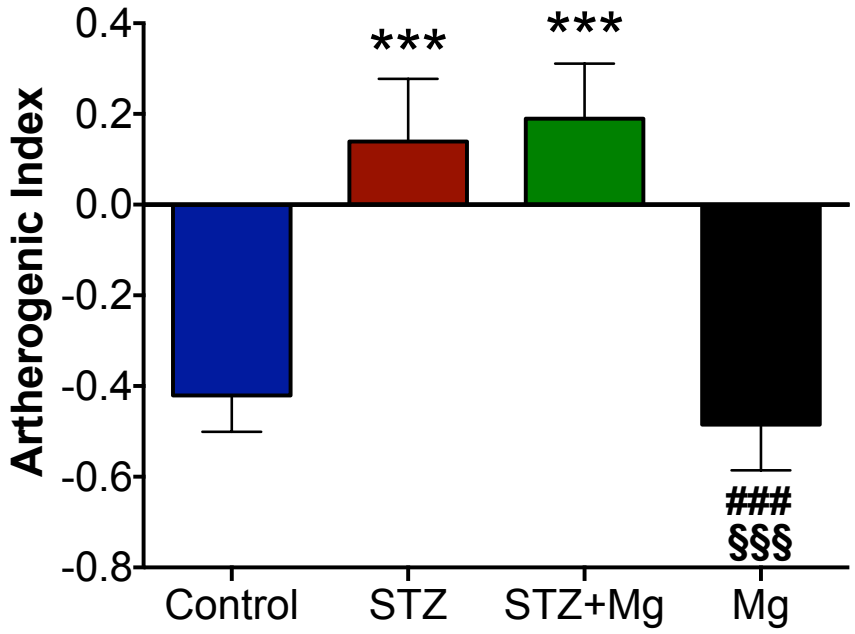

Figure 3-6: Effects of streptozotocin and magnesium on atherogenic index. Diabetic animals had a high atherogenic index, which was not altered by magnesium. *** $P < 0.001$ *vs.* control, ### $P < 0.001$ *vs.* STZ, §§§ $P < 0.001$ *vs.* STZ+Mg, n = 8-10.

3.1.5 The Effects of a Magnesium on Plasma Magnesium levels

There were no significant differences in plasma Mg^{2+} concentration in the non-diabetic control groups due to Mg^{2+} treatment (Mg: 0.95 ± 0.04 mmol/L $P > 0.05$ *vs.* control: 0.91 ± 0.05 mmol/L). Neither were there differences in plasma Mg^{2+} concentration due to Mg^{2+} or STZ treatment in the diabetic animals (STZ+Mg: 1.08 ± 0.05 mmol/L, $P > 0.05$ *vs.* control; $P > 0.05$ *vs.* STZ: 1.03 ± 0.03mmol/L; figure 3-7). However, it appears that the Mg^{2+} levels in diabetic animals were higher than the levels seen in non-diabetic animals.

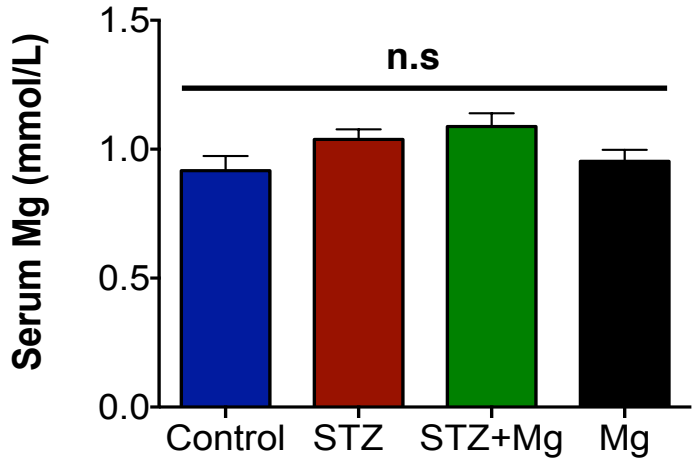

Figure 3-7: Plasma magnesium levels after 7 days of treatment.
No significant differences were observed among the treatment groups. *n* = 5-6.

3.1.5 The Effects of Magnesium on Cardiac Tissue Magnesium Concentration

There were no significant differences in cardiac tissue Mg^{2+} concentration in the diabetic animals (STZ+Mg: 0.63 ± 0.02 mmol/g, $P > 0.05$ *vs*. control; $P > 0.05$ *vs*. STZ: 0.56 ± 0.01 mmol/g; figure 3-8); and neither was there any difference due to Mg^{2+} treatment in non-diabetic animals (Mg: 0.64 ± 0.01 mmol/g $P > 0.05$ *vs*. control: 0.62 ± 0.05 mmol/g). However, it appears that the cardiac tissue Mg^{2+} concentration in diabetic animals were higher than that of non-diabetic animals.

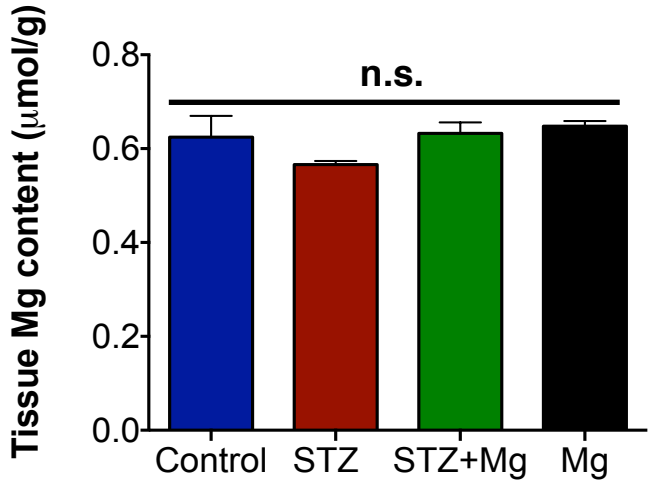

Figure 3-8: Effects of streptozotocin and magnesium on plasma and cardiac tissue Mg^{2+} concentration. No significant differences were observed. *n* = 5-6.

3.2 MAGNESIUM PREVENTS IMPAIRMENT OF HEART RATE VARIABILITY IN STZ-INDUCED DIABETES

3.2.1 The Effects of Magnesium on the Time Domain analysis of HRV

Control and Mg group animals had a normal heart rate of about 420 beats per min (bpm) with a good amount of beat-to-beat variability in heart rate as reflected in the thick fluctuating baseline (figure 3-9). The STZ group demonstrated a significant reduction in resting heart rate to about 370 bpm, with a poor beat-to-beat variability as reflected in the thin, steady baseline. The STZ+Mg group showed an improvement in the resting heart rate and variability to near non-diabetic levels.

Statistical analysis confirmed that Mg^{2+} treatment in non-diabetic animals caused no significant difference in heart rate (Mg: 420 ± 7.1 bpm; Control: 415 ± 6.2 bpm, $P > 0.05$; figure 3-10 (A) or RMSSD (Mg: 18.4 ± 2 ms, $P > 0.05$ *vs*. control: 23.5 ± 2 ms; figure 3-10 (C). Untreated diabetes caused a significantly reduced heart rate (STZ: 370 ± 10.2 bpm, $P < 0.01$ *vs*. control) and RMSSD (STZ: 8.3 ± 1.1 ms, $P < 0.001$ *vs*. control). Both of these were improved by Mg^{2+} treatment (heart rate, STZ+Mg: 417 ± 8.9 bpm, $P < 0.05$ *vs*. STZ; and RMSSD: STZ+Mg: 19.6 ± 2.4 ms, $P > 0.01$ *vs*. STZ) and not significantly different from the controls (STZ+Mg $P > 0.05$ *vs*. control & *vs*. Mg.)

The heart rate *ex vivo* was in concordance with the *in vivo* recordings. Control animals showed a normal heart rate of 229 ± 18bpm and STZ-treated animals had a significantly reduced heart rate (STZ: 160 ± 17bpm, $P < 0.01$ *vs*. control; $P < 0.01$ *vs*. Mg: 243 ± 22bpm; figure 3-10 (B); which was improved by Mg treatment (STZ+Mg: 222 ± 28bpm, $P < 0.05$ *vs*. STZ).

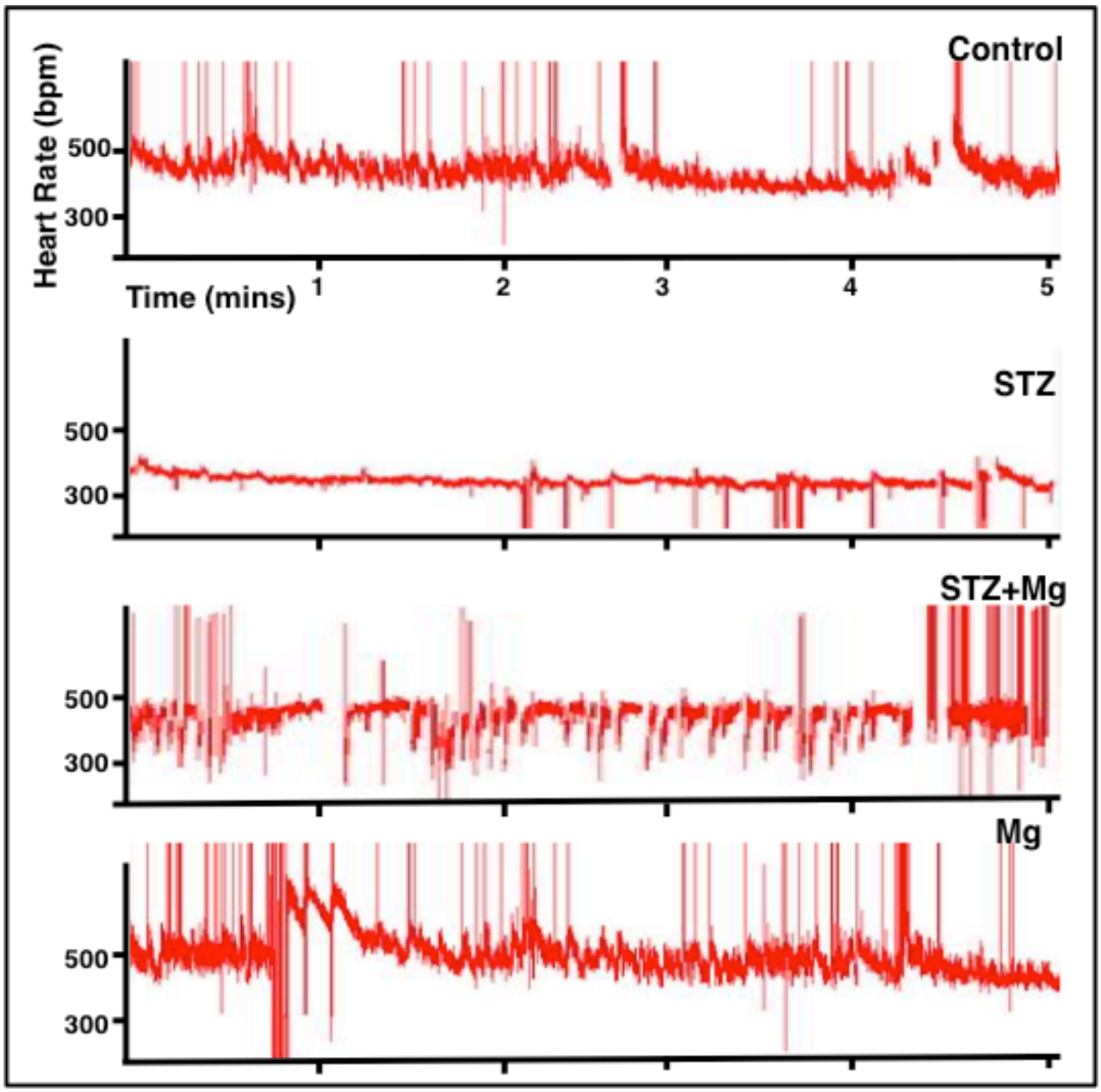

Figure 3-9: Representative examples of 5-min recordings of resting heart rate. Note STZ (second from the top) shows a reduced heart rate baseline and a poor beat-to-beat variability compared to control animals, which was improved by magnesium treatment in the STZ+Mg example (third from the top).

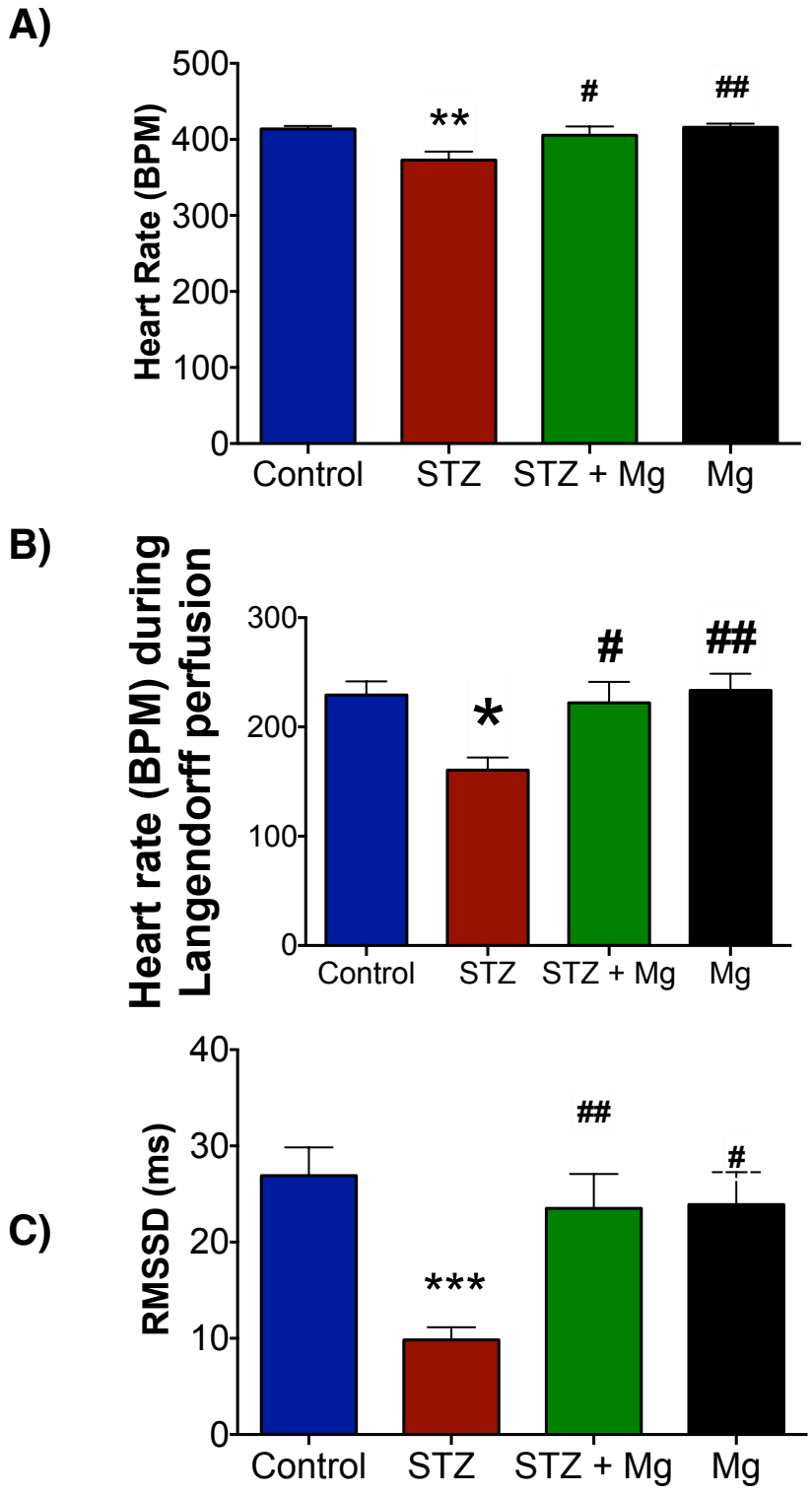

Figure 3-10: Effects of streptozotocin and magnesium on time domain heart rate variability. (A) Resting heart rate during 5 min recording. (B) Heart rates during Langendorff perfusion. (C) Analysis of the root mean square of successive NN interval differences (RMSSD) of *in vivo* recordings. STZ-only treated animals showed significantly reduced heart rates and less beat-to-beat variability compared to controls, which was improved by magnesium treatment. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ *vs.* control; # $P < 0.05$, ## $P < 0.01$ *vs.* STZ. $n = 8$-10.

3.2.2 The Effects of Magnesium on Frequency Domain analysis of HRV

Control animals demonstrated a normal large amount of high frequency (HF) power and about half that power for the low frequencies (LF) (figure 3-11). Mg-treated rats showed a similar pattern of power spectral density with no overt differences in distribution due to Mg^{2+} treatment in non-diabetic animals. In STZ-treated animals, there was a considerable reduction in power of both LF and HF. This reduction was prevented by Mg^{2+} treatment, STZ+Mg.

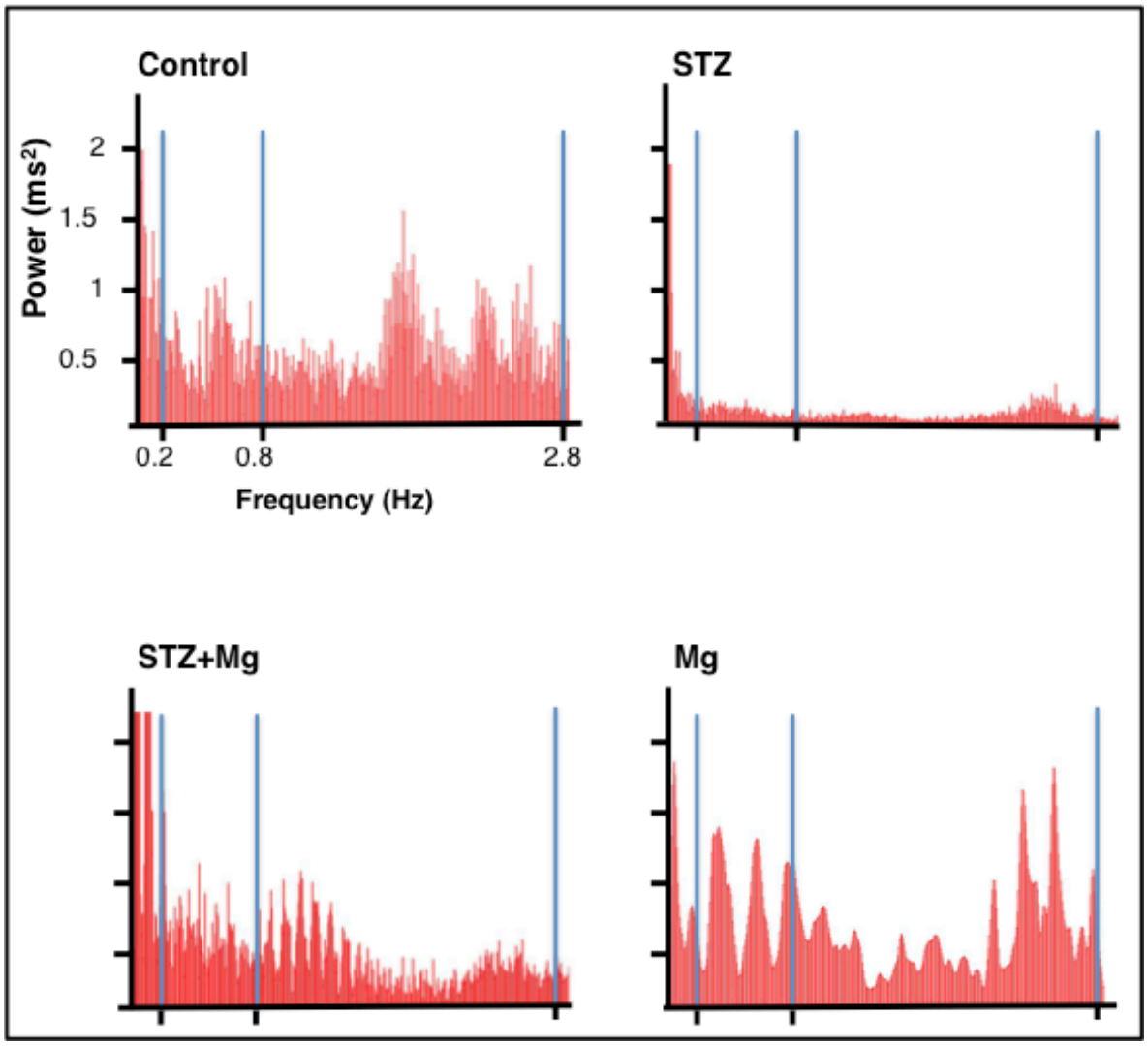

Figure 3-11: Representative examples of frequency-power spectral distribution analysis of the baseline, 5-min recordings of resting heart rate. Note that the blue lines demarcate the frequency cut off values for: very low frequencies (VLF): <0.2; low frequencies (LF): 0.2-0.8Hz; and high frequencies (HF): 0.8-2.8Hz. The STZ shows a significantly reduced frequency power compared to the controls, which was improved by magnesium treatment.

The HF power, which is indicative of parasympathetic activity, was significantly reduced in the untreated diabetic animals compared to control (STZ: 23 ± 8 Hz, $P <$ 0.001 *vs*. control). Mg^{2+} treatment prevented this reduction in HF power (STZ+Mg: 69 ± 12 Hz, P = n.s. *vs*. control). In non-diabetic animals, there was no alteration by Mg^{2+} treatment (Mg: 70 ± 7 Hz, $P > 0.05$ *vs*. control: 82 ± 13 Hz; figure 3-12 (A). The findings were consistent even after normalizing the values to the total power, figure 3-12 (B) and (D).

The power distribution of LF/HF power ratio, which is indicative of sympathetic balance, in non-diabetic animals, was not significantly altered by Mg^{2+} treatment alone (Mg: 0.65 ± 04 Hz, $P > 0.05$ *vs*. control: 0.58 ± 0.06 Hz; figure 3-12 (C). STZ-treated rats had significantly larger LF/HF power ratio than control animals (STZ: 1.6 ± 0.3 Hz, $P < 0.001$ *vs*. control). Mg^{2+} treatment reduced the high LF/HF power ratio seen in diabetes (STZ+Mg: 0.55 ± 0.04 Hz, $P < 0.01$ *vs*. STZ) to control levels ($P >$ 0.05 *vs*. control).

3.2.3 The Effects of Magnesium on Baroreceptor Reflex

The orthostatic stress tilt test compared changes in heart rate and LF/HF power ratio changes from the resting position to the 70° HUT position. Non-diabetic animals, control and Mg groups, showed a normal baroreceptor reflex tachycardia (increase in heart rate) in response to the orthostatic position ($P < 0.05$ vs HUT; figure 3-13 (A)). Diabetic animals had impairment in the baroreceptor reflex tachycardia (HUT: 376 ± 10 bpm, $P = 0.9$ *vs*. rest: 370 ± 10.2 bpm). The loss of the baroreceptor reflex tachycardia was prevented by Mg^{2+} treatment in diabetes (STZ+Mg: 417 ± 8.9 bpm, $P < 0.05$ *vs*. rest: 439 ± 7 bpm).

Non-diabetic animals showed a normal change in sympathetic balance in response to orthostatic stress as represented by a shift in the LF/HF ratio towards an increase in the sympathetic innervation (figure 3-13 (B)). This change in sympathetic balance was impaired in untreated diabetics (STZ: HUT *vs*. rest, $P > 0.05$). Mg^{2+} treatment prevented this aberration (STZ+Mg: HUT *vs*. rest, $P < 0.01$).

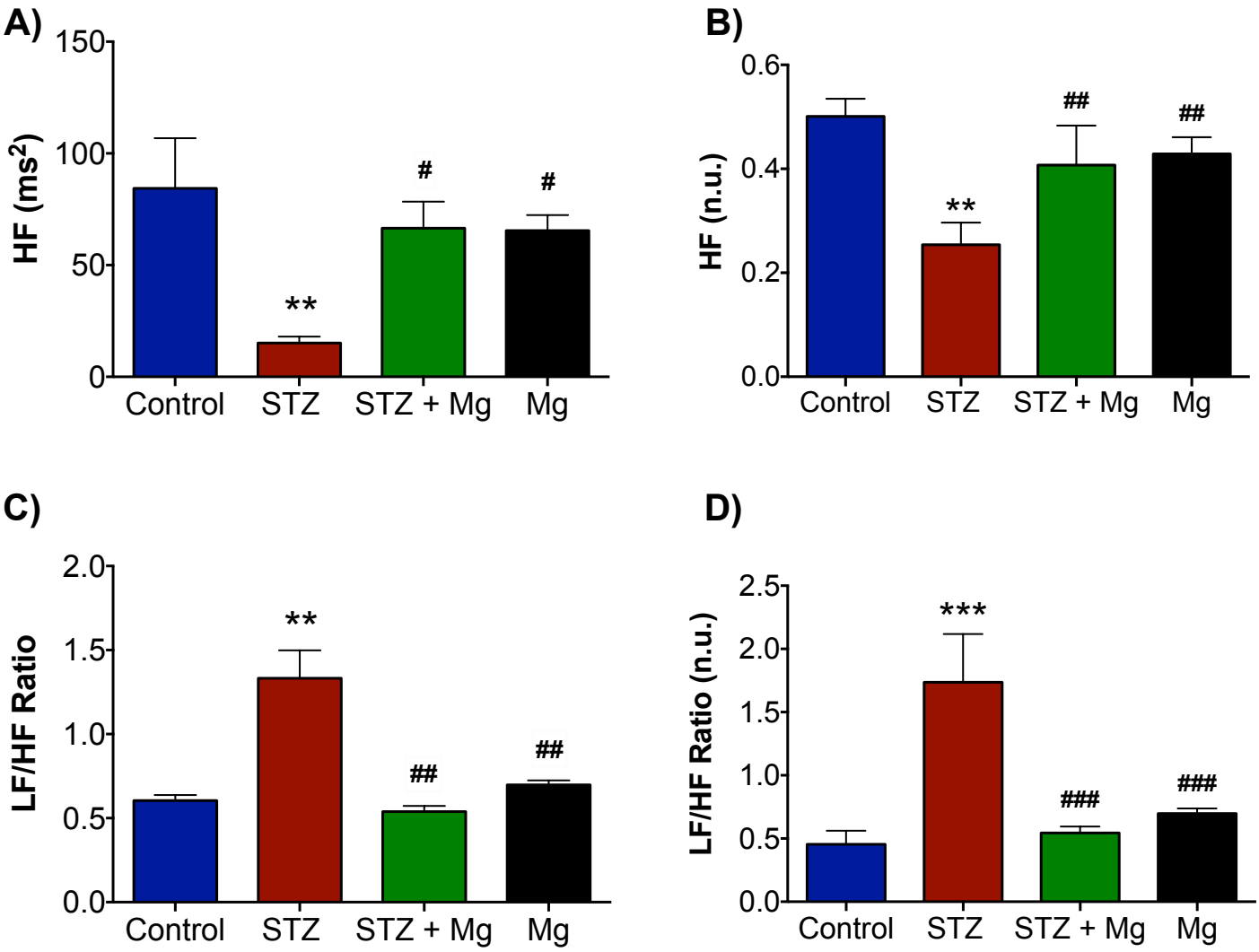

Figure 3-12: Analysis of frequency domain heart rate variability.

(A) High Frequency (HF) power spectral distribution; STZ showed a significantly reduced HF. (B) Normalisation of HF to total power. (C) Analysis of the LF/HF power ratio. STZ-only treated animals showed an abnormally high ratio, which was prevented by magnesium treatment. (D) Normalisation of LF/HF power ratio to total power. n.u. – normalised units.

** $P < 0.01$ *vs.* control; # $P < 0.05$ *vs.* STZ; ## $P < 0.01$ *vs.* STZ. $n = 8\text{-}10$.

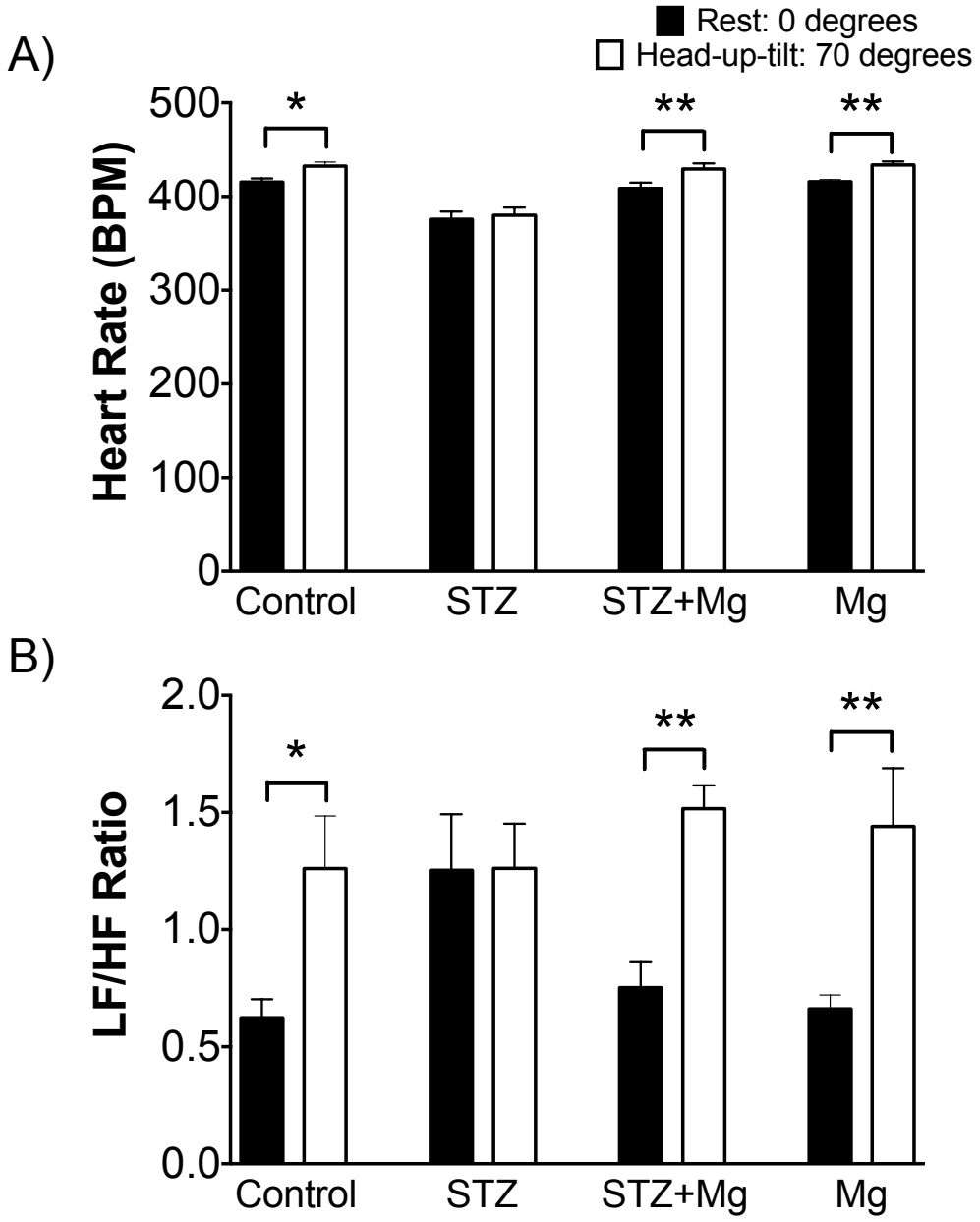

Figure 3-13: Effects of streptozotocin and magnesium on orthostatic stress test responses.

(A) Heart rate changes, STZ-only treated animals showed impairment in normal tachycardia response to HUT. (B) LF/HF ratio changes due to orthostatic. STZ-only treated animals showed impairment in increase in ratio in response to HUT.

* $P < 0.05$, ** $P < 0.01$, HUT *vs.* Rest (0°) position. $n = 8$-10.

3.3 MAGNESIUM IMPROVES DIASTOLIC DISTENSIBILITY AND ELASTANCE IN STZ-INDUCED DIABETES

In the P-V relationship studies, Control and Mg group animals showed a normal stepwise increase in pressure responses to volume (figure 3-14). The untreated diabetic animals (STZ) showed slower progression in pressure responses to volume as demonstrated by the EDP remaining at 0 until 60µL. This was improved by Mg^{2+} treatment (STZ+Mg), which showed a similar pattern to the control animals.

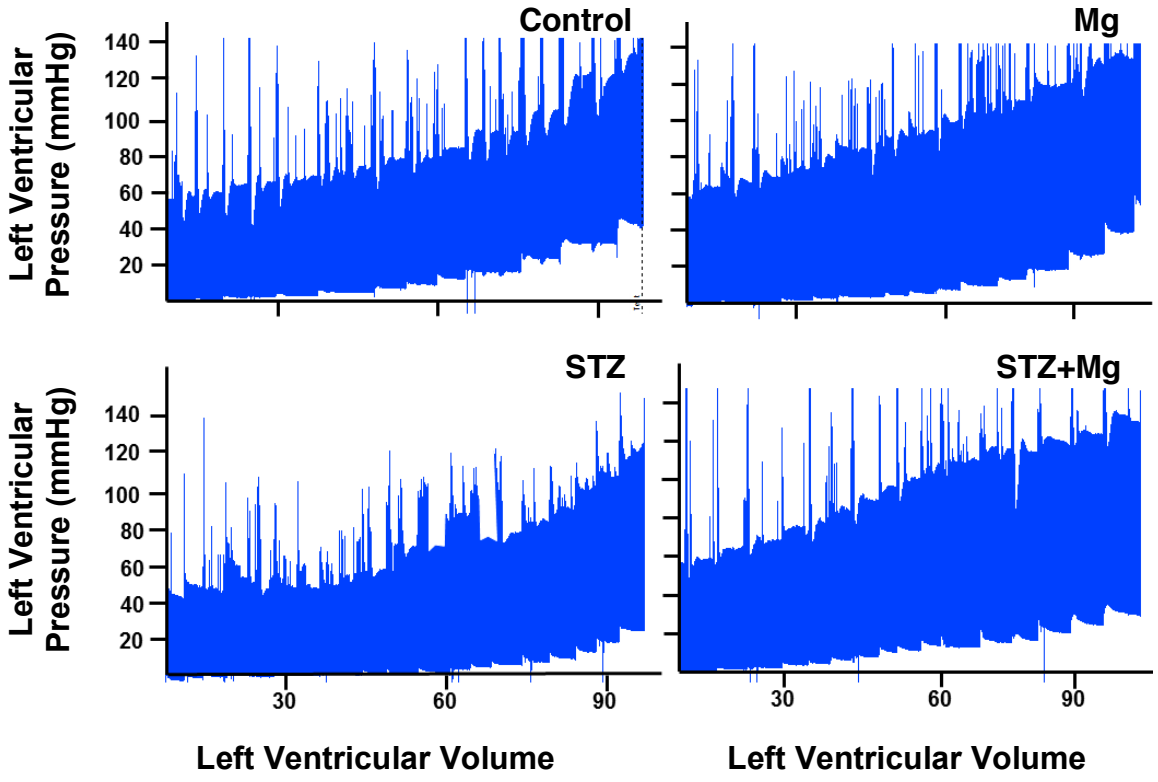

Figure 3-14: Representative examples of left ventricular pressure-volume relationship studies. Note that STZ (bottom left) showed a slow rising end-diastolic pressure response.

3.3.1 The Effects of Magnesium on End-Diastolic Pressure-Volume Relationship

The non-diabetic animals had a normal diastolic volume intercept-normalised to heart weight, which was not different due to Mg^{2+} treatment (Mg: 12 ± 3.7 µL, $P >$

0.05 *vs*. control: 16 ± 2.4 µl; figure 3-15). Untreated diabetic animals (STZ) had a significantly larger intercept compared to control animals (STZ: 30 ± 3.2 µL, $P < 0.01$ *vs*. control), which was reduced by Mg^{2+} treatment (STZ+Mg: 15 ± 3.6 µL, $P < 0.01$ *vs*. STZ; $P > 0.05$ *vs*. control; figure 3-15 insert). Furthermore, the pressure-volume relationship curve of the untreated diabetics had a right shift and followed a slower rising trajectory than both the control and the treated diabetic animals.

The end-diastolic elastance, which is the gradient of the pressure-volume relationship graph between 50 and 70 µL, showed that untreated diabetic animals had a significantly larger end-diastolic elastance than non-diabetic control animals (STZ: 0.1 ± 0.01 mmHg/µL, $P < 0.01$ *vs*. control: 0.37 ± 0.06 mmHg/µL; figure 3-17 (A)). Mg^{2+} co-treatment in diabetes, STZ+Mg group, restored elastance to control values, significantly less than untreated diabetics (STZ+Mg: 0.42 ± 0.05 mmHg/µL, $P > 0.05$ *vs*. control; $P < 0.01$ *vs*. STZ).

3.3.2 The Effects of Magnesium on the End-Systolic Pressure-Volume Relationship

There were no significant differences in the end systolic pressure-volume (ESP-V) relationship plots of the various treatment groups as they followed a similar trajectory (figure 3-16). The end-systolic elastance, which is the gradient of this graph between 50 and 70 µL (figure 3-17 (B)), also showed no differences among the various treatment groups.

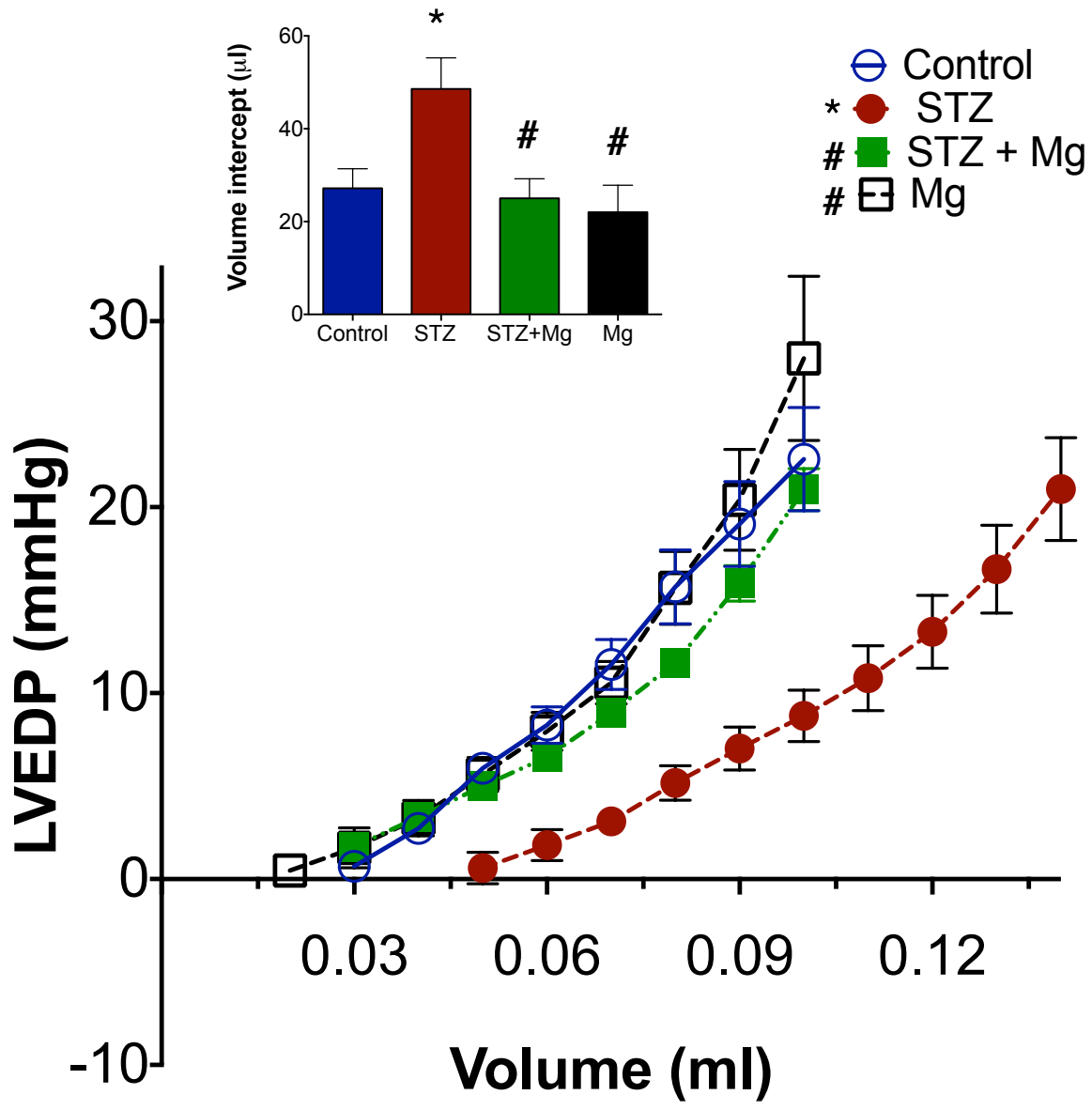

Figure 3-15: The effects of streptozotocin and magnesium on end-diastolic pressure-volume relationship. STZ animals showed a right shift in pressure volume relationship, significantly different from control and Mg treated animals.

(Insert) Summary of the end-diastolic volume intercept (V_0), which is the left ventricular volume that produces an end-diastolic pressure of zero.

* $P < 0.05$ *vs.* control; # $P < 0.05$ *vs.* STZ. n = 6-8

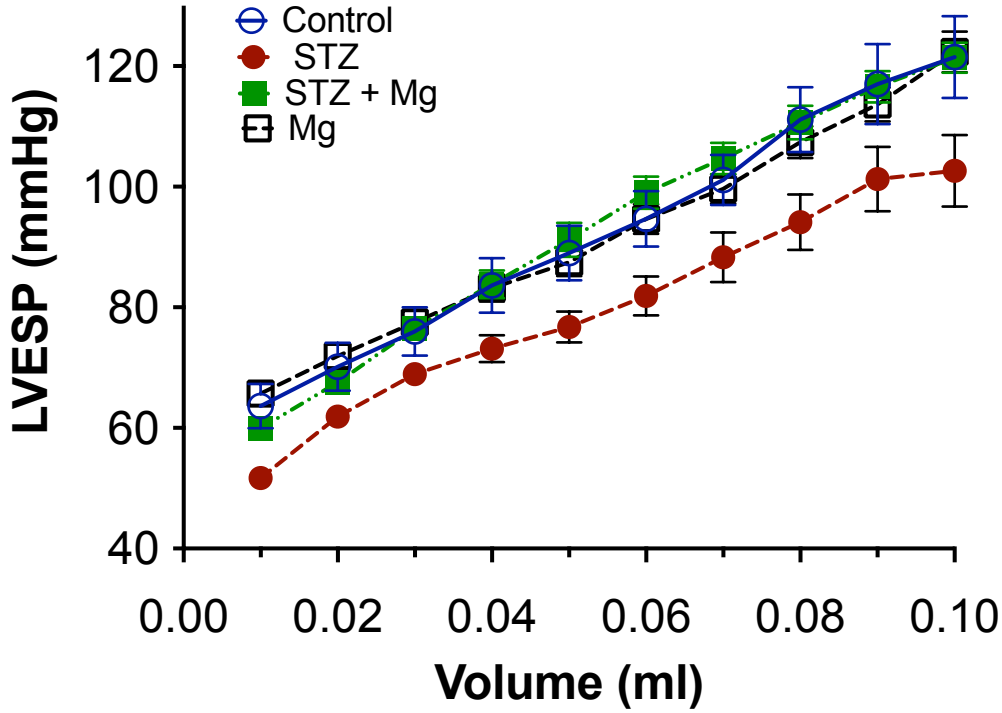

Figure 3-16: The effects of streptozotocin and magnesium on the end-systolic pressure-volume relationship. No significant differences were observed among the various treatment groups. *n* = 6-8.

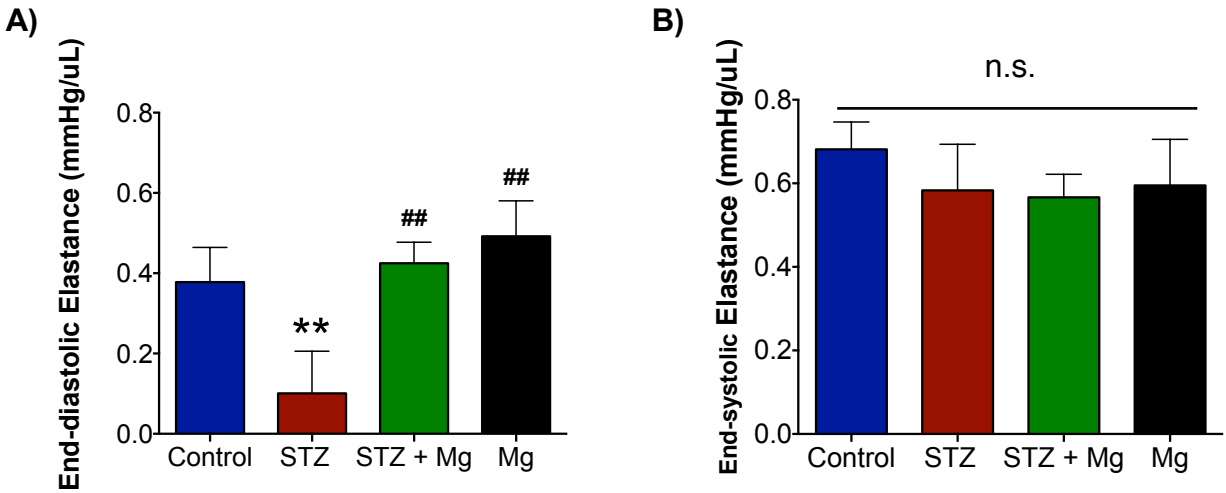

Figure 3-17: The elastance analysis of: (A) the end-diastolic elastance STZ had significantly reduced end-diastolic elastance compared to control that was improved by Mg treatment. ** $P < 0.01$ *vs.* control; ## $P < 0.01$ *vs.* STZ. *n* = 6-8, *n* = 6-8. (B) The end-systolic elastance of the various treatment groups. No significant differences were observed in the end-systolic elastance analysis. *n* = 6-8.

3.3.3 The Effects of Magnesium on Left Ventricular Developed Pressure-Volume Relationship In STZ-Induced Diabetes

The left ventricular developed pressure (LVDP) relationship to volume, which was derived as a computation of the EDP-V and ESP-V relationships showed no differences in the contractile function between diabetics and controls, and this was not altered by Mg^{2+} treatment (figure 3-18).

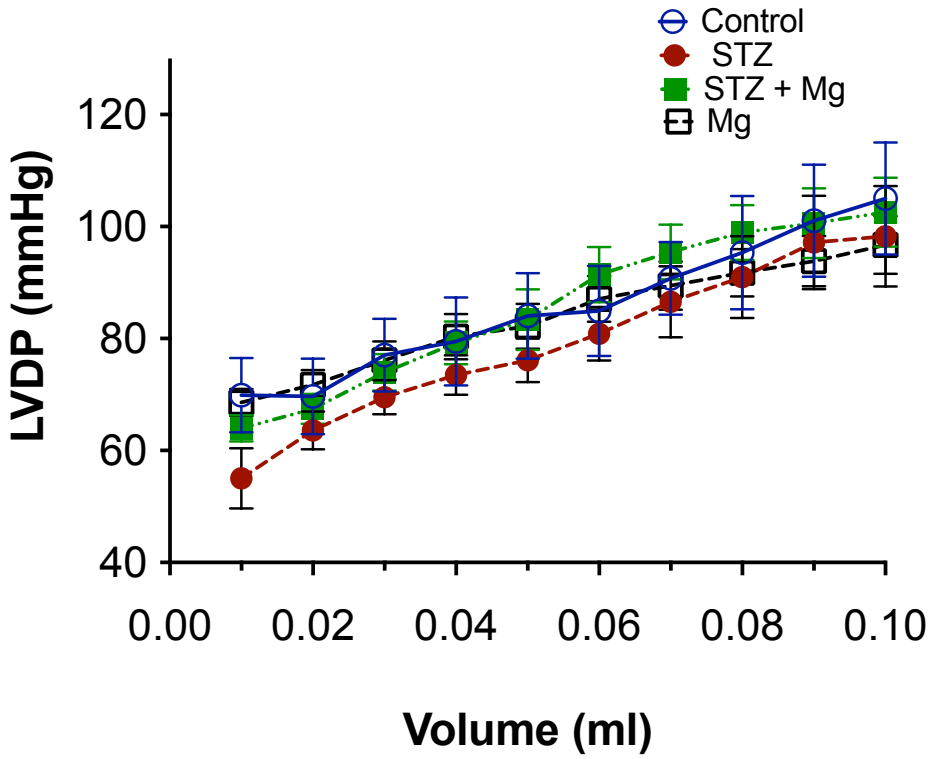

Figure 3-18: The effects of streptozotocin and magnesium on left ventricular developed pressure (LVDP)-volume relationship. No significant differences were observed. *n* = 6-8.

3.4 THE EFFECTS OF DIABETES AND MAGNESIUM ON CARDIAC STRUCTURE

3.5.1 Effects of Magnesium and Streptozotocin on Heart Weight

At the end of perfusion, the hearts were weighed to give an indication of any gross changes in cardiac morphology. The heart weight was normalised to initial body weight (HW/BW ratio). There were no significant differences observed in the HW/BW ratio among the groups (figure 3-19).

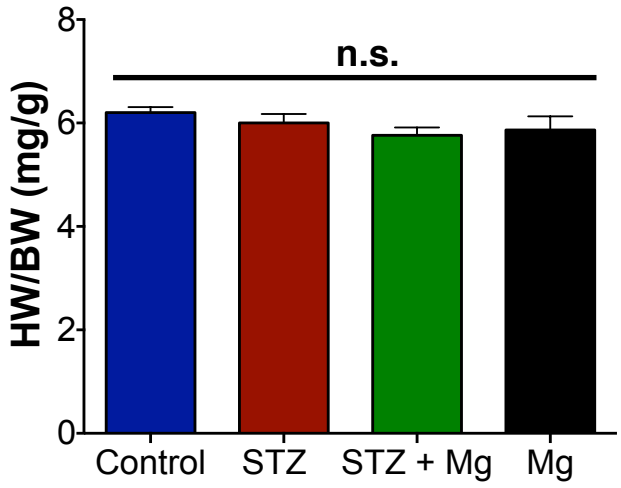

Figure 3-19: The effects of streptozotocin and magnesium on heart weight-to-body weight ratio (HW/BW). No differences were observed among the groups, n = 6-7.

3.5.2 Effects of Magnesium and Streptozotocin on Myocyte Size

Cardiac histological staining by haematoxylin and eosin (H&E) was used to assess myocyte size (figure 3-20). There were no differences observed in myocyte width due to STZ (STZ: 10.6 ± 0.63 µm, $P = 0.96$ *vs*. control: 9.3 ± 0.39 µm; STZ, $P = 0.98$ *vs*. Mg: 9.7 ± 0.7 µm; figure 3-20 (B)) or Mg^{2+} treatment (STZ+Mg: 10 ± 0.82 µm, $P = 0.95$ *vs*. STZ; $P = 0.90$ *vs*. control). Neither was there differences observed in myocyte length due to STZ (STZ: 61.6 ± 1.7 µm, $P = 0.99$ *vs*. control: 62.3 ± 3.3 µm; $P = 0.88$ *vs*. Mg: 64.2 ± 1.5 µm; figure 3-20 (C)) or Mg^{2+} treatment in diabetic animals (STZ+Mg: 62.2 ± 3.5 µm $P = 0.96$ *vs* STZ, $P = 0.99$ *vs*. control).

3.5.3 Effects of Magnesium and Streptozotocin on interstitial fibrosis

Masson's trichrome staining was performed to investigate the amount of interstitial fibrosis (figure 3-21). The amount of fibrosis is presented as a percentage of the ventricular area in the section sampled (figure 3-22). There were no differences observed in the amounts of collagen in the hearts of STZ treated animals (STZ: 7.4 ± 0.13%, $P = 0.94$ *vs*. control: 6.6 ± 0.1%; $P = 0.99$ *vs*. Mg: 7 ± 0.24%) or Mg^{2+} treated animals (STZ+Mg: 6.8 ± 0.2%, $P = 0.84$ *vs*. control; $P = 0.98$ *vs*. STZ).

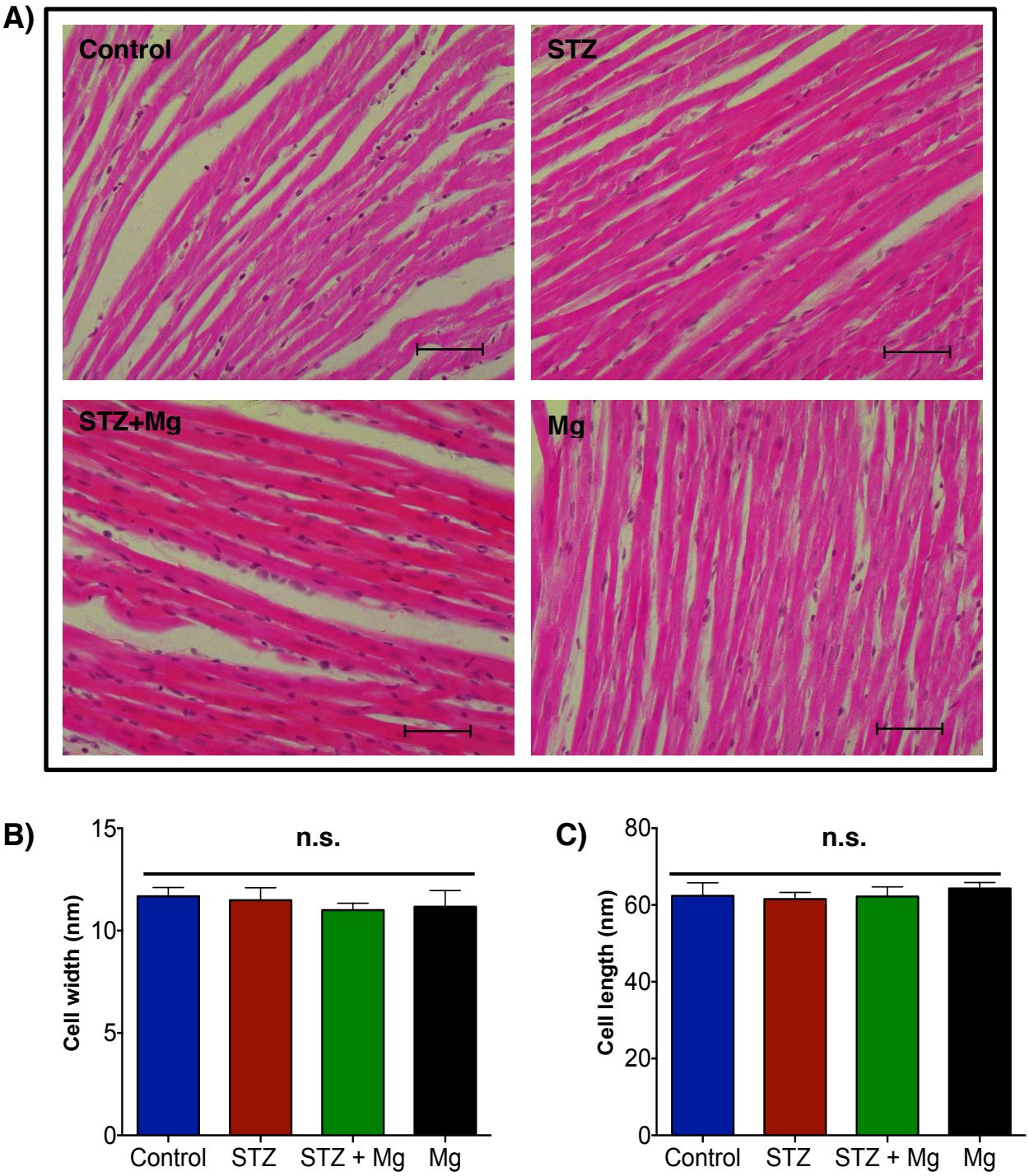

Figure 3-20: The effects of streptozotocin and magnesium on cardiac cell size.

A) Representative examples of haematoxylin and eosin (H&E) stained sections from the various treatment groups. Scale bars in the bottom right corner represent 200µm. Analysis of myocyte width (B), and myocyte length (C) revealed that there were no changes in gross cardiac cell size due to the various treatments. $P = 0.84$, n = 5-7.

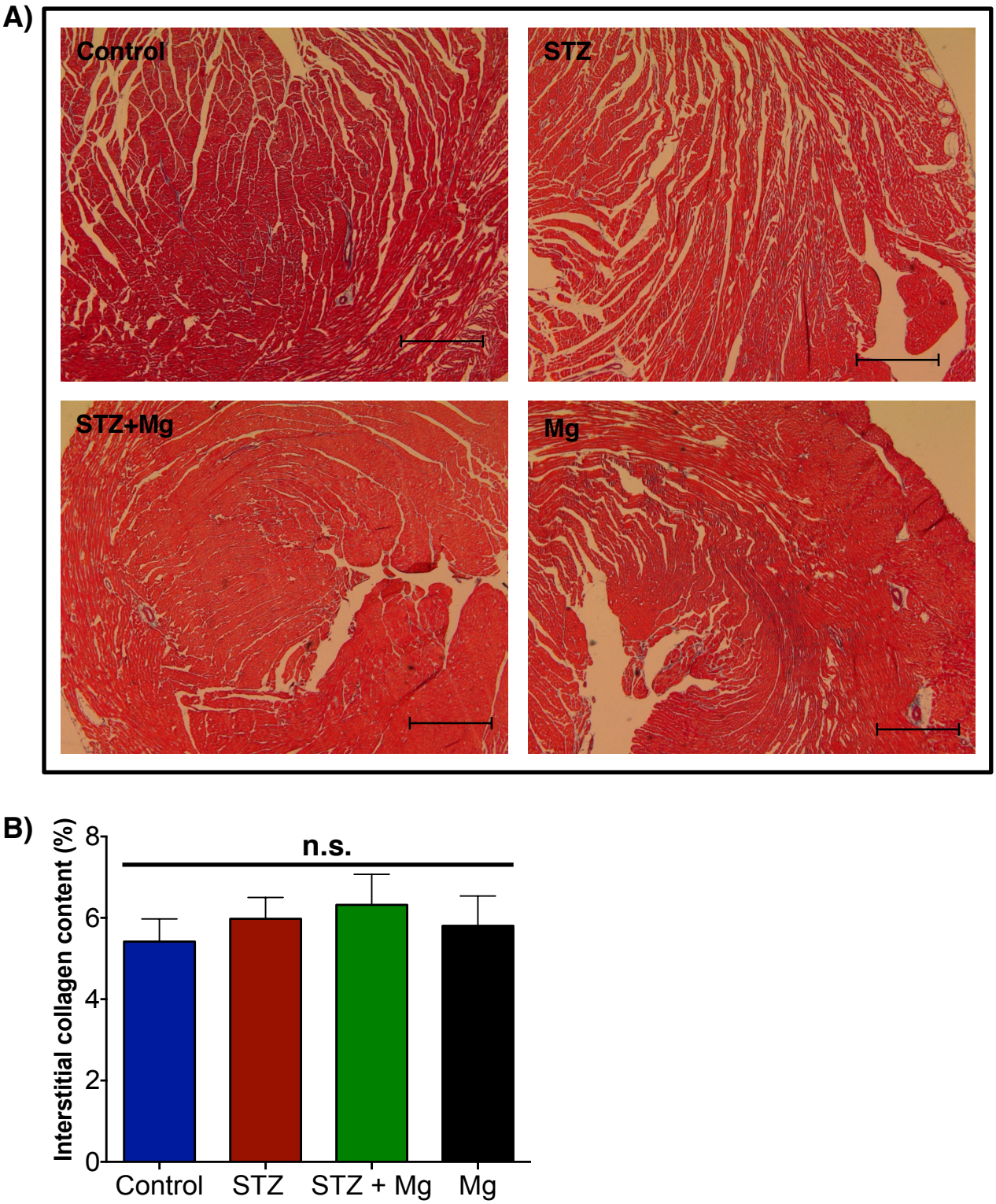

Figure 3-21: The effects of streptozotocin and magnesium treatment on the amount of interstitial fibrosis. (A) Representative examples of Masson's trichrome stained sections from the various treatment groups. Scale bars in the bottom right corner represent 50µm. (B) Quantification analysis of the amount of interstitial fibrosis. No significant differences were seen among the various treatment groups. $P = 0.82$, n = 5-7.

CHAPTER 4: DISCUSSION

In this study we demonstrated that short-term diabetes is associated with the early cardiovascular complications of impairments in heart rat variability and the diastolic ventricular dysfunction of abnormal distensibility. These impairments induced by diabetes were ameliorated by daily Mg^{2+} treatment. However, the metabolic derangements of diabetic dyslipidaemia and hyperglycaemia were unaltered by Mg^{2+} treatment.

4.1 Preservation of cardiac autonomic function and baroreceptor reflex sensitivity in early diabetes

Analysis of HRV is a validated method of assessing cardiac autonomic function and baroreceptor reflex sensitivity (Malliani et al., 1991; Bedette, Santos & Fontes, 2008). Tail pulse plethysmography was demonstrated to be an effective method that could be used to assess early impairments in HRV, in the time domain, frequency domain and orthostatic stress test; and that these defects are prevented by Mg^{2+} treatment.

In the time domain, diabetes-induced impairments in HRV were characterised by reduced RMSSD (figure 3-10), which are markers of vagal tone. Reduction in time domain parameters reflect reduction and impairment in parasympathetic innervation, but may not reflect alterations in sympathetic innervation (Dabiré et al., 1998; Giudice et al., 2002). However, reduction in heart rate was also observed (figure 3-10), a finding which would be discordant with the other time domain parameters, as impaired vagal tone would lead to sympathetic dominance of automaticity and the sinoatrial (SA) node, and thus an increased HR (Japundzic et al., 1990). Therefore, there may also be impairment in the sympathetic innervation and SA node function. Such an effect was demonstrated by analysis of the frequency domain in which diabetic animals exhibited reduced HF power (which is characteristic of impaired vagal tone) as well as an increased LF/HF power ratio (a marker of impaired

sympathetic balance, figure 3-12) (Chiou & Zipes, 1998; Howarth et al., 2005). Therefore, these results in the present study suggest that in early severe diabetes, both vagal and sympathetic autonomic activity to the heart is impaired. What then needs to be further addressed is the finding of significant bradycardia in diabetic animals, as knocking out both divisions of autonomic modulation should result in a faster heart rate at the intrinsic rate of the SA node (Borges et al., 2006). The same bradycardia was observed in diabetic animals in the isolated perfused heart (figure 3-10) (Hicks et al., 1998). In this model, the heart is under sole SA node control. Thus, these findings may suggest that the diabetes-induced bradycardia is most likely due to intrinsic electrophysiological defects in the SA node due to diabetes, which remain to be further clarified.

Diabetes also induced impairments in the orthostatic stress test (figure 3-13), a validated marker of baroreceptor reflex sensitivity (Steinback et al., 2005). Diabetic animals failed to produce the reflex increase in heart rate (tachycardia) in response to orthostatic stress. The impairment in baroreceptor reflex was confirmed to be due to impairment of autonomic activity by analysis of the LF/HF power ratio, which was unchanged during the test. Dall'Ago and colleauges (2002) demonstrated by invasive assessment that the baroreceptor reflex was impaired in short-term, 10 days, STZ-induced diabetes, and localised the defect to the effector sympathovagal nerves; confirming earlier studies that proposed axonal myelin defects in diabetes to underlie neuropathy (Schmidt, Nelson & Johnson, 1980). DeAndrade and collaborators demonstrated that the baroreceptor sensitivity could indirectly be assessed non-invasively. In a model of a central CNS HR regulatory centre defect, by microinjections of cobalt chloride and muscimol, a calcium channel blocker that prevents the release of transmitters and a GABA-A receptor agonist that inhibits the neuronal action potentials respectively. They showed that the LF/HF power ratio was unchanged following a HUT orthostatic stress test, representing impaired sympathovagal activity. Our similar findings in a diabetes model represent a novel demonstration of non-invasive detection of impaired baroreceptor sensitivity in short-term diabetes.

Mg^{2+} is beneficial to the nervous system as demonstrated by Sameshima and collaborators (1999), who showed that Mg^{2+} treatment has a neuroprotective effect in

hypoxic-ischemic brain damage. Moreover, our novel finding of an autonomic neuroprotective effect of Mg^{2+} treatment is supported by evidence from experiments conducted by Begon and associates (2000), who demonstrated that i.p. Mg^{2+} treatment attenuated diabetes-induced peripheral neuropathy in rats. These findings were further validated by Rondón and colleauges (2010) who showed that Mg^{2+} and its deficiency play a role in the pathogenesis of diabetic spinal neuropathy and proposed that Mg^{2+} treatment attenuated diabetic neuropathic pain via allosteric effect on inositol pathways and N^{+}/K^{+} ATPase activity. Although our results are different from these in that our findings relate to autonomic nerves, the principles demonstrated by these researchers are similar to ours, being that Mg^{2+} protects against metabolic injury to neurones in early diabetes.

4.2 Improvement of ventricular dysfunction in early diabetes

In the pressure-volume (PV) response studies, features of early diastolic dysfunction were observed as evidenced by an increase in end diastolic equilibrium volume intercept (V_0), a right shift in the PV response curve and a decrease in its gradient – the end diastolic elastance in diabetic animals (figure 3-15). These results are in agreement with Litwin and colleagues (1990) who observed a right shift and increased time constant of relaxation with increased –dP/dt in a 1-week STZ-induced diabetes model; these abnormalities were reversed by insulin treatment illustrating reversibility of early dysfunction. This study is in contrast to Penpargkul and associates (1980) who observed the same pattern of diastolic dysfunction that was not corrected by acute administration of insulin and glucose in the perfusion medium, suggesting that the dysfunction is due to a remodelling process that cannot be acutely corrected as would be a dysfunction due to a metabolic abnormality. Riva and collaborators (1998) performed similar PV studies on long-term diabetic rats and observed an increased V_0 compared to control animals, which are comparable to our results. PV response experiments have been used in other animal models of ventricular remodelling, to demonstrate cardiac dilatation (increase V_0) and pump

dysfunction (right shifted PV curves) (Osadchii et al., 2007; Hodson et al., 2014); supporting the postulate that our findings of ventricular dysfunction represent evidence of early diabetic remodelling of the left ventricle.

In contrast to the diastolic parameters, systolic parameters of the PV studies including the systolic intercept, LVESP-volume relationship (figure 3-16), end systolic elastance (figure 3-17) and developed pressure (figure 3-19) were unaltered in diabetes and Mg^{2+} treatment. These results are in concert with other researchers who observed diastolic dysfunction without concomitant systolic dysfunction in early or short-term diabetes (Litwin et al., 1990; Borges et al., 2006), but observed systolic dysfunction following long-standing diabetes (Riva et al., 1998; Hoit et al., 1999). Moreover, these findings are in agreement with clinically observed patterns of diabetic cardiomyopathy in patients, which is characterized by early diastolic dysfunction with preserved systolic function that precedes other overt cardiovascular complications (Chaudhary et al., in press; Cosson & Kevorkian, 2003).

In this study, Mg^{2+} also prevented early ventricular dysfunction observed in short-term diabetic rats, which is a novel finding in this study. These findings are similar to our prior studies in which we described a cardioprotective effect of Mg^{2+} in preserving ventricular function in non-diabetic animal model of ventricular remodelling (Amoni et al. 2015). Furthermore, findings that Mg^{2+} treatment improves ritonavir-induced ventricular dysfunction in rats by improving carbohydrate metabolism and oxidative stress (Mak et al., 2013) support its beneficial effects. However, Ogunyankin and colleagues (1995) demonstrated that 200 µmol/kg intravenous Mg^{2+} does not prevent ventricular dysfunction and remodelling following ischaemia-reperfusion injury, which highlights the necessity of clarifying the mechanisms by which the observed beneficial Mg^{2+} effects are observed. The first consideration to be addressed in discussing the underlying mechanisms and factors involved in early autonomic- and ventricular dysfunction is to explore whether they are a reflection of alterations in structure. Neuronal structural studies are complex and beyond the scope of this study, thus, we focussed on investigating cardiac histological structure.

4.3 Gross cardiac structure: unaltered in short-term diabetes

Our histological studies found no evidence of structural alterations in cell size (figure 3-20) or interstitial fibrosis (figure 3-21) in diabetic or Mg^{2+} treated animals. These findings are consistent with studies by Litwin and colleagues (1990) who observed impairments in LVEDP-volume relationship without histological changes of cell morphology or collagen content in short-term diabetes. The findings are also supported by Dent and associates (2001) who observed diastolic dysfunction by echocardiographic assessment in early diabetic rats, without changes in myocardial collagen content. Based on their modelling, it was suggested that the impairment was functional and related to diabetic effects of uncoupling of the contractile apparatus probably related to protein modifications. Furthermore, in chronic diabetes, ventricular dysfunction has been linked to the structural ventricular remodelling of increased interstitial fibrosis; further supporting the theory that in early diabetes, which is not characterised by ventricular remodelling and fibrosis, the ventricular dysfunction is likely to be due to functional rather than functional abnormalities (Norton, Candy & Woodiwiss, 1996; Riva et al., 1998; Miric et al., 2001). This suggests that the early ventricular dysfunction may be a result of early reversible functional remodelling that precedes permanent structural remodelling; which has been attributed to AGEs and oxidative stress (Van Heerebeek et al., 2008; Patel, Raghunathan & Porwal, 2014). The role of AGEs or oxidative stress was thus not investigated in this study, given that the study sought mechanisms associated with dysfunction in short-term diabetes. Other possible metabolic derangements that could help us understand these early complications as well as the beneficial effects of Mg^{2+} treatment were thus investigated.

4.4 Unchanged hyperglycaemia and gross magnesium levels point to alternate mechanism of magnesium effects

STZ-induced diabetes was found to cause a sustained hyperglycaemia that was unaltered by Mg^{2+} treatment in the present study (figure 3-2). On the one hand, Mg^{2+} is suggested to improve glycaemic control and insulin profiles in diabetic patients (Lal et al., 2003; Barbagallo, Dominguez & Resnick, 2007); this is supported by evidence presented by Soltani and colleagues (2005), Hasanein and partners (2006), and other researchers who observed that 1g/L oral Mg^{2+} therapy prevents STZ-induced hyperglycaemia. Moreover, Soltani presented histological evidence that Mg^{2+} prevented STZ-induced β-cell destruction that occurred in untreated animals. The protection of β-cells from destruction as a protective mechanism from hyperglycaemia and its adverse effects is supported by evidence presented by Patel and others (2014), who showed that Mg^{2+} valproate is effective at preventing STZ induced hyperglycaemia and cardiac complications; although this was attributed to the action of valproate as a histone deacytylase inhibitor preventing STZ-induced pancreatic β-cells destruction rather than an action of Mg^{2+}.

On the other hand, there is sufficient evidence that Mg^{2+} does not prevent hyperglycaemia and the induction of diabetes: Pelit and associates (2013), and Rondón and collaborators (2010), among other authors observed that Mg^{2+} supplementation in the dosage of 1 g/L and 296 mg/L respectively, did not prevent hyperglycaemia or return glucose levels to normal. This argument is supported by deLordes Lima and co-workers (1998), who presented evidence that the beneficial effects of Mg^{2+} treatment on hyperglycaemia in diabetic patients are dose dependant with a narrow therapeutic range. Therefore, while consideration is given to the thought that Mg^{2+} may prevent pancreatic β-cell destruction due to STZ, this was beyond the aims and scope of this study that sought to investigate the effects on cardiovascular complications in the presence of diabetes. Thus, it may suggest that single dose Mg^{2+} may exert effects on metabolic and signalling processes.

A key metabolic process by which Mg^{2+} treatment could exert beneficial effects is by modulating Mg^{2+} homeostasis. Hypomagnesaemia is a commonly observed metabolic derangement in diabetes implicated as an underlying mechanism involved in development of complications of neuropathy (Engelen et al., 2000), ventricular dysfunction (Freedman et al., 1990), abnormal lipid metabolism (Mak et al., 2013) and cellular Mg^{2+} homeostasis (Fagan, Cefaratti & Romani, 2004). This makes the effect of Mg^{2+} treatment on plasma and tissue Mg^{2+} levels of great interest. The findings of this study revealed that in short-term diabetes, there were no changes in plasma- or cardiac tissue Mg^{2+} concentration (figure 3-7 & 3-8). The absence of hypomagnesaemia was probably in keeping with the short-term diabetes model used in this study, because hypomagnesaemia is reported to be more common in chronic, poorly controlled diabetes (Ward et al. 2001; Soltani et al. 2005). Thus, it is unlikely that the effects of Mg^{2+} observed in our study were related to a correction of plasma hypomagnesaemia in diabetes, as there were no differences in plasma Mg^{2+} levels among the Mg^{2+} treatment groups at the time of measurement. Furthermore, in the cardiac tissue Mg^{2+} concentrations, although no significant differences were observed, the untreated diabetic hearts had the lowest Mg^{2+} levels, suggesting the presence of a certain degree of Mg^{2+} deficiency in diabetic hearts that could be modulated by Mg^{2+} treatment. This is congruent with findings of Reed and others (2008) that diabetic animals exhibited worsening cardiac tissue Mg^{2+} levels, which although not apparent after 1 week were evident at 2 weeks and statistically significant after 4 weeks. Therefore, we cannot rule out a relative hypomagnesaemia, which may occur in the intracellular compartment. This is in accord with Paolisso & Barbagallo (1997) who proposed that Mg^{2+} deficiency may occur in the presence of normal serum Mg^{2+} concentrations and may be attenuated via intracellular shift by Mg^{2+} supplementations. This has been supported by evidence from studies by Soltani and collaborators (2005), and Pelit and associates (2013) who observed beneficial effects of Mg^{2+} treatment despite lack of significant changes in plasma Mg^{2+} concentrations.

4.5 Early diabetic dyslipidaemia – continuing the search to clarify the role of Mg^{2+}

Investigation of the diabetic lipid profile and effect of Mg^{2+} on it was sparked by an observation of an abnormality in turbidity of the plasma of diabetic animals compared to non-diabetic animals. The plasma of diabetic animals was milky and opaque due to high triglyceride concentration, in comparison to non-diabetics, which had straw coloured and clear plasma (figure 3-3). Biochemically, diabetic animals had considerably increased triglycerides (figure 3-4), and had raised total cholesterol and LDL-C with trend towards low HDL-C; further analysis of the HDL-C subfractions revealed there was a trend towards reduced large subfractions and increased intermediate subfractions (figure 3-5). These parameters were used for risk stratification, which showed that diabetic animals had a high atherogenic index that was unaltered by Mg^{2+} treatment (figure 3-6).

The diabetic dyslipidaemia observed in this study is parallel to the typical diabetic dyslipidaemia observed in other short-term and long-term STZ-induced diabetes models (Soltani, Keshavarz & Dehpour, 2007; Ojezele, 2011), as well as in clinical practice, of elevated triglycerides and LDL-C, and reduced HDL-C (Taskinen, 2003; Colhoun, 2005). However, our observations of a lack of effect of Mg^{2+} treatment on these lipid derangements in short-term diabetes are different from studies such as Olatunji & Soladoye (2007) and Soltani and others (2007), which show that Mg^{2+} supplementation reduces plasma total cholesterol, triglycerides, and ameliorates the atherogenic risk ratio in chronic long-term diabetes. Moreover, although beneficial effects of the normalisation of diabetic dyslipidaemia by Mg^{2+} supplementation have been demonstrated in clinical studies (Djurhuus et al., 2001), the effects of Mg^{2+} on the lipid profile and atherogenic risk of patients is controversial (Lal et al., 2003). The lack of beneficial effects in short-term Mg^{2+} supplementation was observed by De Valk and collaborators (1998), supporting our findings and the theory that the regulatory role of Mg^{2+} on lipid metabolism may be indirect via its modulatory role as a co-factor on metabolic pathways that impact on lipogenic gene expression over time (Mak et al., 2013).

HDL subfraction analysis has received growing interest in the pathophysiology of CVD and cardioprotection (Barter & Rye, 1996; Frias et al., 2012). The function and roles of HDL subfractions in CVD are still being clarified, but preliminary evidence suggests that it is the quality of the subfractions and not the quantity of HDL-C that is an important cardiovascular risk factor (Rizzo et al., 2014). The, novel results in this study represent the first attempt at HDL-C subfraction analysis in STZ-induced diabetes in rats. While the small dense subfractions are close to undetectable, it was interesting to note a trend towards reduced large subfractions. These results are in keeping with recent studies that demonstrated increased large HDL-C subfractions to be associated with obesity and increased CVD risk (Woudberg et al., 2016). Further investigations into the functionality and importance of the HDL-C subfractions and relationship with Mg^{2+} are warranted to further inform our understanding of their relevance in diabetic cardiovascular complications.

4.6 The streptozotocin experimental model of diabetes

Several models of diabetes exist that are comparable to Type I (Insulin dependent diabetes mellitus/IDDM) and Type II diabetes (Non-Insulin dependent diabetes mellitus/NIDDM). In this study, we used an IDDM model, which is widely used to study the early cardiovascular complications of diabetes due to the rapid onset of diabetes and the early effects on metabolism and the cardiovascular system (De Angelis, Irigoyen & Morris, 2009; Deeds et al., 2011). Several methods of diabetes induction to create this model are used in laboratory animals with varying success and benefits. For induction of diabetes (IDDM), 90-95% of the pancreas needs to be removed which can be done surgically or pharmacologically. The surgical option has the advantage of certainty, however, it is a highly technical skill with numerous risks due to surgery, anaesthesia and infection; furthermore, if small pieces of the pancreas are not resected, they soon confound the experiment as they regenerate and begin insulin production. Therefore, the pharmacological ‘pancreatectomy’, such as by the β-cell toxin STZ provides a cost effective, expeditious and nontechnical

method of diabetes induction. This induces IDDM that is similar to the pathology of type 1 IDDM seen clinically characterized by severe hypoinsulinaemia and hyperglycaemia (Deeds et al., 2011; Kumar et al., 2012).

The STZ-treated animals in this study exhibited overt hyperglycaemia (figure 3-2), well exceeding the ubiquitous threshold of 15mmol/L, from the first day following STZ administration. This rapid onset of diabetes is consistent with the literature and attributed to the rapid action of STZ in destroying the pancreatic β-cells (Deeds et al., 2011; Ojezele, 2011). STZ induced diabetes successfully in 90% of cases (n = 50), while the vehicle did not induce hyperglycaemia in any cases (table 1). These results are remarkably close to current literature where success rates of 90-100% are observed (Norton, Candy & Woodiwiss, 1996; Howarth et al., 2005; Choi et al., 2013). The minor failure rate could be attributable to the process of injection and absorption, an inherent challenge of the intraperitoneal injection; such as if the substance is injected into intestinal lumen, wall or any other structures in the peritoneal cavity (Deeds et al., 2011). Further consideration should be given to the fact that a minor population of animals display inherent resistance to low dose STZ (Abeeleh et al., 2009).

The toxicity was assessed by body weight changes and mortality rate, which took into account all animals that deteriorated and had to be euthanized due to severe systemic toxicity. Diabetic animals showed a trend of weight loss that was not altered by Mg^{2+} treatment (figure 3-1). This is a common observation as the animals exhibit weight loss most likely due to insulin deficiency and the failure to utilize glucose in addition to the barrage of metabolic derangements accompanying the onset of hyperglycaemia and diabetes (Ward et al., 2001; Borges et al., 2006). Moreover, it was noted that STZ-treated animals seemed to be less active than control animals. This attribute was a confirmation of observations by Howarth and colleauges (2005), who noticed that the STZ animals show a rapid decline in activity in the first 5 day following STZ treatment that was significantly lower than healthy animals.

In this study, we observed that 10% of STZ treated animals suffered severe systemic toxicity of STZ and diabetes and had to be euthanized (table 1). According to the literature, other authors report similar or lower mortality rates of about 5-10% (Deeds

et al., 2011; Salum et al., 2012). However, it is difficult to contextualize this aspect of our model in the literature, because, although this phenomenon is well known, authors often neglect to account for this preferring to make general statements or not report mortality data. The larger mortality rate seen in this study was most likely due to optimisation of induction protocol, specifically, the starvation time prior to drug administration that enhances the STZ sensitivity by depleting glucose stores and reducing competition at GLUT transporter sites for STZ to be maximally absorbed into pancreatic β-cells (West, Simon & Morrison, 1996). By using a shorter starvation time of 6 hours, as compared to the commonly used 12 hours the severity of the negative effects of STZ was reduced, while maintaining the effectiveness of the induction of diabetes. Some of the negative effects seen have been attributed to an immediate post-STZ hypoglycaemia, followed by an overwhelming hyperglycaemia with ketosis, which may overcome the animal's adjustment capacity. Some authors suggest giving the animals a supplemented 10% glucose solution as drinking water for the 24hours subsequent to the starvation and STZ administration to further prevent hypoglycaemia and toxicity (Brosius, 2003).

4.7 Limitations and future studies

A key limitation of this study is that the mechanisms of the beneficial effects of Mg^{2+} treatment observed were not clarified. Histological studies by Sanyal and associates (2012) and Li and co-workers (2015), suggest that the mechanisms underlying the features of autonomic neuropathy observed in diabetes are due to damage and remodelling of autonomic nerves via the modulation of enzymes involved in neurotransmitter synthesis and depletion of cholinergic neurotransmitter vesicles in cardiac parasympathetic nerve endings. To investigate this in our model of diabetes and Mg^{2+} treatment, in future experiments, western blot analysis of key proteins in both the SA node and the terminal nerve endings such as hyperpolarisation-activated channel (HCN) and syndophinin could be performed. Furthermore, western blot studies of the Mg^{2+} transporter TRPM7 that is implicated in in ventricular remodelling (Antunes et al., 2016) will provide further clarity on the role of Mg^{2+} in diabetic complications.

Another limitation of this study is that, although the STZ-induced diabetic rat is a good model of human diabetic complications, there is controversy regarding the transferability of results, such as differences in HRV profiles, to clinical situations because of species-related differences. Moreover, the artificial nature of the diabetic model and by extension the beneficial effects of Mg^{2+} observed may not be present in the human disease model; highlighting the need for corroboration by clinical trials in humans. Finally, the results of this study provide a strong basis for the investigation of the sustainability of cardiovascular effects of Mg^{2+} in chronic diabetes.

CONCLUSION

The results of this study suggest that Mg^{2+} treatment may prevent impairments in autonomic function, as demonstrated by HRV and baroreceptor reflex sensitivity assessment; and improve left ventricular pressure-volume responses, a reflection of ventricular function, in early diabetes. The observation that Mg^{2+} modulates these diabetes-induced cardiovascular abnormalities without preventing diabetes-induced dyslipidaemia or hyperglycaemia, as well as the lack of significant changes in cardiac microstructure, plasma- and tissue Mg^{2+} content suggests that the mechanism is independent of these factors. While this study did not clarify the exact mechanisms of the protective action of Mg^{2+}, the trends of reduced tissue Mg^{2+} content suggest that changes could have been mediated intracellularly; an aspect that should be explored in future studies. Furthermore, whether these effects would be translatable to chronic diabetes is an important next question. Clinically, the results imply that Mg^{2+} supplementation therapy in early diabetic states may attenuate the onset of cardiovascular complications in the early stages of diabetes, a foundation for translational clinical studies.

CHAPTER 5: REFERENCE LIST

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PUBLICATIONS & AWARDS

Papers in Peer Reviewed Journals

Matthew Amoni, Dee Blackhurst, Roisin Kelly-Laubscher and Asfree Gwanyanya. ***Beneficial Effects of Magnesium Treatment on Heart Rate Variability and Cardiac Ventricular Function in Diabetic Rats.*** Journal of Cardiovascular Pharmacology and Theraputics. 2016 Jun 8. DOI: 10.1177/1074248416653831.

Abstracts in Peer Reviewed Journals

Matthew Amoni, Roisin Kelly-Laubscher and Asfree Gwanyanya. ***Improvement of diabetes-induced cardiac autonomic dysfunction and left ventricular diastolic pressure-volume response by magnesium pretreatment in streptozotocin-induced diabetic rats.*** SA Heart Journal, Volume 12, Number 4, Spring 2015.

AMONI Matthew, KELLY-LAUBSCHER Roisin, GWANYANYA Asfree. ***Preservation of Heart Rate Variability and Improvement of Cardiac Ventricular Distensibility by Magnesium Treatment In Diabetic Rats.*** African Journal of Physiology.

Awards

Best speaker. 3rd EU-SASCAR Cardiovascular Conference.

CHAPTER 6: APPENDICES

APPENDIX 1: Animal ethics approval letter

UNIVERSITY OF CAPE TOWN

Health Sciences Faculty
Research Ethics Committee
Room E53-24 Groote Schuur Hospital Old Main Building
Observatory 7925
Telephone [021] 406 6338 • **Facsimile** [021] 406 6411
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06 May 2014

AEC REF NO: 014/014

Dr A Gwanyanya
Human Biology
Anatomy Building

Dear Dr Gwanyanya

PROJECT TITLE: DIABETES MELLITUS AND THE HEART: EFFECTS OF MAGNESIUM AND OTHER ION CHANNEL MODULATORS ON DIABETES AND MYOCARDIAL INJURY IN DIABETIC WISTAR RAT HEARTS

Thank you for submitting your study to the Faculty of Health Sciences Animal Ethics Committee for review.

It is a pleasure to inform you that the FHS AEC has authorised your study specifically for the use of 252 Wistar rats for the period of three years.

Please note that the first annual progress report is due in May 2015.

Please quote the REC REF in all your correspondence

Yours sincerely

Signed

PROF PJ COMMERFORD
CHAIR, HSF AEC

APPENDIX 2: Streptozotocin preparation and Injection

A1.2.1: Reagent and solution preparation

0.1 M sodium citrate:

Dissolve 0.294 g of sodium citrate tribasic dihydrate (Sigma C0909, MW 294.10) in 10 ml ddH2O and store at room temperature. Mix on vortex.

0.1 M citric acid:

Dissolve 0.210 g of citric acid monohydrate (Sigma C1909, MW 210.14) in 10 ml ddH2O and store at room temperature. Mix on vortex.

A1.2.2: STZ Preparation and injection protocol

1. IMPORTANT: Prepare reagents prior to experiments and treat with Mg/Saline at least an hour prior to induction of diabetes with streptozotocin.

2. Remove the food from rats (DO NOT remove the water bottle) and fast the rats overnight starting around 8 pm for injection at 8am (~12 hours).

3. Prepare Citrate buffer fresh before use by combining 5.3 ml of 0.1 M Sodium citrate with 4.7 ml of 0.1 M Citrate acid to produce 10ml of 0.1 M Citrate buffer (See Fig 19 below). Measure pH and adjust to 4.5 if necessary. Filter-sterilize the Citrate buffer and store it in a sterile tube on ice.

4. Weigh the rats and tag/tail label them.

5. Add Citrate buffer to Streptozotocin (STZ) in a sterile Eppendorf covered with aluminium foil to prevent light entry in a biosafety hood right before use to a final concentration of 50 mg/ml and keep on ice. For example, add 1 ml of Citrate buffer to 50 mg of Streptozotocin. Mix on vortex.

6. Inject STZ at 50 mg/kg, i.p. (0.01ml/10 g; therefore if animal: 250g = 0.25; 275 = 0.27; 300 = 0.3)

7. Inject Citrate buffer at 0.01ml/10 g for the **control groups**.

8. Remove the glucose and feed the rats with fresh water the next day.

9. Monitor body weight and blood glucose after injection daily.

10. Harvest tissue samples after euthanizing the rats with 70mg/kg i.p. pentobarbital.

A1.2.3: Citric Acid – Sodium Citrate Buffer Solutions recipe

To create a buffer of pH 4.5 mix

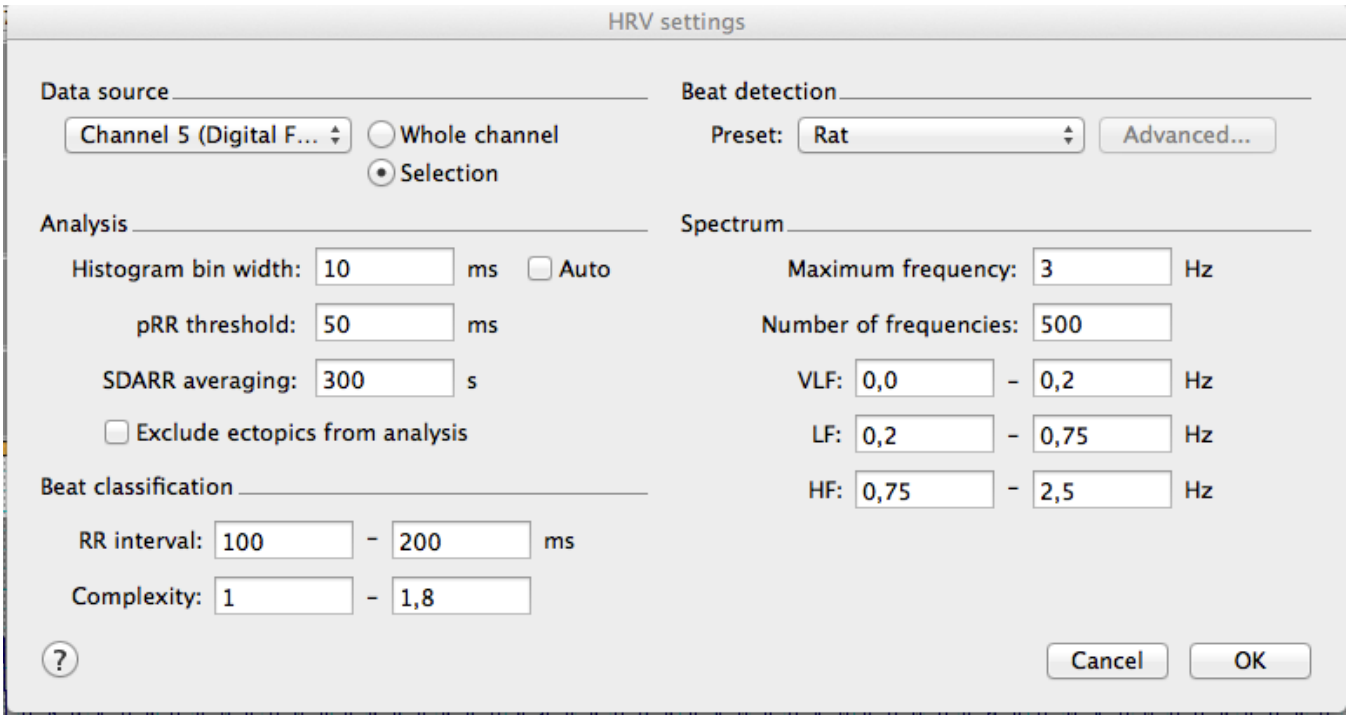
- 47.5ml of Citric acid monohydrate C6H8O7.H2O, M.wt 210.14 (0.1M solution containing 21.01g/l)
- 53.5ml of Trisodium citrate dehydrate C6H5O7Na3.2H2O M.wt 294.12 g/l (0.1M solution containing 29.41 g/l)

or equivalent ratios.

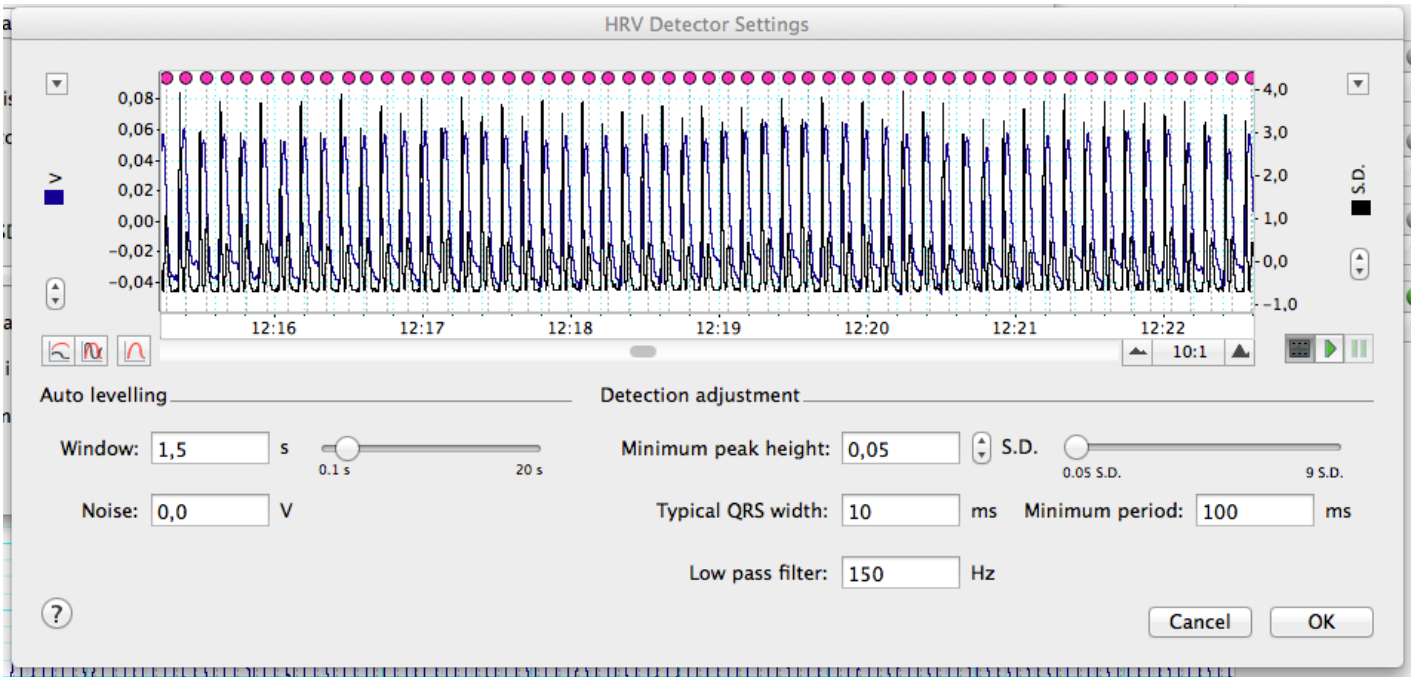
Adapted from Sigma-Aldrich Buffer Reference Centre (http://www.sigmaaldrich.com/life-science/core-bioreagents/biological-buffers/learning-center/buffer-reference-center.html)

APPENDIX 3: Heart rat variability analysis settings

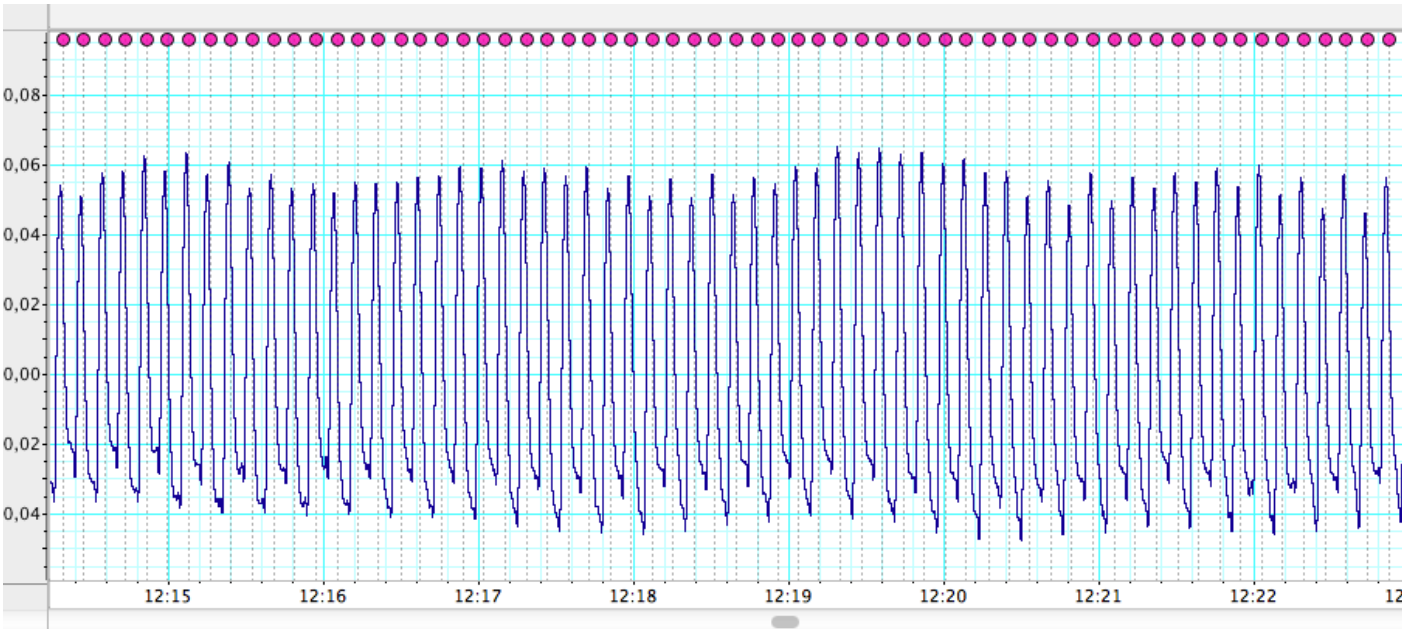
To ensure that all pulse wave peaks/'R-waves' were picked up accurately by the LabChart software, the following settings were used. The first figure below is a screenshot of the HRV Settings module with the detection set to the 'rat' pre-set parameters and spectral power analysis according to the Task Force of The European Society of Cardiology and The North American Society of Pacing and Electrophysiology (1996) guidelines: very low frequencies (VLF) < 0.2 low frequencies (LF) <0.8 high frequencies (HF) < 2.8:



After the beat detection, the tracing was manual scanned to ensure that all peaks or beats were accurately detected. If not, the advanced beat detection was used to manipulated the threshold for detection (i.e. the amplitude in mV reduced to 0.02mV for weak signals) as shown below:



This resulted in clean tracing identifying all beats accurately as shown below:



APPENDIX 4: Lipid Profile Assessment

A3.1: Protocol for colorimetric quantification of triglycerides

TRIGLYCERIDE ASSAY: Wako (3 µg/ µl; Linear 0-6 µg/ µl)

Generate a standard curve by adding the following components to successive wells of a 96 well microplate (Lasec, SA):

Well No.	1	2	3	4	5	6	7	8	9	10	11	12
Standard (µg)	0	2	4	10	14	20	30	40	50		?	?
Standard (µl)	0	1	2	5	7	10	15	20	25		10	10
Saline (µl)	50	49	48	45	43	40	35	30	25		40	40
											Ctrl	Ctrl

Fill the rest of the wells with: Saline 10 µl + 40 µl plasma samples.

Add 250 µl of chromogenic reagent to all samples. Allow the plate to stand for 30 min at room temperature to ensure complete reaction and colour change. Then read the absorbance at 510 nm.

A3.2: Protocol for colorimetric quantification of Cholesterol

CHOLESTEROL ASSAY: Wako (2 µg/ µl; Linear 0-6 µg/ µl)

Generate a standard curve by adding the following components to successive wells of a 96 well microplate (Lasec, SA):

Well No.	1	2	3	4	5	6	7	8	9	10	11	12
Standard (µg)	0	2	4	6	10	14	20	26	32		?	?
Standard (µl)	0	1	2	3	5	7	10	13	16		10	10
dH2O(µl)	50	49	48	47	45	43	40	37	34		40	40
											Ctrl	Ctrl

Fill the rest of the wells with: dH2O 5 µl + 45 µl plasma samples / dH2O 30 µl + 20 µl of HDL sample. Add 250 µl of chromogenic reagent to all samples. Allow the plate to stand for 30 min at room temperature to ensure complete reaction and colour change. Then read the absorbance at 600 nm.

A3.3: Quantification of High-density lipoprotein cholesterol (HDL)

High-density lipoprotein cholesterol (HDL-C) can be separated from low-density lipoprotein cholesterol (LDL-C) and very low-density lipoprotein cholesterol (VLDL-C) by single precipitation. The principle of this method is that LDL-C and VLDL-C are apolipoprotien(apo)-B containing lipoproteins whereas HDL (apo-A lipoprotein containing) is the only non-apo-B containing cholesterol. Furthermore, it is well established that apo-B lipoproteins form insoluble complexes with sulphated polysaccharides in the presence of divalent cations, most stably with Mn2+. Therefore, sodium heparin sulphate, a sulphated polysaccharide and Mn2+ can be used to bind and precipitate LDL and VLDL-C from plasma thereby separating HDL-C which remains dissolved in plasma and thus can be quantified by simple colorimetric methods.

A3.3.1: Modified Gidez Method

Add the following components in an eppendorf tube:

Sample plasma ……. 0.5 ml
Heparin sodium (5 000 IU/ml) ……...……20 µl
Manganese chloride ……...…… 50 µl

Mix briefly by vortexing and allow to stand at 4°C for 30 min

Centrifuge at 1500 rpm at 4°C for 20 min

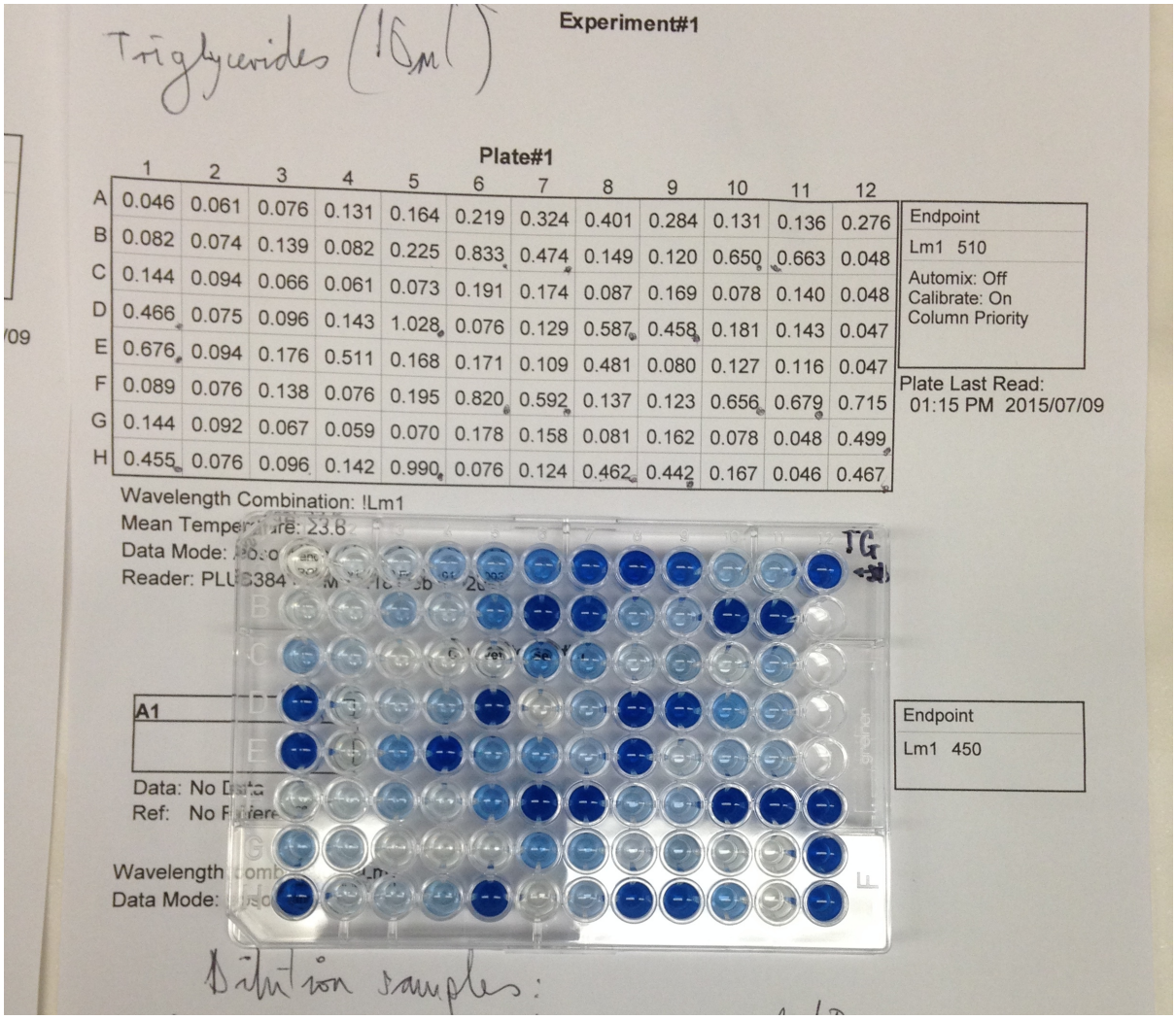
Carefully transfer or pour out the supernatant (HDL-C) into a fresh eppendorf leaving the pellet of precipitated apo-B lipoproteins (LDL- and VLDL-C).

Chelate the excess Mn+ ions by adding ethelyne-diamine tetracetic acid (EDTA) 0.4mol/L pH 7.5.

Quantify the HDL-C using the cholesterol quantification assay described above.

A3.4: Protocol for the determination of triglyceride/cholesterol concentration from standard curve

The absorbance determined by the spectrophotometer will be produced as a representative table of a block for each column showing the absorbance of the well in nanometres as follows:



Triglycerides (16m)

Experiment#1

Plate#1

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.046	0.061	0.076	0.131	0.164	0.219	0.324	0.401	0.284	0.131	0.136	0.276
B	0.082	0.074	0.139	0.082	0.225	0.833	0.474	0.149	0.120	0.650	0.663	0.048
C	0.144	0.094	0.066	0.061	0.073	0.191	0.174	0.087	0.169	0.078	0.140	0.048
D	0.466	0.075	0.096	0.143	1.028	0.076	0.129	0.587	0.458	0.181	0.143	0.047
E	0.676	0.094	0.176	0.511	0.168	0.171	0.109	0.481	0.080	0.127	0.116	0.047
F	0.089	0.076	0.138	0.076	0.195	0.820	0.592	0.137	0.123	0.656	0.679	0.715
G	0.144	0.092	0.067	0.059	0.070	0.178	0.158	0.081	0.162	0.078	0.048	0.499
H	0.455	0.076	0.096	0.142	0.990	0.076	0.124	0.462	0.442	0.167	0.046	0.467

Endpoint
Lm1 510
Automix: Off
Calibrate: On
Column Priority

Plate Last Read:
01:15 PM 2015/07/09

Wavelength Combination: !Lm1
Mean Temperature: 23.6
Data Mode:
Reader: PLUS384

TG

A1

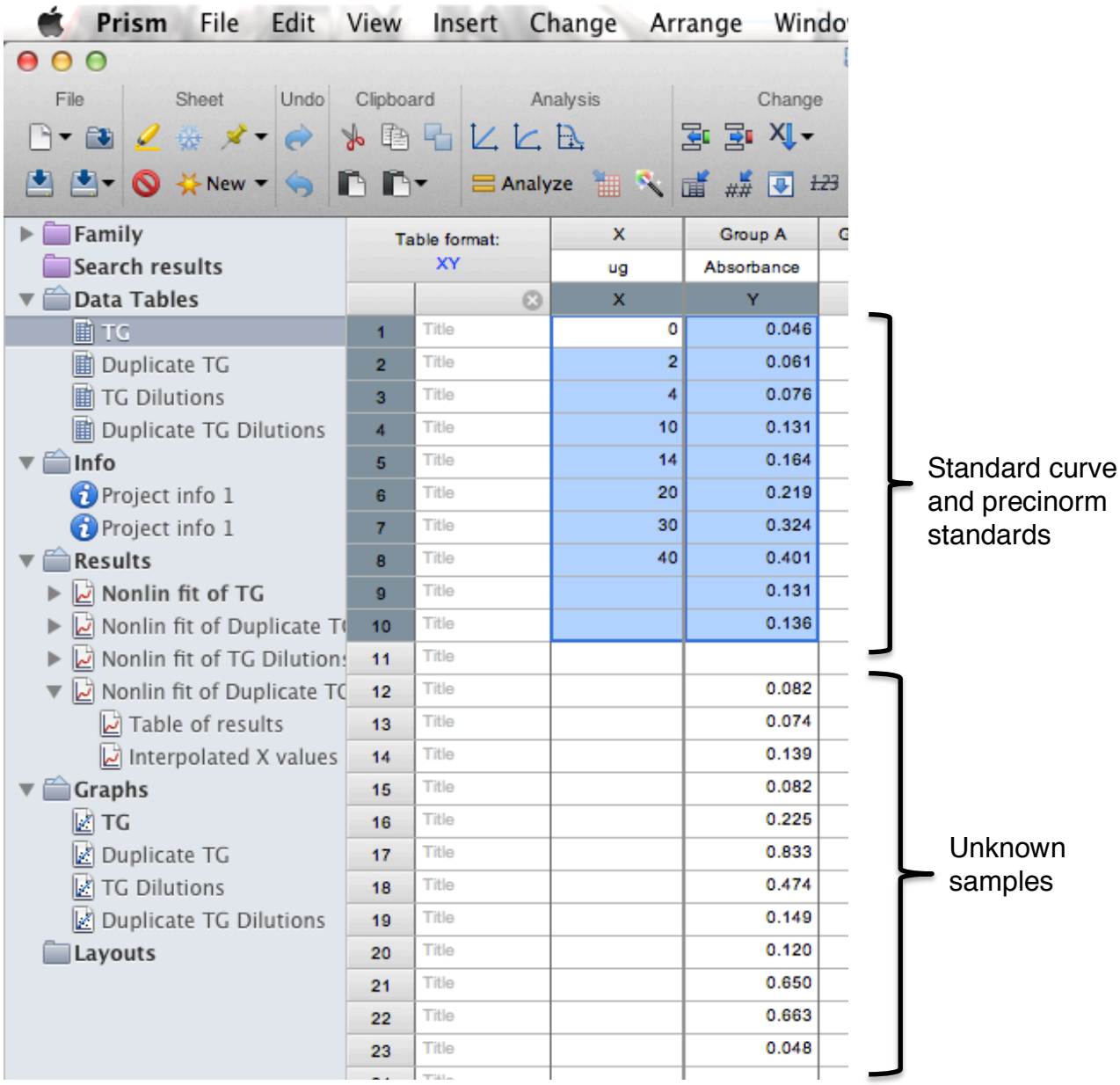
Data: No Data
Ref: No Ref

Endpoint
Lm1 450

Wavelength
Data Mode:

Dilution samples:

Each well is then correlated to its respective sample identity and inputted into an excel sheet. These values are then input into an “XY graph” table in GraphPad Prism software in order with the standard curve (known concentration of cholesterol/TG and respective absorbance) first then the unknown samples below it:



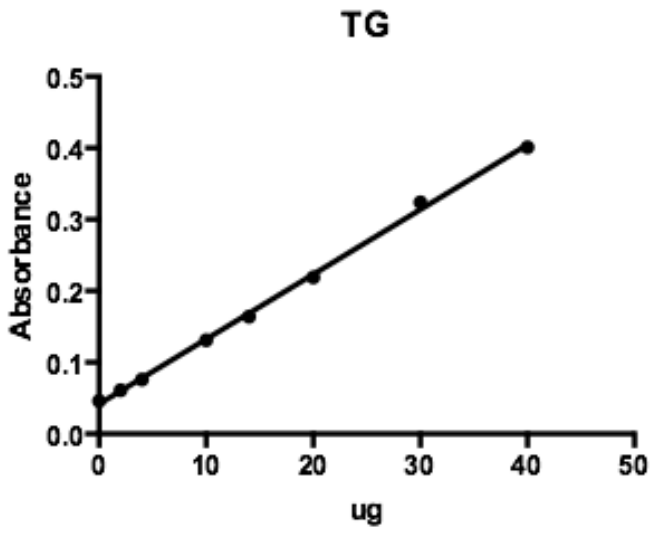
The standard curve graph is then generated by analysing the standard curve concentration and respective absorbance by a non-linear regression equation programmed as “ACAT”:

ACAT: $y = a*x/[b+x] + C$ where initial values set at: a = 0.5; b = 1.0 and C = 1.0

Values are then fitted by least squares, completing the generation of the standard curve. The unknown values are then interpolated from the standard curve.

The final output is in the form of a 4 panel layout showing the standard curve values, the standard curve graph, the linear regression analysis results highlighting the R-square value (the closer to 1, the more accurate the curve), and the interpolated unknown values determined.

ug	Absorbance
0	0.046
2	0.061
4	0.076
10	0.131
14	0.164
20	0.219
30	0.324
40	0.401
	0.131
	0.136



	Absorbance
ACAT	Ambiguous
Best-fit values	
a	Value too large
b	~ Value too large
C	0.04171
Std. Error	
a	4.052e+037
b	~
C	0.002126
95% Confidence Intervals	
a	
b	(Very wide)
C	0.03625 to 0.04718
Goodness of Fit	
Degrees of Freedom	5
R square	0.9985
Absolute Sum of Squares	0.0001807
Sy.x	0.006012
Number of points	
Analyzed	8

ug (Interpolated)	Absorbance (Entered)
9.845	0.131
10.397	0.136
4.442	0.082
3.560	0.074
10.727	0.139
4.442	0.082
20.210	0.225
	0.833
47.665	0.474
11.830	0.149
8.632	0.120
	0.650
	0.663
0.693	0.048

The concentration determined by the interpolation represents the amount of cholesterol/TG (in µg) per total volume of sample used (e.g. 10µl) therefore, the concentration is acquired by dividing the volume of the sample by the amount of cholesterol/TG determined.

i.e. 10.4 µg in 10µL = 1.04 µg/µL

This is then converted to mg/dL or mmol/L as follows:

Triglyceride calculations:

a) conversion of µg/µL to mg/dL	b) conversion of µg/µL to mmol/L
mg/dL = µg/µL* 100	mmol/L = µg/µL / 0.885
i.e. 1.04 µg\µL*100 = 104mg/dL	i.e. 1.04 µg/µL / 0.0885 = 1.18mmol/L

Total cholesterol calculations:

a) conversion of µg/µL to mg/dL	b) conversion of µg/µL to mmol/L
mg/dL = µg/µL* 100	mmol/L = µg/µL / 0.3861
i.e. 1.04 µg\µL*100 = 104mg/dL	i.e. 1 µg/µL / 0.3861 = 2.586 mmol/L

The known triglyceride/cholesterol standards used to confirm the success of the standard curve used were Precinorm Standard (Roche systems, SA). The reference range for the precinorm standard is: TG (92 – 128 mg/dL or 1.06 – 1.42 mmol/L); and cholesterol (77.4 – 106.4 mg/dL or 1.99 – 2.11 mmol/L). Our standard curve was successful with an R-square value of 0.9985 for TG and 0.995 for Cholesterol. Furthermore, the Precinorm standard control was determined as TG = 103 mg/dL or 1.13mmol/L and 87 mg/dL or 2,2 mmol/L, therefore, supporting the validity of our results.

A3.5: Lipoprint HDL subfraction separation system and protocol

HDL-C is separated into 10 subfractions grouped by size into large, intermediate and small sub-fractions by the Quantimetrix Lipoprint System HDL subfractions kit (Quantimetrix, California, USA). The kit consists of : precast linear polyacrylamide gel (stacking and separating) in cylindrical glass tubes; a liquid loading gel with hydrophilic dye; buffer salts with a complete tank and power source similar to a western blot electrophoresis apparatus. Therefore, this is a polyacrylamide gel electrophoresis test that separates and measures HDL-C subfractions in plasma.

A3.5.1: Assay Procedure

1. The tank buffer solutions are prepared by dissolving the 2 buffer salts in 1200mL of distilled water each.
2. The gel tubes are placed in the preparation rack and the storage buffer solution carefully poured out to make space for the sample and loading gel taking care to remove all bubbles.
3. Add 25µL of sample to each tube.
4. Add 300µL of the prepared lipoprint HDL loading gel to each tube.
5. Cover the top of the tubes and preparation rack with parafilm and invert several times to mix.
6. Place the preparation rack against the photopolymerizing light for 30 min (this allows the loading gel to photopolymerize)
7. Remove the gel tubes from the rack and insert each into the silicone adapter for the tank buffer (fill any empty slots with the glass tubes provided).
8. Add the 1000mL electrolyte buffer solution to the lower tank chamber; place the silicone adapter and gel tubes on top; and then use the 200mL buffer to fill the upper chamber (freshly made at room temperature).
9. Remove any bubbles and place the electrophoresis lid at the top, connecting the leads to the power source. Set the power source to a to total of 3mA per gel tube (i.e. for 6 tubes set power to 6 × 3mA =12mA) and the voltage to maximum 500V.

10. Run the electrophoresis for 50 min and confirm that the albumin has migrated approximately 35mm from the top of the separating gel.
11. Dry the gel tubes and allow them to sit for at least 30 min before placing them in the scanner.

Quantitation

The electrophoresed HDL gels are scanned and analysed using the Lipoprint HDL software, which is automated and outputs a graph showing the peaks of quantities for the various HDL-C subfractions shown below: