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Early cardiac effects of a high-sucrose diet in a rat model of prediabetes

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Dissertação apresentada à Faculdade de Ciências e Tecnologia da Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Bioquímica, realizada sob a orientação científica do Doutor Flávio Reis (Universidade Coimbra)

Sara Raquel Nunes

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–Teixeira-Lemos E, **Nunes S**, Teixeira F, Reis F. Regular physical exercise training assists in preventing type 2 diabetes development: focus on its antioxidant and anti-inflammatory properties. Cardiovasc Diabetol. 2011; 10: 12. Review.

–Ferreira L, Teixeira-de-Lemos E, Pinto F, Parada B, Mega C, Vala H, Pinto R, Garrido P, Sereno J, Fernandes R, Santos P, Velada I, Melo A, **Nunes S**, Teixeira F, Reis F. Effects of sitagliptin treatment on dysmetabolism, inflammation, and oxidative stress in an animal model of type 2 diabetes (ZDF rat). Mediators Inflamm. 2010;592760.

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LIST OF ACRONYMS AND ABBREVIATIONS

ADA - American Diabetes Association

AGEs - Advanced Glycation End Products

AGR - Abnormal Glucose Regulation

Ang II - Angiotensin II

AR - Aldose reductase

AT1-R - Angiotensin Type 1 Receptors

AUC - Area Under the Curve

BCA - Bicinchoninic Acid Assay

BHT - Butylated Hydroxytoluene

BNP - Brain Natriuretic Peptide

BSA - Bovim Serum Albumin

CAD - Coronary Artery Disease

CRP - C-Reactive Protein

CTGF - Connective Tissue Growth Factor

CV - Cardiovascular

CVD - Cardiovascular Disease

DAG - Diacylglycerol

DBP - Diastolic Blood Pressure

DCM - Diabetic Cardiomyopathy

DM - Diabetes Mellitus

ECF - Enhanced Chemifluorescence

eNOS - Endothelial nitric oxide synthase

ERK - Extracellular signal-Regulated Kinase

FFAs - Free Fatty Acids

FPG - Fasting Plasma Glucose

G-6-P - Fructose-6-Phosphate

GAPDH - Glyceraldehyde-3-Phosphate Dehydrogenase

GTT - Glucose Tolerance Test

GLUT-4 - Glucose Transporter-4

GOD - Glucose Oxidase

GSH - Reduced Glutathione

GSK-3β - Glycogen Synthase Kinase -3 Beta

H_2O_2 - Hydrogen Peroxide

HNE - 4-hydroxy-2-nonenal

HbA1c - Glycated hemoglobin

HOMA-IR - Homeostasis Model Assessment of Insulin Resistance

HDL-c - High-Density Lipoprotein Cholesterol

HF - Heart Failure

HR - Heart Rate

HTA - Arterial Hypertension

ICAM-1 - Intracellular Adhesion Molecule-1

IFG - Impaired Fasting Glucose

IGF-1 - Insulin-like Growth Factor-1

IGF-1R - Insulin-like Growth Factor-1 Receptor

IGT - Impaired Glucose Tolerance

IL1-β - Interleukin-1 beta

IL-6 - Interleukin-6

iNOS - inducible Oxide Nitric Synthase

IR - Insulin Receptor

IRS - Insulin Receptor Substrate

IRS-1 - Insulin Receptor Substrate-1

ITT - Insulin Tolerance Test

JNK - c-jun NH2-terminal kinase

LDL - Low-Density Lipoproteins

LV - Left Ventricle

LVH - Left Ventricle hypertrophy

MAPK - Mitogen Activated Protein Kinase

MBP - Mean Blood Pressure

MDA - Malondialdehyde

mTOR - mammalian Target Of Rapamycin

NADPH - Nicotinamide Adenine Dinucleotide Phosphate in Reduced Form

NEFAs - Nonesterified Fatty Acids

NHANES - National Health And Nutrition Examination Survey

NF-κB - Nuclear Factor-kappa B

NO - Nitric oxide

OGTT - Oral Glucose Tolerance Test

OLEFT - Otsuka-Long-Evans-Tokushima Fatty

PAI-1 - Plasminogen-activator inhibitor 1

PDK1 - Phosphoinositide-dependent Protein Kinase-1

$PI4,5P_2$ - Phosphatidylinositol 4,5-bisphosphate

$PI3,4,5P_3$ - Phosphatidylinositol 3,4,5-trisphosphate

PI3K - Phosphoinositide 3-Kinase

PPARs - Peroxisome Proliferator-Activated Receptors

POD - Peroxidase

PKB - Protein Kinase B

PKC - Protein Kinase C

PVDF - Polyvinylidene Difluoride

RAAS - Renin-Angiotensin Aldosterone System

RAGE - Receptor for Advanced Glycation End-Products

ROS - Reactive Oxygen Species

SBP - Systolic Blood Pressure

SERCA - Sarcoplasmic/Endoplasmic Reticulum Ca2+-ATPase

SHR - Hypertensive Rats

sRAGE - soluble Receptor for Advanced Glycation End-Products

T1DM - Type 1 Diabetes Mellitus

T2DM - Type 2 Diabetes Mellitus

TAS - Total Antioxidant Status

TBA - Thiobarituric Acid

TBARs - Thiobarbituric Acid Reactive substances

TGF-β - Transforming Growth Factor-Beta

TGs - Triglycerides

TNF-α - Tumor Necrosis Factor-Alpha

TRB - Mammalian Tribbles Homologs

TSP-1 - Thrombospondin 1

VCAM-1 - Vascular Cell Adhesion Molecule-1

VEGF - Vascular Endothelial Growth Factor

VLDL – Very Low-Density Lipoproteins

WHO - World Health Organization

RESUMO - ABSTRACT

RESUMO

A cardiomiopatia diabética (CMD) é vista como uma cardiomiopatia específica e definida por alterações estruturais e funcionais no miocárdio devido a anomalias metabólicas e celulares induzidas pela diabetes mellitus (DM). A transição das anomalias metabólicas precoces que precedem a DM, como a glicemia de jejum alterada e a intolerância à glicose, para um estado de diabetes pode levar muitos anos; no entanto, as estimativas actuais indicam que a maioria dos indivíduos neste estado de pré-diabetes eventualmente desenvolve DM. Durante o estado pré-diabético, o risco de eventos cardiovasculares está já aumentado e as anomalias cardíacas podem aparecer antes do diagnóstico da DM. A identificação precoce de alterações cardíacas em pacientes pré-diabéticos/resistentes à insulina poderá ser a melhor estratégia para prevenir a evolução para fases mais graves da doença.

Para elucidar se estados iniciais de disfunção cardíaca estão já presentes na pré-diabetes com resistência à insulina, e os mecanismos envolvidos, testamos um possível modelo animal que pode mimetizar um estado pré-diabético em humanos com resistência à insulina, sem outros factores associados que podem levar à lesão/disfunção cardíaca, consistindo numa dieta rica em açúcares (35% de sacarose) durante 9 semanas.

Dois objectivos específicos principais foram estabelecidos: a) caracterizar este modelo animal (em rato), no que concerne ao perfil glicídico, lipídico e insulínico; b) avaliar possíveis alterações cardíacas e os mecanismos subjacentes, determinando a pressão arterial e a frequência cardíaca, indicadores de trofismo cardíaco e do ventrículo esquerdo e a expressão proteica e/ou génica de marcadores de fibrose, hipertrofia, proliferação, apoptose, angiogénese, função endotelial, inflamação e stresse oxidativo.

Este modelo caracteriza-se por uma normoglicemia em jejum, acompanhada por hiperinsulinemia, resistência à insulina e tolerância à glicose alterada, em conjunto com hipertrigliceridemia, o que pode ser definido como um modelo animal de pré-diabetes. Este modelo animal poderá ser uma ferramenta útil para avaliar o impacto das perturbações metabólicas precoces no tecido cardíaco, independentemente de outros factores como a obesidade e a hipertensão, que não se verificaram.

Relativamente ao impacto da dieta rica em sacarose no tecido cardíaco, os nossos resultados indicaram que 9 semanas de tratamento pode estar associado a alterações iniciais a nível cardíaco, como sugerido pelo aumento da razão entre o peso do ventrículo esquerdo e massa corporal e pelo aumento da expressão génica de BNP, juntamente com uma tendência para um aumento da expressão de outros mediadores de fibrose / hipertrofia (como TGF-β1, pró-colagénio III, TSP-1 e CTGF) e de angiogénese e lesão endotelial (VEGF e iNOS, respectivamente). Além disso, o stresse oxidativo parece ser um potencial mecanismo que contribui para as iniciais alterações cardíacas. Esta hipótese, que deverá merecer uma atenção posterior mais aprofundada, é sugerida pela tendência para valores aumentados de MDA no soro e no tecido cardíaco. Contudo, em nosso entender, poderá ocorrer um mecanismo de compensação em resposta à formação de espécies reactivas, evidenciado pelo aumento do estado antioxidante total sérico e pela expressão aumentada da SOD no tecido cardíaco, bem como pela expressão génica reduzida de HNE e RAGE.

Estes resultados sugerem que 9 semanas de consumo de sacarose poderá ser um período curto para promover alterações mais profundas na disfunção cardíaca, mas as alterações iniciais poderão estar já em curso. Contudo, estudos adicionais (incluindo outros tempos de exposição e marcadores de miopatia) são necessários para uma melhor compreensão dos mecanismos moleculares e celulares subjacentes ao efeito da dieta rica em sacarose numa fase inicial da CMD.

ABSTRACT

Diabetic cardiomyopathy (DCM) is viewed as a specific cardiomyopathy and defined as structural and functional changes in the myocardium due to metabolic and cellular abnormalities induced by diabetes mellitus (DM). The transition from the early metabolic abnormalities that precedes diabetes, for example impaired fasting glucose (IFG) and impaired glucose tolerance (IGT), to diabetes may take many years; however, current estimates indicate that most individuals with these pre-diabetic states eventually develop DM. During the prediabetic state, the risk of cardiovascular events is already increased and myocardial abnormalities might appear prior to the diagnosis of Type 2 DM. Thus, the earlier identification of cardiac changes in prediabetic/insulin resistance patients could be a better strategy to prevent the evolution to most serious stages of the disease.

To elucidate whether the initial stages of cardiac dysfunction are already present in a prediabetic state with insulin resistance, and the mechanisms involved, we tested a putative animal model that might mimic a human prediabetic state of insulin resistance, without other complicating factors that could lead to cardiac events, consisting on a high sugar diet (35% of sucrose) during 9 weeks.

Two main specific goals were pursued: a) characterize the animal (rat) model, concerning glycemic, lipidic and insulinic profile; b) evaluate possible cardiac alterations and the underlying mechanisms, assessing by blood pressure and heart rate, heart and left ventricle trophism indexes and protein and/or mRNA expression of markers of fibrosis, hypertrophy, proliferation, apoptosis, angiogenesis, endothelial function, inflammation and oxidative stress.

This model is characterized by the presence of fasting normoglycemia, accompanied by hyperinsulinemia, insulin resistance and IGT, together with hypertriglyceridemia, which

might be defined as a prediabetic animal model. Since obesity and hypertension were not present, this animal model seems to be a useful tool to evaluate the impact of earlier metabolic perturbations on cardiac tissue and the existence of cardiomyopathy, independently of these confounding factors.

Concerning the impact of the sucrose diet on cardiac tissue, our results indicated that 9 weeks of treatment might be associated with initial changes at cardiac level, as suggested by the increased left ventricle mass/body weight ratio and increased of BNP mRNA expression, together with a trend to upregulation of other mediators of fibrosis/hypertrophy (TGF-β1, procollagen III, TSP-1 and CTGF) and of angiogenesis and endothelial lesion (such as VEGF and iNOS, respectively). In addition, oxidative stress seems to be a putative mechanism contributing to earlier cardiac changes. This hypothesis, which deserves further elucidation, is suggested by the trend to increased values of MDA in serum and heart tissue. However, according to our view, a compensatory mechanism against the generation of reactive species might occurs, evidenced by the increased serum total antioxidant status and cardiac overexpression of SOD, as well as by the downregulation of HNE and RAGE mRNA.

The results suggested that 9 weeks of sucrose exposure might be a short period to promote pronounced changes related to cardiac dysfunction, but the initial changes might be already in course. However, additional studies (including other times of exposure and markers of myopathy) are needed to better understand the molecular and cellular mechanisms underlying the effects of HSu on this earlier phase of DCM.

I. INTRODUCTION

1. Diabetes Mellitus – "*the road to diabetic cardiomyopathy*"

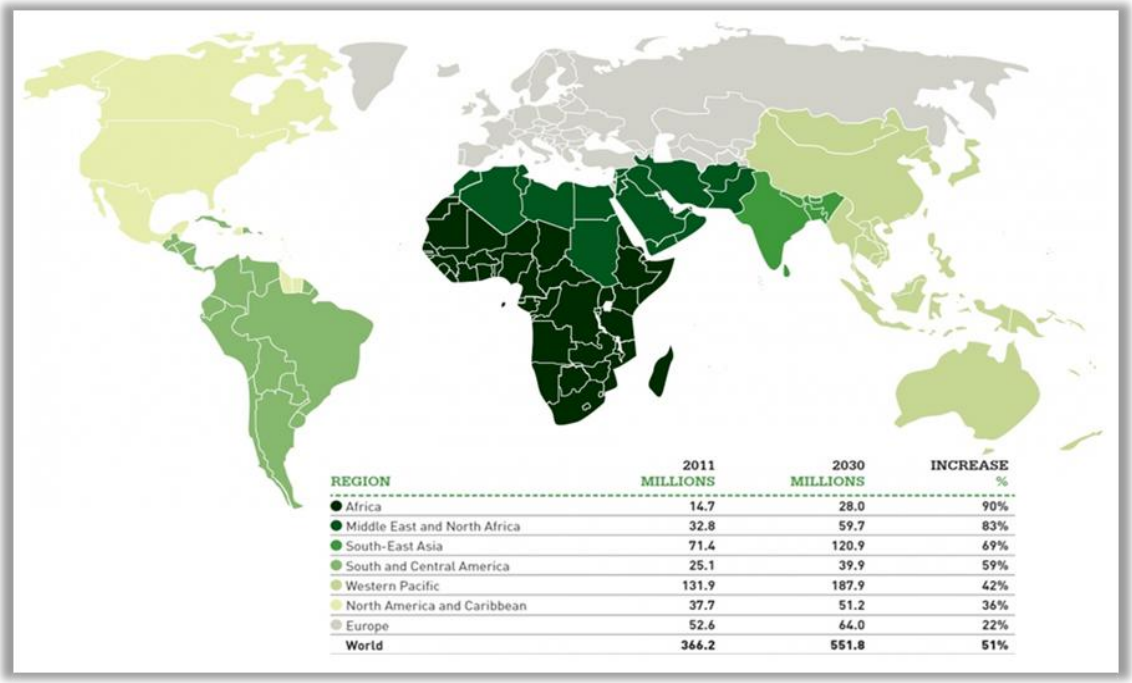
1.1. General definition and epidemiology of Diabetes Mellitus

Diabetes Mellitus (DM) describes a metabolic disorder of multiple aetiology, characterized by chronic hyperglycemia, with abnormal metabolism of carbohydrates, lipids and proteins, resulting from defects in the secretion and/or insulin action. The consequences of DM, although in the long-term, are various and includes chronic injury with dysfunction and failure of various organs and tissues. According to the World Health Organization (WHO) and the American Diabetes Association (ADA), DM can be classified into four different categories: type 1, type 2, other specific types of diabetes and gestational diabetes (WHO, 1999; ADA, 2004*).* Within these categories, the most frequent are type 1 DM (T1DM) and type 2 DM (T2DM), which are briefly described below.

T1DM, also called insulin-dependent, or immune-mediated, is characterized by a deficiency of insulin production, due to an autoimmune destruction of insulin-secreting pancreatic islet beta (β)-cell, which are related to multiple genetic predispositions and environmental factors, or unknown etiologies, called idiopathic diabetes. T1DM patients are, therefore, dependent on exogenous insulin ("insulin dependent") requiring treatments based on multiple daily insulin injections to maintain adequate insulin and to control glucose levels. The onset of T1DM usually occurs in children and young ages and, therefore, can also be referred to as juvenile diabetes; however, it can also appear at any age. This type of diabetes represents about 5-10% of the population affected by the disease.

T2DM is the most common endocrine deregulation worldwide, representing about 90-95% of the diabetic population. In recent years, the global prevalence of T2DM has increased in such a way that has achieved epidemic proportions. Currently, there are over

336 million cases of diabetes and an increase to 552 million by 2030 is estimated by the International Diabetes Federation (IDF) (Figure 1). Statistically, DM affects 8.3% of global adult population and it is expected to rise to 9.9% in 2030 (IDF, 2011).



REGION	2011 MILLIONS	2030 MILLIONS	INCREASE %
Africa	14.7	28.0	90%
Middle East and North Africa	32.8	59.7	83%
South-East Asia	71.4	120.9	69%
South and Central America	25.1	39.9	59%
Western Pacific	131.9	187.9	42%
North America and Caribbean	37.7	51.2	36%
Europe	52.6	64.0	22%
World	366.2	551.8	51%

Figure 1. Regional and global projections for the number of people with diabetes (20-79 years) from 2011 to 2030. Taken from IDF Diabetes Atlas, 5th Edition (IDF, 2011).

The prevalence of DM, in percentage, is higher among men; however, there are more women with diabetes worldwide. The most important demographic change to the increased prevalence of DM is the worldwide aging population, translated into an increase in population over 65 years of age (Wild et al., 2004). Despite the prevalence of DM is increasing overall, the prevalence of T2DM has increased more than the prevalence of T1DM, due to sedentary lifestyle with lack of physical activity and increasing population of obesity, mainly in developed countries, but also in developing countries that are becoming increasingly industrialized (IDF, 2011).

In Portugal, T2DM has also reached an epidemic status, with overwhelming consequences at various levels, including personal, social, family and economic.

According to data of the Annual Report of National Diabetes Observatory ("*Observatório Nacional de Diabetes"* - *OND*), the prevalence of diabetes in 2010 was 12.4% in the portuguese adult population, people aged 20 to 79 years, which corresponds to a total of about 901 thousands, with some difference between men (14.7%) and women (10.2%). The prevalence of undiagnosed diabetes is 5.4%, and 26% of portuguese population aged between 20 and 79 years has already a large predisposition to disease development (SPD, 2012).

Type 2 diabetes occurs predominantly in adults (people older than 40 years); however, there has been a rise in diagnosed cases of T2DM at increasingly younger ages, even adolescents, suggesting a linkage with the increase of childhood obesity (Schwartz and Chadla, 2008). This aspect is even more alarming when considered that over the past 20 years its prevalence has increased dramatically among children and adolescents (Coopeland et al., 2005).

1.2. Prediabetes and metabolic syndrome as predictors of diabetes

T2DM is nowadays viewed as part of a continuum of metabolic abnormalities, which includes prediabetes and metabolic syndrome, involving specific defects in insulin signaling, as well as other metabolic perturbations. The term metabolic syndrome describes a group of metabolic alterations that predisposes to cardiovascular disease (CVD) and diabetes, associating risk factors, such as insulin resistance, hypertension, decreased circulating high-density lipoprotein cholesterol (HDL-c) and high triglycerides (TGs) levels. In the presence of these factors, there is a raised risk of about 1.5 to 3 fold greater to develop coronary heart disease (CAD) and a risk 3-5 fold higher for diabetes (Figure 2). As metabolic syndrome develops, there is a rise of prevalence of associated risk factors that

are observed many years before the diagnosis of T2DM. The high prevalence of the metabolic syndrome is a great motif of concern worldwide.

Initially, T2DM is characterized by a state of insulin resistance, in which cells do not properly respond to insulin and, as a consequence, there is a compensatory increase of insulin secretion by pancreatic β-cells (hyperinsulinemia), in order to maintain normoglycemia, a process termed β-cell compensation. This phase is now viewed as a prediabetes state and is mainly characterized by two conditions: impaired fasting glucose (IFG) and/or impaired glucose tolerance (IGT). Over the years, the excessive function of β-cells ("the β-cells overactivity") may lead to their exhaustion and impaired secretory capacity, contributing to the β cells failure and, therefore, to a decreased insulin secretion which then results in hyperglycemia (Figure 2). Thus, T2DM ensues and its pathophysiology is characterized by peripheral insulin resistance, abnormal regulation of hepatic glucose production and decline in β-cell function, leading to eventual failure of these cells (Virally et al., 2007).

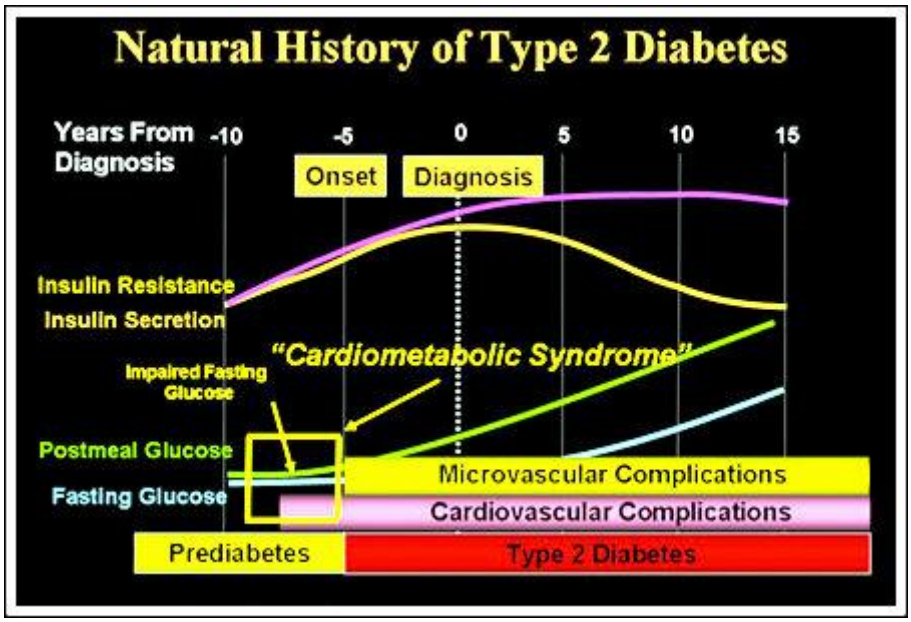


Figure 2. Schematic representation of the metabolic syndrome development in the natural history of T2DM. As metabolic syndrome develops, there is a rise of prevalence of associated risk factors that are observed many years before the diagnosis of T2DM. Taken from Ramlo-Halsted and Edelman, (1999).

Prediabetes is an increasingly common condition, typically defined as blood glucose concentrations higher than normal, but lower than diabetes thresholds, being a high-risk state for diabetes development. Diagnostic criteria for prediabetes have changed over time and vary depending on the institution of origin. According to WHO, people are at high risk of developing diabetes if they have one of two distinct states: IFG, defined as a fasting plasma glucose (FPG) concentration of ≥6.1 and <7.0 mmol/L, without IGT; and IGT, defined as an FPG concentration of <7.0 mmol/L and a 2 h postload plasma glucose concentration of ≥7.8 and <11.1 mmol/L, measured during a 75 g oral glucose tolerance test (OGTT) (WHO, 2006). The ADA applies the same thresholds for IGT, but uses a lower cutoff value for IFG (FPG 5.6 – 6.9 mmol/L), and has introduced glycated haemoglobin A1c (HbA1c) 5.7 – 6.4% as a new category for high diabetes risk (ADA, 2011). The term prediabetes has been criticised because many people with prediabetes do not progress to diabetes, and it might imply that no intervention is necessary because no disease is present. Furthermore, diabetes risk does not necessarily differ between people with prediabetes and those with a combination of other diabetes risk factors. Indeed, WHO use the term intermediate hyperglycemia and an International Expert Committee convened by the ADA prefers the "high-risk state of developing diabetes" than prediabetes (WHO, 2006; International Expert Committee, 2009).

The population-based US National Health and Nutrition Examination Survey (NHANES) suggests that 35% of US adults older than 20 years and 50% of those older than 65 years had prediabetes in 2005–08, defined by FPG or HbA1c concentrations. Application of these percentages to the entire US population in 2010 yielded an estimated 79 million adults with prediabetes (Centers for Disease Control and Prevention, 2011). Prevalences of IFG and IGT vary between ethnic groups and both disorders are more common in people older than 40 years (Cowie et al., 2009). Additionally, IFG is more

prevalent in men than in women, although the reasons for this difference are poorly understood (Cowie et al., 2009). Figure 3 shows worldwide projections of IGT prevalence for 2030, according to the International Diabetes Federation (2011). The number of adults with IGT is expected to increase worldwide, reaching 472 million by 2030.

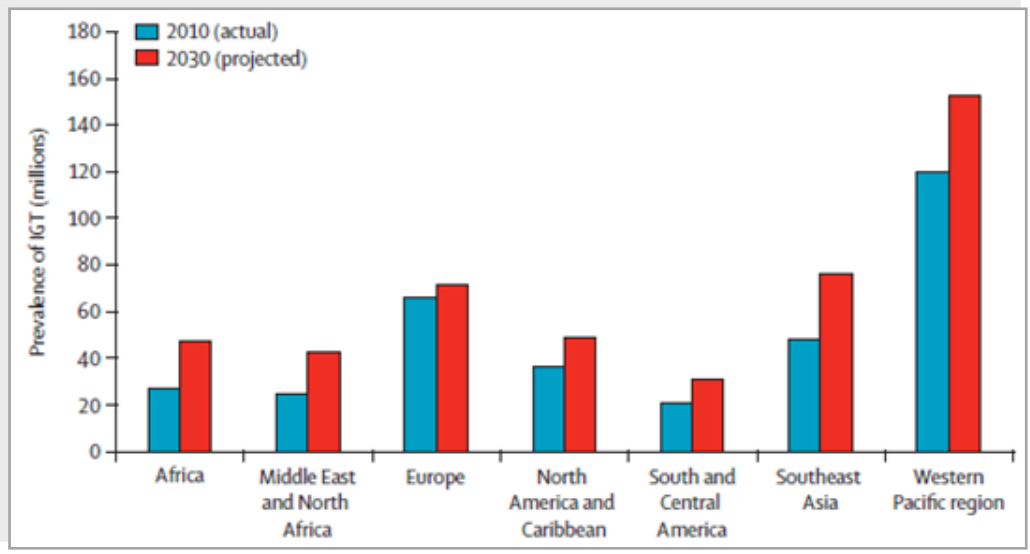


Figure 3. Actual and projected prevalence of impaired glucose tolerance (IGT) by region in adults aged 20-79 years in 2010 and 2030. Taken from Tabák et al. (2012).

Prediabetes was recognized is an independent predictor of conversion to type 2 diabetes in "The Strong Heart Study", which showed that most of them can be identified through a fasting glucose measure (Wang et al., 2010). Nguyen et al. (2010) reported in another study (The Bogalusa Heart Study) that fasting plasma glucose levels within the normoglycemic range in childhood is a predictor of prediabetes and T2DM in the adulthood. Several longitudinal studies support the hypothesis that prediabetes is a risk factor for diabetes and CVD (Meigs et al., 2002; DECODE study group, 1999; Unwin et al., 2002; Gabir et al., 2000; Stern et al., 2002).

Prediabetes in Portugal reached, in 2010, 26% of the population aged between 20 and 79 years, meaning that this percentage of individuals already has probably a large predisposition to disease development. Furthermore, about 38.4% of this population suffers

from diabetes or prediabetes, corresponding to more than one third of the portuguese population (Figure 4).

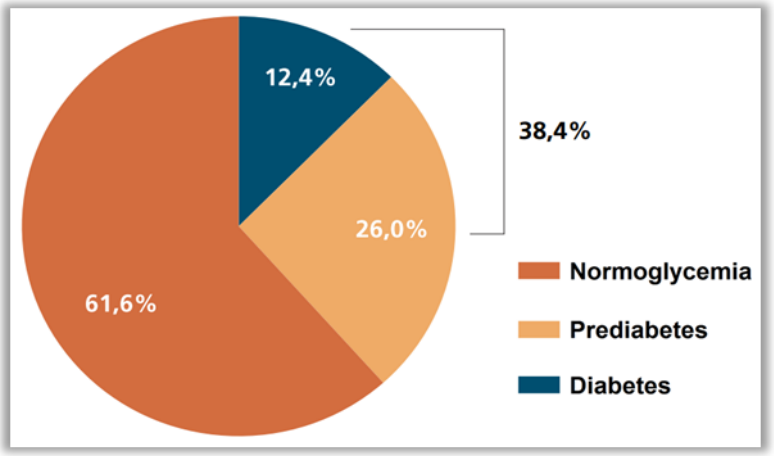


Figure 4. Prevalence of diabetes and prediabetes in Portugal in 2010. Adapted from SPD (2011).

1.3. Diabetic complications

The frequency, severity and progression of diabetic complications are related to levels of hyperglycemia, metabolic changes associated with the duration of disease and exposure to risk factors, and also with the genetic environment. Over the years, an alarming number of 40% of diagnosed patients with diabetes develop late complications of the disease in several organs, which are frequently not timely detected. Thus, it is essential that patients with diabetes make a control of the disease to prevent further complications**.**

Patients with a diagnosis of diabetes can have many complications, either at the microvascular (retinopathy, nephropathy or peripheral neuropathy) and macrovascular (stroke, coronary heart disease, acute myocardial infarction or peripheral vascular disease) level (Figure 5), causing human suffering and significant costs in the context of economics of global health (Fowler, 2008).

Although microvascular complications are the major risk in T1DM, are also often present in patients with T2DM. These complications have been directly associated with glucose toxicity, with amplification of intracellular metabolic pathways, including the proteins glycosilation, the abnormal activation of signaling cascades, such as protein kinase C (PKC), the hexosamine and the polyol pathways, and elevated production of reactive oxygen species (ROS). The microvascular complications increase the risk of cardiovascular (CV) events in diabetic patients, which implies that these are related indirectly to the macrovascular diabetic complications. Several studies shows that different type of these complications, such as diabetic retinopathy (Son et al., 2011), diabetic polyneuropathy (Chung et al., 2011) and diabetic nephropathy (Gerstein, et al., 2001), are independently linked to a high risk of CV events.

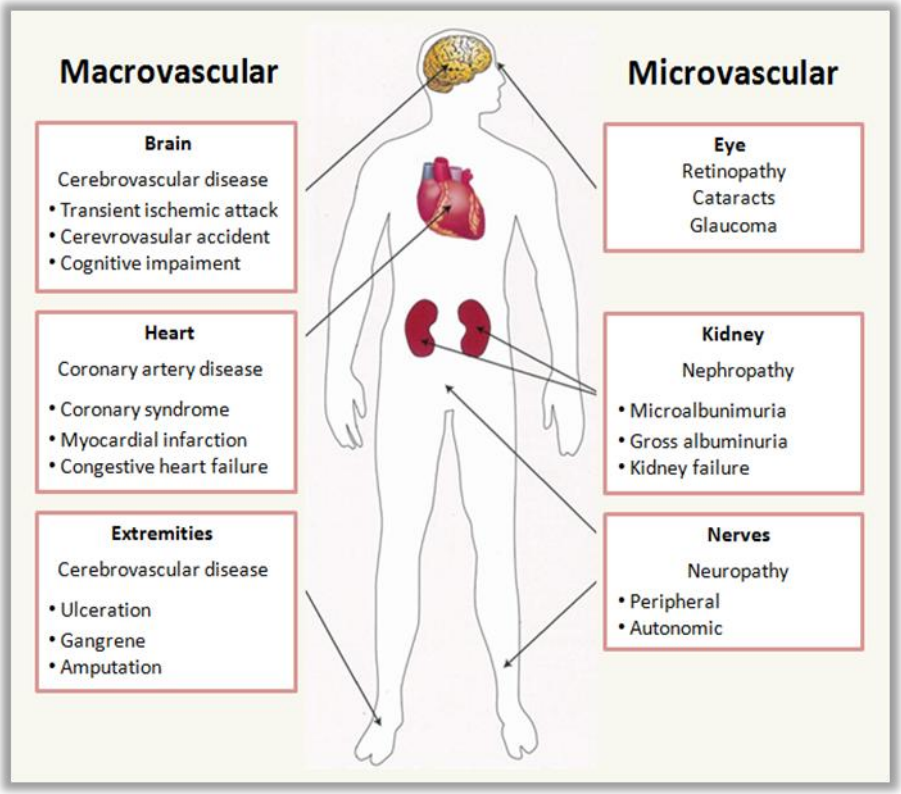


Figure 5. Schematic diagram of macro and microvascular complications of diabetes. Taken from Cefalu and Canon (2007).

Concerning the macrovascular complications, which are the leading cause of mortality in T2DM (representing 75-80% of deaths), there are no evidences of reduction of its evolution when the therapeutic intervention consists only on a better glycemic control (Castagno et al., 2011; Duckworth et al., 2009); thus, the control of other factors seem to be crucial.

As obesity is strongly associated with T2DM, the excessive release of free fatty acids (FFAs) gives rise to a dyslipidemic phenotype typical of diabetes (hypertriglyceridemia and reduced HDL-c), which is related to a higher CV risk (Toth, 2004). Associated with dyslipidemia, there is frequently hypertension, present in 60% of the T2DM patients, thus increasing the rates of myocardial infarction and stroke. Although the strict control of hyperglycemia, per se, does not significantly reduce the incidence of myocardial infarction and mortality, most of the epidemiological and pathophysiological data suggest that hyperglycemia increases the rate of CV disease and aggravates the consequences. In any way, the attenuation of microvascular consequences justifies, by itself, an energetic effort to tightly control blood glucose (Beckam et al., 2002).

To better recognize and evaluate the CVD, other factors should be considered, including the peaks of hyperglycemia, the oxidative stress and the endothelial dysfunction. The finding that the common element of the micro and macrovascular complications is the vascular endothelium and the fact that, in this, the entry of glucose into the cells do not require insulin leads to assign greater importance to the peak postprandial hyperglycemia, with consequent oxidative stress. The knowledge of these factors could lead to assess the insulin-resistance as a phenomenon of protection triggered by the body. Interestingly, the organs do not protected against the insulin resistance are precisely the most affected.

Older age, high blood pressure and smoking history are also major risk factors for the development of macrovascular complications. Due to the accumulation of different risk

factors within one individual, the risk of developing macrovascular complications is substantially increased.

In addition to the high prevalence rates, chronic complications of diabetes mean that the disease has a strong impact on the health budgets related with disease treatment and hospitalization, as well as with early disability, inability to work and decreased quality of life; thus, it is becoming a serious public health problem.

1.3.1 Cardiac complications of T2DM

T2DM is a major risk factor for the development of CVD. It is responsible for up to 65% of all deaths in people with T2DM, as well as for substantial morbidity and loss of quality of life. T2DM can lead to CV damage in a number of ways. As T2DM progresses, the heart and blood vessels undergo changes and lead to a number of different CV complications (Tuomilehto and Lindstrom, 2003). Although CAD is two to three times more common in diabetic patients that in non diabetic individuals (Fisher, 2003), it is not the only CV complication. Several experimental, pathological, epidemiological, and clinical studies have shown an association between diabetes mellitus and cardiomyopathy. The term diabetic cardiomyopathy (DCM) has been introduced in 1972 by Rubler et al., based on postmortem observations of heart failure (HF) in diabetic patients free of detectable CAD, which was further confirmed in numerous other clinical studies (Galderishi et al., 1991; Shrestha et al., 2009). This fact suggests that diabetes is able to lead to damage of cardiac tissue without depending on the co-existence of other CV risk factors.

Diabetes is typically associated with multiple metabolic and physiologic abnormalities, such as hyperglycemia, peripheral insulin resistance, dyslipidemia, hypertension, and overweight or obesity, activation of multiple hormone and cytokine systems, each of which are most known risk factors for cardiac failure. However, it

remains unknown whose factors are more important to the overall incidence of cardiac failure in diabetic patients.

2. The diabetic cardiomyopathy

DCM, a distinct clinical entity, is currently viewed as a result of complex relationships between metabolic abnormalities that accompany diabetes and its cellular consequences, resulting in functional and structural changes in the myocardium that, in combination with other features, impair cardiac performance, ultimately resulting in cardiac failure (Fang et al., 2004; Tziakas et al., 2005) (Figure 6).

Diabetic cardiomyopathy is defined as functional and structural changes at the level of myocardium, independent of hypertension, CAD, or any other known cardiac diseases, leading to HF (Zarich and Nesto, 1989; Hayat et al., 2004). Although diabetic patients are at increased risk of structural heart disease due to vascular complications, the concept of diabetic cardiomyopathy suggests a direct cellular insult to the myocardium. Therefore, patients with hypertension and CAD may well have myocardial changes related to these disease processes, but a specific cardiomyopathy may also affect the myocardium secondary to diabetes causing a synergistic adverse effect.

2.1. Epidemiological data

Diabetes has accounted for a significant percentage of patients with a diagnosis of HF in numerous epidemiologic studies (Tang, 2007). The Framingham study (Kannel et al., 1974), the United Kingdom Prospective Diabetic Study (Stratton et al., 2000), the Cardiovascular Health Study (Gottdiener et al., 2000) and the Euro Heart Failure Survey (Nieminen et al., 2006) all suggested that the presence of diabetes may independently

increase the risk of developing HF. Several clinical studies (Galderisi et al., 1991; Devereux et al., 2000; Bella et al., 2001; Liu et al., 2001) have suggested that there is a consistent association between DCM and the presence of cardiac hypertrophy and myocardial stiffness, both independent of hypertension. Such associations have provided a credible existence of DCM as a unique clinical entity.

DCM has evolved from a nebulous concept to concrete reality over the years. Evolving evidence supports a strong association between diabetes and cardiomyopathy. In one of the largest epidemiologic studies, involving over 800.000 patients, diabetes was found to be independently associated with the occurrence of congestive HF after adjusting for left ventricular hypertrophy (LVH), hypertension, CAD, and atrial fibrillation (Movahed et al., 2005). Furthermore, higher prevalence of biventricular cardiomyopathy in diabetes patients (Movahed et al., 2007) is also suggestive of diabetes as an independent cause of cardiomyopathy. Whether there are other confounding factors that could lead independently to cardiomyopathy in diabetics is unclear. However, it is important to note that actually no specific histologic and/or biochemical markers for so-called ‘‘diabetes cardiomyopathy’’ has been found; so, other mechanisms of damage can coexist or be responsible of a cardiomyopathy developing in a diabetic patient. Hyperglycemia, insulin resistance, increased fatty acid metabolism, microcirculatory changes, sympathetic dysfunction, and fibrosis are considered to collectively contribute to its pathology. It is hoped that as the mechanisms of this cardiomyopathy in diabetics continue to be elucidated, they will provide the impetus for generating novel therapies tailored to reduce the risk of HF in patients with diabetes mellitus.

DCM may be subclinical for a long time, before the appearance of clinical symptoms or signs. According to the molecular theory of DCM, hyperglycemia is the main pathogenetic factor, which causes abnormalities at the cardiac myocyte level,

eventually leading to structural and functional abnormalities. However, other factors seem to be involved in the evolution of the disease and several substrates have been suggested. During the last years, the structural functional, pathological and molecular aspects of the disease have been increasingly investigated, but the issue is far to be elucidated.

The next section briefly review the main pathophysiological mechanisms underlying DCM on the light of the current knowledge.

2.2. Cardiac changes

Accumulating data from experimental, pathologic, epidemiologic, and clinical studies have shown that diabetes results in structural and functional cardiac changes.

2.2.1. Structural changes

A number of studies have shown structural changes in diabetic hearts in the absence of hypertension, CAD, and valvular heart diseases. One of the most important structural hallmarks of DCM is cardiac hypertrophy, and this, in turn, is a powerful predictor of CV events (Figure 6).

Hypertrophy is a form of growth characterized by an increase in the average cell size of the constituting organ (in contrast to hyperplasia, in which the number of cells increases). Although LVH is more common, the right ventricle can also become enlarged or both may be affected. The common factor is that in physiological cardiac hypertrophy the intermittent load induces an increase in chamber wall thickness, which is compensated by an increase in ventricular volume, thus balancing wall thickness to chamber volume ratio (Dorn, 2007). Numerous studies have shown that patients with T2DM have LVH independent of other confounding factors, including hypertension (Devereux et al., 2000).

LVH in the diabetic patient generally represents a more advanced stage of the disease and may manifest after a longer period. The causes and mechanisms underlying LVH are poorly understood, and the predictive contribute of LVH to DCM is not entirely clear. Recently, however, clinical and animal studies have reported evidences implicating the diabetic milieu of hyperinsulinemia, insulin resistance, hyperglycemia, and increased nonesterified fatty acids (NEFAs) in the pathophysiology of LVH in DM patients. For instance, insulin may act as a growth factor in the myocardium, which is supported by the experimental observation that sustained hyperinsulinemia leads to increased myocardial mass and decreased cardiac output in rats (Karaso et al., 2003; Holmäng et al., 1996). Higher circulating levels of the hormone leptin have also been linked to the development of LVH in obese diabetic humans (Barouch et al., 2003). The consistency of results demonstrates a clear impact of DM, *per se*, on increased left ventricule (LV) mass that encompasses the development of diabetes-related LVH.

Another mechanism proposed to explain cardiac alterations in DCM is related to the development of myocardial fibrosis (Figure 6). Cardiac fibrosis is the accumulation of interstitial glycoproteins and increased extracellular collagen matrix, leading to increased stiffening and reduced relaxation of the ventricles. Interstitial and perivascular fibrosis with significant increase in collagen deposition has frequently been observed in heart biopsy samples from diabetic patients without significant CAD (van Heerebeek et al., 2008). Similar findings have also been observed in animal models of DM (Mizushige et al., 2000; Tschöpe et al., 2004).

The echocardiographic features of increased LV fibrosis appear in the form of impaired relaxation and diastolic dysfunction and it has been suggested that collagen is a major determinant of ventricular stiffness. Consequently, alterations in collagen phenotype may play an important role in the impaired LV diastolic filling that is typical of DCM (van

Heerebeek et al., 2008). In a study with prediabetic Otsuka-Long-Evans- Tokushima Fatty (OLEFT) rats, a correlation between increased extracellular collagen content and decrease in early mitral peak flow (decreased E/A ratio) was reported (Mizushige et al., 2000). The cause for the accumulation of cardiac fibrosis in diabetes is believed to result from decreased degradation of glycosylated collagen by matrix metalloproteinases and, conversely, from excessive production of collagen by fibroblasts due to increased renin-angiotensin aldosterone system (RAAS) activation (Fang et al., 2004).

In addition, an increased formation of advanced glycation end-products (AGEs) has also been reported to occur in the myocardium of DM patients, as a result of hyperglycemia (Basta et al., 2004). It has been demonstrated that collagen is particularly susceptible to AGEs cross-linking and this association not only leads to myocardial stiffness but also impairment of collagen degradation, leading to further collagen accumulation or fibrosis (van Heerebeek et al., 2008; Aronson, 2003). Furthermore, the impairment of LV diastolic function of patients with diabetes may be secondary to alterations in collagen structure, specifically increased collagen cross-linking or AGEs (Aronson, 2003), thus contributing to the myocardial fibrosis and decreased compliance characteristic of DCM.

2.2.2. Functional changes

In many cases, it has been found that abnormalities of diastolic function may advertise the subsequent progressive deterioration of cardiac function. The noninvasive assessment of diastolic dysfunction mainly relies on Doppler studies of diastolic transmitral inflow, flow velocities, flow patterns, isovolumic relaxation time, and deceleration time, which are the most common criteria used in its evaluation. Numerous authors have

suggested that the most frequent and earliest functional effect observed by echocardiography in type 2 diabetic hearts is impaired diastolic function (Shehadeh and Regan, 1995; Fang et a., 2003; Karamitsos et al., 2007).

Diastolic dysfunction can be defined as a condition in which myocardial relaxation and filling are impaired and incomplete. It can manifest as reduced early diastolic filling, increased atrial filling, extended isovolumetric relaxation, and as an increased number of supraventricular premature beats (Schannwell et al., 2002). There is also an increased LV end-diastolic pressure and a decreased LV end-diastolic volume (Hamblin et al., 2007).

It is important to note that the impairment of diastolic performance is non-specific and frequently observed in many diseases, such as hypertension, hypertrophic cardiomyopathy and CAD, while systolic function remains intact. However, several studies have shown that changes in diastolic function are common, in diabetic animals (Loganathan et al., 2006; Seneniuk et al., 2002) and patients (Vaninen et al., 1992; Boyer et al., 2004; Di Bonito et al., 1996), without any clinical manifestations of congestive HF. Indeed, evidence of diastolic dysfunction has been found in patients with impaired glucose tolerance (Celentano et al., 1995), with newly diagnosed diabetes or a short duration of the disease, and free of microvascular complications. Thus, the detection of diastolic dysfunction may be a useful marker for the prognosis of cardiac mortality in diabetic subjects.

There are also numerous studies that have reported an association between diabetes and changes in systolic function. Systolic dysfunction is defined as the loss of the heart´s ability to pump arterial blood in the peripheral circulation and it is associated with a reduction of the LV ejection fraction, fractional shortening and cardiac output. These alterations of systolic function were proven in both human and animal models of T1DM

and T2DM (Mihm et al., 1991; Mytas et al., 2009). However, Radovits et al. (2009) suggest that systolic dysfunction may be more pronounced in type 1 DCM.

In DCM, systolic dysfunction occurs late, often when patients have already developed significant diastolic dysfunction. The prognosis in patients with depressed systolic dysfunction is poor with an annual mortality of 15%–20%. The presence of systolic dysfunction in the early years of diabetes is still controversial, while diastolic dysfunction is more easily detected by Doppler echocardiography. The controversy of the early existence of systolic dysfunction in diabetes is probably related with the fact that current techniques used for systolic function evaluation are less sensitive than those used for diastolic dysfunction assessment. For this reason, more sensitive techniques for systolic assessment have been developed, such as strain, strain rate, and myocardial tissue Doppler velocity, which have allowed the detection of preclinical systolic abnormalities in diabetic patients (Murarka and Movahed, 2010).

2.3. Pathophysiology of diabetic cardiomyopathy

T2DM causes metabolic deregulation and is associated with numerous risk factors which are coupled with cardiomyopathy and HF. Extensive cellular and molecular studies have been focused on the putative process of metabolic disturbances in the pathogenesis of cardiac dysfunction found in diabetes (Figure 6). The metabolic and structural disturbances putatively underlying the development of cardiomyopathy are following briefly discussed.

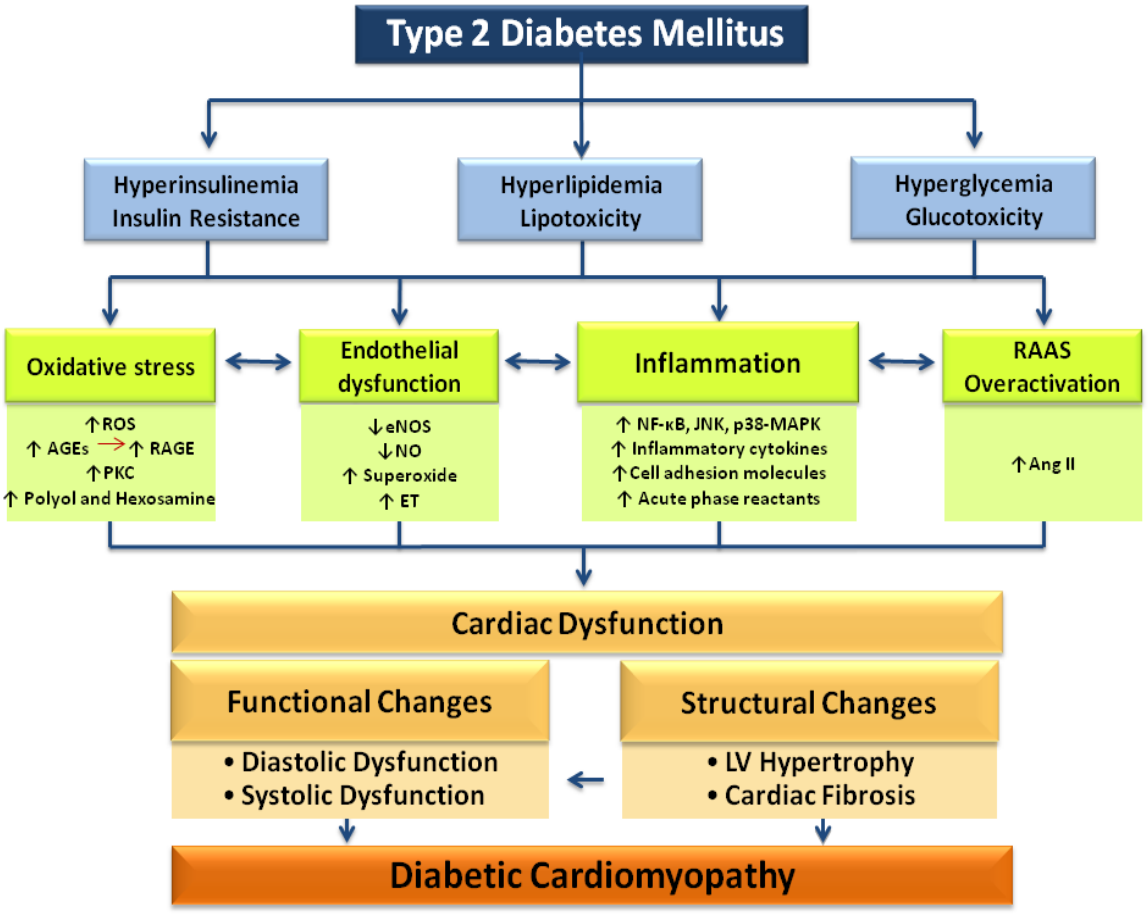


Figure 6. Metabolic and structural abnormalities associated with type 2 diabetes mellitus that might underlie the development of diabetic cardiomyopathy. Taken from Nunes et al. (2012).

2.3.1. *Myocardial hyperinsulinemia and insulin resistance*

Insulin is an anabolic hormone produced by pancreatic β-cells in the islets of Langherhans that plays a fundamental role in the regulation of glucose homeostasis. It is of great importance that blood glucose levels remain constant as persistently high blood sugar concentrations over many years can lead to the development of several clinical complications.

Insulin acts on insulin sensitive tissues, such as brain, fat, muscle and liver, to allow the transport of glucose into cells. Insulin is released by the pancreas in response to various stimuli; however, the transport of insulin from the β-cells is mainly determined by the plasma glucose levels and the concentrations of amino acids and fatty acids (Waselle et al., 2005).

Although the main role of insulin is the regulation glucose levels, it is also an important mediator of metabolic and mitogenic cellular actions, including glycolysis, glycogenesis, lipid metabolism, DNA synthesis, gene transcription, mRNA turnover, protein synthesis and degradation, as well as cellular growth and differentiation.

Impaired insulin action (or insulin resistance) is characterized by the impaired ability of insulin to stimulate glucose uptake in peripheral tissues, inhibition of lipolysis in the adipose tissue and hepatic glucose production, which are major metabolic dysfunctions associated with the early stages of T2DM. Elevated plasma insulin levels can lead to numerous metabolic and pathophysiological derangements in various tissues. In the heart, insulin resistance involves defects in insulin signaling and glucose transport in cardiomyocytes. Moreover, insulin resistance is also considered to be one of the main risk factors for CVD.

Insulin exerts its effects through initial binding to its cell-surface receptor. The insulin receptor (IR) consists of two extracellular α-subunits and two transmembrane β-subunits which are linked to form α2β2 heterotetrametric complex (Ottensmeyer et al., 2000). The binding of insulin to the α-domain of IR initiates a rapid conformational change in the receptor that leads to activation of the intracellular tyrosine kinase domain β-subunits of the receptor. This allows the receptor to undergo a series of intramolecular transphophorylation reactions in which one β-subunit phosphorilated its adjoining partner on specific tyrosine residues.

IR shares a similar structure with the insulin-like growth factor (IGF-1) receptor (IGF-1R), explaining cross-reactions and partially overlapping functions between the two receptors and their ligand. Once activated, IR phosphorylates a variety of important intracellular substrates on tyrosine, including IR substrate family members (IRS-1-4), isoforms of Src-homology-2-containing (Shc), Grb2-associated binder 1 (Gab1), Cas-Br-M (murine) ecotropic retroviral transforming sequence homologue (Cbl), the adaptor protein APS. However, IRS1 and Shc isoforms are those who play a key role in insulin signal transmission, serving as docking sites for connecting a variety of proteins and leading to adaptation of the signaling cascade (White, 2002). The tyrosine phosphorylation of these proteins creates recognition sites for additional effector molecules containing Src-Homology-2 (SH2) domains, to form the ultimate productive insulin receptor complex. Accordingly, each scaffold protein can interact with different SH2-containing proteins that direct a pathway of signal transduction.

In addition to tyrosine phosphorylation, both the insulin receptor and the IRS proteins undergo serine phosphorylation, which may attenuate signaling by decreasing insulin-stimulated tyrosine phosphorylation. These alterations in phosphorylations provide negative feedback to insulin signaling and serve as a mechanism for cross-talk from other

pathways that are involved in the development of insulin resistance (Pessin and Saltiel, 2000).

The insulin signaling involves two major signaling cascades: the phosphoinositide 3-kinase (PI3K) and the extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) pathways.

PI3K is a heterodimeric protein composed of a p110 catalytic and p85 regulatory subunit. This regulatory subunit contains SH2 domain that bind to phosphorylated tyrosine residues of IR or IRS adaptor proteins, which allosterically activates the preassociated p110 catalytic subunit to generate the lipid product phosphatidyl-inositol 3,4,5-trisphosphate ($PI3,4,5P_3$) from the substrate phosphatidyl-inositol 4,5-bisphosphate ($PI4,5P_2$). The increase of $PI3,4,5P_3$ at the plasma membrane induced the recruitment and co-localization of the phosphoinositide-dependent protein kinase-1 (PDK1). Once recruited, PDK1 phosphorylates and activates other downstream serine-threonine kinases, including protein kinase B (PKB; also known as Akt) and atypical PKC isoforms (Alessi et al., 1997; Vanhaesebroeck and Alessi, 2000).

The activation of the PI3K pathway and consequent increased activations of PKB/Akt permits the transport of glucose through the translocation and recruitment of glucose transporter GLUT-4 containing vesicles to the cell membrane in order to augment the uptake of glucose by facilitated diffusion. GLUT-4 is the major insulin-responsive glucose transporter, with a greater expression protein in the heart (Zorzano et al., 1997; Abel, 2004). Glucose transport and utilization by cardiac myocytes is critical for the maintenance of normal morphology and function. In agreement, GLUT4-knockout mice exhibit cardiac hypertrophy characterized by vascular sclerosis, interstitial fibrosis and concentric hypertrophy (Katz et al., 1995; Stenbit et al., 200).

The PI3K/Akt pathway culminates in many metabolic effects of insulin (Zecchin et al., 2004). Therefore, activation of PI3K/Akt has been associated with growth factor mediated cell survival and inhibition of apoptosis in response to various stimuli. Akt promotes cell survival by directly phosphorylating transcription factors that control the expression of pro-and antiapoptotic genes. Furthermore, in response to insulin, Akt promotes glycogen synthesis via serine phosphorylation and inactivation of glycogen-synthase kinase-3 beta (GSK-3β), a well-recognized inhibitor of nuclear transcription governing the hypertrophic process, which is also involved in the regulation of apoptotic events. In addition, Akt activates the mammalian target of rapamycin (mTOR) that activates the p70 ribosomal subunit S6kinase-1, leading to increased protein synthesis (Khamzina et al., 2005; Manning, 2004; Shan et al., 2004). However, these actions mediated through the insulin receptor may be mitigated when insulin signaling through the PI3K/Akt-1 pathway is impaired during chronic hyperinsulinemia

In the heart, the MAPK cascade represents an important mechanism for cellular stress response (Rose et al. 2010). This pathway involves tyrosine-phosphorylated Shc binding to SH2 domain of the adaptor protein growth factor receptor bound 2 (Grb-2), that results in activation of the next signal transduction protein Sos. Sos is a guanosine nucleotide exchange protein which continues the cascade of activation by converting a monomeric small G protein called Ras from its GDP-inactive mode to its GTP-active mode (Gibson and Harris, 2002), which then initiates a kinase phosphorylation cascade involving RAF, which function as an upstream kinase for MAPK kinase (MEK). MEK phosphorylates ERK 1/2 to activate MAPK on threonine and tyrosine residues, resulting in the activation and regulation of gene expression.

The four most thoroughly investigated MAPKs include ERK1/2, ERK5, JNK and p38. p38 MAPK is activated by angiotensin II (Ang II), inflammation, oxidative stress and

ischemia (Gao et al. 2002; Zhang et al. 2004). Previous studies suggest that long-term, but not short-term, activation of the MAPK is deleterious as it increases fibrosis, hypertrophy and apoptosis (Kompa et al., 2008). Previous studies have shown that p38 induces collagen formation, connective tissue growth factor (CTGF) mRNA expression and increase in the pro-inflammatory cytokine interleukin-6 (IL-6) in the heart (Tenhunen et al., 2006).

So, while the PI3K/Akt pathway plays an important role in the metabolic effects of insulin, mediates glucose uptake and, after activation, is associated with the development of physiological hypertrophy, the MAPK pathway is important on nonmetabolic mitogenic and growth effects of insulin, mediating the pathological hypertrophy.

LVH may be associated with insulin resistance. Therefore, insulin resistance or chronic hyperinsulinemia is characterized by a specific impairment of PI3K-dependent pathway and by an insulin-induced activation of the MAPK pathway, which results in cardiac hypertrophy. It is known that activation of PI3K pathway results in increased endothelial nitric oxide synthase (eNOS) activity and nitric oxide (NO) production and that the activation of the MAPK pathway is involved in stimulation of the vasoconstrictor endothelin-1. In fact, this represents a mechanism associated with insulin resistance, thus contributing to endothelial dysfunction and increased blood pressure due to the impaired vasodilator action of insulin (Figura 6).

Heart insulin resistance has been associated with changes of TRB3. TRB3 is a member of Mammalian tribbles homologs (TRB) protein family which is characterized by the presence of a domain homologous to serine/threonin kinase in the middle of molecule [kinase homology (KH) domain] (Hegedus et al., 2007). The KH domain of TRB lacks a functional ATP binding site and contains the variant amino acid residues that are essential for kinase catalytic activity. Thus, members of the TRB family are referred to as pseudokinase to reflect the view that they are not functional kinases (Boudeau et al., 2006).

Despite lacking kinase activity, KH domain of TRBs still contain substrate-binding domain through which TRBs interact with different factorsTRB3 is an inducible gene whose expression is modulated by metabolic stresses including endoplasmic reticulum stress, nutrient stress (e.g. amino acid and glucose deprivation) and insulin (Ohoka et al., 2005; Ding et al., 2008). By interacting with Akt, TRB3 negatively regulated insulin signaling, and consequently result in increased insulin resistance and diabetes-associated complications. Expression of TRB3mRNA is induced in the liver by fasting and is elevated in db/dbmice. Hepatic overexpression of TRB3 in amounts comparable to those in db/db mice promoted hyperglycemia and glucose intolerance (Du et al., 2003).These observations have led to the suggestion that TRB3 elevation contributes to insulin resistance.

TRB3 also serves as a molecular switch and regulates the activation of the three classes of MAPKs. TRB3 binds to and regulates MAPK kinase, thus controlling the activation of MAPK by incoming signals (Kiss-Toth et al., 2004). However, the TRB3/MAPK signal-transduction pathway has not been investigated in vivo on cardiac tissues directly. Akt and MAPK are the most important pathways involved in selective insulin resistance (Jiang et al., 1999), and activated MAPK contributes to the development of cardiac fibrosis (Papakrivopoulou et al., 2004; Tang et al., 2008).

Recently, the relationship among TRB3 expression, and development of diabetic cardiomyopathy was established (Ti et al., 2011). In this study, the authors found TBR3 overexpression in rats with diabetes, which was paralleled by increased ER1/2 and JNK expression which is key mediator of the inflammatory pathway, whereas phosphorylarion of p38 MAPK was decrease, suggesting that the TRB3/MAPK signaling pathways participated in the pathogenesis of DCM. In addition, with TRB3 silencing, enhanced

phophoryation of ERK/MAPK was suppressed, whereas the impaired Akt activity was upregulated, resulting in improvement of selective insulin resistance.

2.3.2. *Glucotoxicity and oxidative stress*

Hyperglycemia, a consequence of decreased glucose clearance and augmented hepatic gluconeogenesis, has been indicated as one of the major causes of DCM, due to activation of several mechanisms, leading to an increase in oxidative stress, which is defined as an imbalance between the production of ROS and antioxidant defense mechanisms.

ROS are chemical compounds that result from the activation or reduction of molecular oxygen or derivatives of the products of this reduction, such as the superoxide anion and hydroxyl radical (Irani, 2000). ROS may have harmful consequences for the heart cells, including DNA damage and apoptosis, which in turn may promote cardiac dysfunction leading to structural and functional abnormalities that are associated with DCM (Figure 6).

Hyperglycemia induces oxidative stress by several pathways, such as increased activation of the polyol and hexosamine pathways, increased formation of AGEs and activation of classical isoforms of PKC, which induce increased production of mitochondrial ROS, nonenzymatic glycation of proteins and glucose auto-oxidation. Activation of these pathways corresponds to an increased glucotoxicity, which may cause cellular injury. They are also associated not only with the development of diabetic complications but also with insulin resistance and pancreatic β-cell dysfunction.

i) Polyol pathway

The polyol pathway reduces toxic aldehydes generated by ROS to inactive alcohols. Aldose reductase (AR), via the consumption of nicotinamide adenine dinucleotide phosphate (NADPH), is responsible for the initial and rate-limiting step in the process. Glucose can be reduced to sorbitol, and eventually fructose, through this pathway, but AR has a low affinity for glucose at normal concentrations. Elevated intracellular glucose can increase AR activity, resulting in significantly decreased NADPH. NADPH is also required for glutathione reductase activity, which reduces glutathione (GSH)–a major mechanism for reducing intracellular oxidative stress. Decreased NADPH GSH reductase activity ultimately increases oxidative stress and activates pathways that increase cellular damage (Rambhade et al., 2005)

ii) Hexosamine pathway and glucose auto-oxidation

Hyperglycemia increases flux through the hexosamine pathway by providing more fructose-6-phosphate (G-6-P) for glutamine fructose-6-phosphate amidotransferase, the rate-limiting enzyme of the pathway. The increased flux through the hexosamine pathway increases tissue concentration of a hexosamine metabolite, UDP-N-acetylglucosamine. This may lead to O-linked glycolisation of proteins on serine and threonine residues, which then compete with serine and threonine phosphorylation of these sites (McClain, 2002), providing a mechanism by which hyperglycemia could interfere with insulin transduction pathways. Activation of the hexosamine pathway may also occur by other metabolites, such as FFAs, and the ability of a variety of nutrients to activate hexosamine pathway has led to the theory that this pathway serves as a general nutrient sensing pathway, through

which hyperglycemia or hyperlipidemia could decrease insulin sensitivity when nutrient excess prevails.

The effect of hyperglycemia on flux of the hexoasamine pathway probably reflects increased G-6-P levels, which result from inhibition of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) by ROS. Recently, ROS formation due to glucose auto-oxidation has been hypothesized to play a role in the pathogenesis of DCM in diabetic patients; however, no unifying hypothesis exists to explain how glucose auto-oxidation causes any of the complications seen in diabetes.

iii)Advanced glycation end-products (AGEs)

As already noted, the increase of AGEs is highly associated with myocardial fibrosis in diabetic hearts by affecting the structural components of the extracellular matrix, such as collagen, indicating the potential role of AGEs in DCM (Candido et al., 2003). AGEs are a heterogeneous group of proteins, lipids and nucleic acids that are formed by nonenzymatic glycosylation. They bind to specific cell surface receptors [receptor for AGEs (RAGE)] and, in the cascade of intracellular signal transduction, form oxygen free radicals and promote the activation of gene expression. This receptor ligation increases the production of the transcription factor nuclear factor-κB (NF-κB), also causing increased oxidative stress, and it is critically involved in the mechanisms of endothelial dysfunction and platelet activation. The AGE-RAGE axis besides playing a role on oxidative stress represents a key mediator of inflammation (Cziszar and Ungvari, 2008).

The RAGE is a multi-ligand member of the immunoglobulin superfamily of cell surface molecules that binds to a wide variety of ligands that are susceptible to aggregation and posttranslational modifications. This receptor is widely expressed in the peripheral and central nervous systems. A series of ligands of RAGE, as well as the activation of RAGE

itself, can induce oxidative stress (Irani, 2000) and exert direct effects on cardiac remodeling due to adhesive and growth-regulating properties.

RAGE has an endogenous secretory receptor form, called soluble RAGE (sRAGE), which could exert antiatherogenic effects by acting as a decoy. Reduced sRAGE levels in CAD subjects with normal levels of low-density lipoprotein (LDL) cholesterol could also raise the possibility that the measurement of sRAGE level may improve risk assessment among normolipidemic subjects (Falcone et al., 2005). sRAGE levels were significantly higher in type 2 diabetic patients than in non-diabetic subjects and positively associated with the presence of CAD (Nakamura et al., 2007). Plasma sRAGE levels are positively associated with endothelial function and predict CV events in nondiabetic patients with suspected coronary artery disease, suggesting a pivotal role in atherothrombosis (Chiang et al., 2009). Low levels of sRAGE have also been associated with stroke, especially in subjects without identifiable conventional risk factors, being even proposed as a potential biomarker (Park et al., 2009).

In diabetes patients, elevated serum and tissue contents of AGEs have been found, which then activate RAGE (Yamagishi et al., 2007; Yan et al., 2007). Further, in the heart tissues of diabetic rats, an increased expression of RAGE has been found, which seems to be associated with CTGF (Candido et al., 2003). Overexpression of RAGE in diabetic hearts of animals implies a role for this receptor in mediating AGE-induced myocardial structural alterations. In addition to AGE-induced fibrosis, several lines of evidence have suggested that AGE accumulation is associated with impaired calcium homeostasis and mitochondrial function and altered expression and function of both the ryanodine receptor and sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA), contributing to reduction of calcium entry in the myocyte and, consequently, impaired change myocardial contractility (Bidasee et al., 2003).

iv) Activation of protein kinase C isoforms

PKC is a serine kinase that has a number of potential substrates, including the IR (Newton, 1995). PKC is activated by diacylglycerol (DAG), the concentration of which increases in a glucose-dependent manner during hyperinsulinemia (Chen et al., 1994). Subsequential PKC activation can lead to serine phosphorylation and inhibition of the insulin receptor and IRS proteins (Virkamaki, 1999).

PKC pathway activation is also correlated with biochemical changes underlying the development of DCM. These changes include increased vascular permeability and lead to blood vessel constriction and changes in blood flow, stimulating neovascularization, proliferation and apoptosis of endothelial cells, and activation of several factors, such as transforming growth factor-beta (TGF-β), vascular endothelial growth factor (VEGF) and IGF-1 (Koya and King, 1998; Das Evcimen and King, 2007). Moreover, a recent study indicated that PKC promotes cardiac hypertrophy by activating NF-κB (Min et al., 2009).

2.3.3. *Lipotoxicity*

Diabetic hearts are also characterized by increased myocardial fatty acids uptake and TGs levels. FFAs, once inside the cell, are converted into acetyl coenzyme A derivatives that will activate the isoforms of PKC, an intracellular enzyme with the ability to block the insulin signaling pathways which, in turn, will block the entire cascade of subsequent reactions preventing insulin action (Perseghin et al., 2003). In the presence of elevated levels of FFAs, there is a competition of these with glucose as energy substrate, with a shift in energy production from β-oxidation of FFAs (Rodrigues et al., 1998), leading to decreased glucose utilization and oxidation, with increased glucose levels and release

stimulation, increasing the amount of insulin and promoting insulin resistance (Poitout and Robertson, 2002)

The increase of FFAs and its metabolism leads to intracellular accumulation of toxic fatty acids intermediates, such as ceramide and diacylglycerol, as well as the formation of ROS, which contribute to oxidative stress, cardiomyocyte apoptosis, and increased myocardial oxygen consumption. This results in reduced myocardial high-energy reserves and impaired contractility, promoting mitochondrial uncoupling and leading to decreased adenosine triphosphate availability (Khullar et al., 2010; Eckel and Reinauer, 1990). Therefore, all these mechanisms may be involved with a reduced cardiac performance, thus contributing to the pathogenesis of DCM.

Furthermore, the cardiac lipotoxicity in DCM also involves the activation of transcription factors that further affect FA uptake and oxidation. Therefore, FFAs intracellular deposition is responsible for the saturation of the mitochondrial capacity of oxidation, thus activating transcription factors, including the peroxisome proliferator-activated receptors (PPARs). Although PPARs are key regulators of FA metabolism in the heart, recent studies have shown that cardiac overexpression of PPAR-alpha (PPARα) and PPAR-gamma (PPARγ) causes lipotoxic cardiomyopathy and steatotic cardiomyopathy (Finch et al., 2003; Son et al., 2007).

In animal models of obesity and diabetes an increase in apoptosis has been shown to occur in the heart (Barouch et al. 2006). Several mechanisms have been proposed to lead to the increase in apopotic cell death, including gluco and lipotoxicity, mitochondrial dysfunction and increased RAAS activity. All of these lead to an increase in the build-up of ROS. The high circulating and intracellular stores of fatty acids leads to alternative non-oxidative forms of energy production that result in the accumulation of toxic by-products.

The reactive lipids react with oxygen products to produce toxic reactive lipid species that may lead to increased cell death.

2.3.4. *Endothelial dysfunction*

Endothelial dysfunction is also involved in the pathogenesis of DCM. The endothelium regulates the vascular tone by releasing vasodilator and vasoconstrictor substances. Endothelial dysfunction expresses the malfunction of the vascular endothelium and usually reflects a reduced bioavailability of NO, which is an important vasodilator. Furthermore, NO also has anti-hypertrophic, anti-proliferative, and anti-inflammatory properties and inhibits leukocyte adhesion, limits adhesion and aggregation of platelets, and reduces the expression of plasminogen activator inhibitor 1 (PAI-1), a pro-thrombotic protein associated with vascular homeostasis. Diabetes is associated with decreased expression of eNOS and decreased production of NO through the inhibition of the IRS-PI3K-Akt-eNOS-NO (Figure 7). However, due to increased oxidative stress, an excess of NO becomes cytotoxic. Part of its toxicity is due to its reaction with superoxide, resulting in peroxynitrite formation and decreased of NO bioavailability (Yu et al., 2011), causing endothelial dysfunction and leading to impaired vasodilatation. A relationship between the excessive cardiac production of ROS and endothelial dysfunction has been demonstrated in animal models of type 1 and T2DM and in humans with T2DM (Guzik et al., 2002).

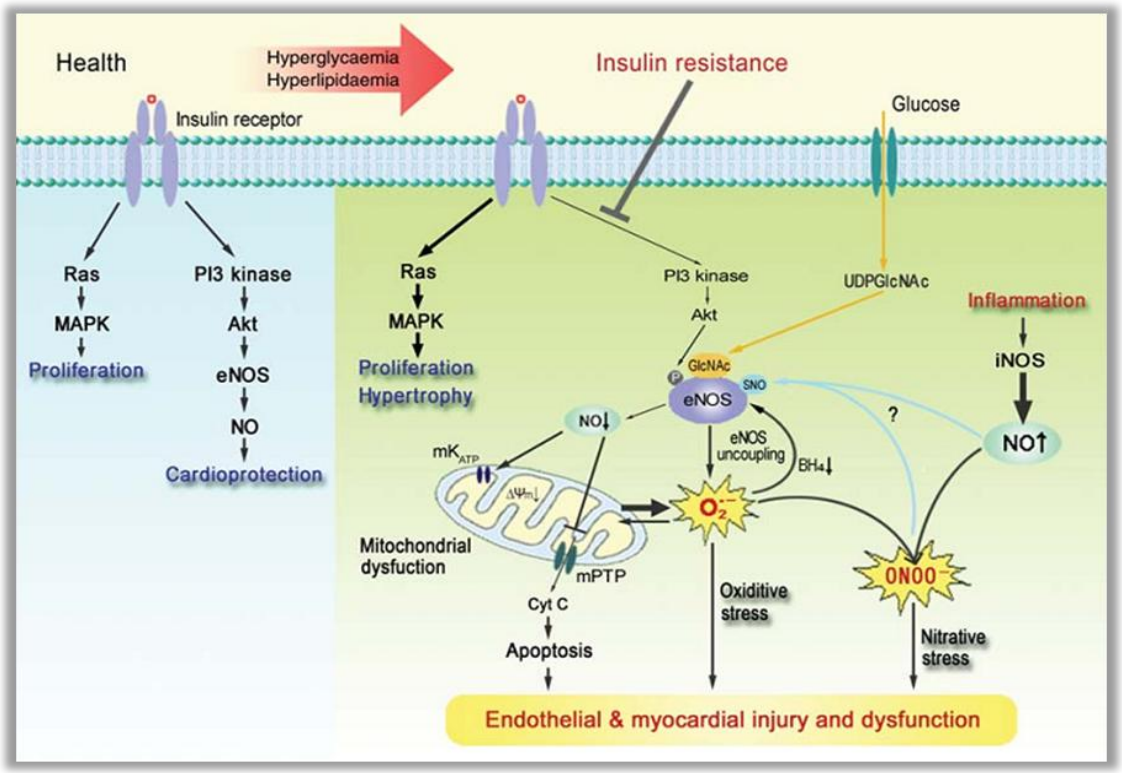


Figure 7. Impairment of insulin – PI3K – Akt – eNOS – NO signaling defines a key characteristic of insulin resistance and myocardiac dysfunction.Taken from de Yu et al. (2011).

2.3.5. *Inflammation*

Chronic low-grade inflammation is commonly associated with obesity and T2DM, and clear evidence has emerged to suggest that inflammatory process also contributes to the pathogenesis of DCM.

The activation of several signaling pathways, such as NF-κB, c-jun NH_2-terminal kinase (JNK) or p38-MAPK, could mediate a state of inflammation, which is linked to insulin resistance, thus playing an important role in diabetic complications (Lorenzo et al., 2011; Kaneto et al., 2004; Li et al., 2007). In fact, NF-κB represents one of the most important mediators of the inflammatory process. Activation of NF-κB is associated with the increased release of cytokines, such as tumor necrosis factor-alpha (TNF-α), which is often involved in cardiac damage and leads to NF-κB activation, thus contributing to the

intensification of adverse effects in diabetic hearts (Lorenzo et al., 2011). Elevated inflammatory mediators cause insulin resistance since they reduce the IRS-1 tyrosine phosphorylation, and the activation of PI3K and AKT, decreasing insulin signaling (Jager et al., 2003). Moreover, elevated inflammatory markers may exacerbate insulin deficiency by impairing β cell function and inducing cell death, which implicate a progressive decline in β -cell mass (Donath et al., 2003).

Elevated inflammatory cytokines, such as TNF-α, IL-6, cell adhesion molecules, including vascular cell adhesion molecule-1 (VCAM-1) and intracellular adhesion molecule-1 (ICAM-1), acute phase reactants, such as C-reactive protein (CRP), PAI-1 and other biological markers of inflammation have been found in circulation and in the diabetic hearts o T2DM patients (Yu et al., 2011; Mano et al., 2011). Accumulating data have been demonstrated that elevated levels of several inflammatory markers, such as interleukin-1 beta (IL-1β) and TNF-α, are implicated in diabetic cardiomyopathy, increases epicardial thickness, promotes myocyte contractile dysfunction, thus depressing myocardial function, contributing to HF (Westermann et al., 2006). Moreover, cardiac overexpression of TNF-α has been associated with cardiac hypertrophy and fibrosis, as well with left ventricular dysfunction (Yokoyama et al., 1997; Sun et al., 2004). Furthermore, IL-6 has been also described as an inducer of myocardial damage. Therefore, excessive production of IL-6 can promote LV dysfunction and cardiac hypertrophy under acute myocardial infarction (Younce et al., 2010).

Although the inflammatory response seems to be linked with DCM development, its presence and influence differs in the early and the long-standing stages of the disease. Therefore, according to Ares-Carrasco et al. (2009), using normotensive and spontaneously hypertensive rats (SHR) with T1DM induced by streptozotocin, myocardial fibrosis and apoptosis are features of myocardial damage secondary to long-term experimental

diabetes, but inflammation was modulated by the expression of anti-inflammatory molecules, in particular IL-10, and antioxidants. The authors also found interesting effects when diabetes coexisted with hypertension. DCM and hypertensive cardiomyopathy share some typical features, including functional and structural changes, which contribute to cardiac tissue impairment.

Additionally, according to the proteomics studies of Ares-Carrasco et al. (2012) in SHR and SHR/DM1 rats, when diabetes and hypertension coexist, hearts present with impaired expression of metabolic, hypertrophic, and apoptotic proteins, against what occurs in the early stages of injury, in which fibrotic and inflammatory rates are not additive. Furthermore, they suggest that in such a stage of diabetes and hypertension, PPAR-α activation, as a compensatory response to the metabolic, apoptotic, and hypertrophic impairment, could reduce cardiac hypertrophy. In this sense, therapeutics with PPARα agonists could have beneficial anti-hypertrophic effects on the hypertensive DCM.

As mentioned, hyperglycemia induced-oxidative stress is strongly correlated with development of diabetic cardiomyopathy. In fact, hyperglycemia has been shown to activate various oxidative stress-responsive/proinflammatory transcription factor, including NF-κB, to induce collagen and fibronectin synthesis, as well as to stimulate the production of inflammatory cytokines.

A recent study suggests that hyperglycemia-induced cardiomyocyte apoptosis is mediated by production of monocyte chemotactic protein-1, providing a molecular link between hyperglycemia, inflammation and DCM (Kaneko et al., 1997). Yu et al. (2011) indicated that hyperglycemia-induced diastolic dysfunction may be mediated partly by the macrophage migration inhibitory factor, suggesting that the NF–κB pathways may be involved in this process. In addition, in the same study, the authors demonstrated the

macrophage migration inhibitory factor effects in abnormal upregulation of G protein–coupled receptor kinase 2, which may be associated with a constant activation of beta1-adrenergic receptor and HF development. The activation of the RAAS seems to be another factor that plays an important role in modulation of inflammation in DCM. Ang II not only induces vasoconstriction, cell growth and oxidative stress but also stimulates inflammation. Ang II is able to induce cytokine release (Schieffer et al., 2000), also stimulating the production of PAI-1andpro-inflammatory transcription factors, such as NF-κB (Hernández-Presa et al., 1997), which in turn regulate adhesion molecule (VCAM-1 and ICAM-1) and the expression of several cytokine, as already mentioned.

2.3.6. *The rennin-angiotensin-aldosterone system (RAAS)*

The rennin-angiotensin-aldosterone system (RAAS) is a hormonal cascade that plays a major role in the homeostatic control of arterial pressure by regulating blood vessel constriction. Deregulation of RAAS plays an important role in pathological origin of renal diseases, CVD and arterial hypertension (HTA) and also contributes to the development of cardiomyopathy.

Activation of the RAAS, locally and systemically, is strongly associated with the development of insulin resistance and the onset of type 2 diabetes (Niklason et al., 2004; de Kloet et al., 2010; Abuissa et al., 2005). In diabetes, excessive activation of the RAAS has been described (Hayashi et al., 2010; McGuire et al., 2008). Since RAAS overactivation has been associated with some of the hallmarks of DCM, such as increased fibrosis, angiogenesis, oxidative damage and cardiomyocyte and endothelial cell apoptosis and necrosis, it has increasingly recognized as an important factor in the progression of the disease (Fang et al., 2004; Privratsky e al., 2003).

Ang II is the main physiological effector molecule of RAAS; its release in the myocardium, due to the upregulation of RAAS, has diverse and widespread actions that affect cardiac function (Fyhrquist and Saijonmaa, 2008). Animal experiments have also shown that Ang II infusion induces insulin resistance (Richey et al., 1999) and increased levels of Ang II and Type 1 receptors (AT1-R) have been show in diabetic rats (Singh et al., 2008; Lee et al., 2008; Siddiqui et al., 2007). Ang II can interfere with all intracellular signaling of insulin receptors, preventing the glucose receptor translocation, thus being one of the pathophysiologic bases of insulin resistance. AT1-R mediate most physiological and pathophysiological and deleterious effects attributed to Ang II. In the adult CV system, Ang II induces its vasoconstrictive effects on cardiomyocytes through ligand binding to the AT1-R (Fiordaliso et al., 2000). The rise of Ang II in diabetic rats has been related to cardiomyocyte hypertrophy and apoptosis, and it stimulates the proliferation of cardiac fibroblast and synthesis of collagen, causing myocardium interstitial and perivascular fibrosis, ventricular myocardium rigidity and impaired diastolic function, leading to the clinical symptoms of DCM (Brilla et al., 1997; Dostal et al., 2000).

2.4. Are there earlier cardiac changes in (pre)diabetes?

A link between DM and HF has been well-recognized for more than a century. HF is also closely linked to abnormal glucose regulation (AGR) and insulin resistance in patients without DM and, similarly, these conditions commonly coexist. In epidemiological studies, each condition appears to predict the other. The prevalence of AGR/IR in HF patients without DM is significantly under recognized and, as yet, the optimal method for screening for these abnormalities in the outpatient setting is unclear (Mamas et al., 2010).

Clinical studies indicate a high prevalence of insulin resistance and impaired fasting glucose among non-diabetic patients with cardiovascular disease (Berry et al., 2007; Bartnik et al., 2004). Furthermore, the prevalence of prediabetes is substantial among adults with CHD and likely underestimated because of suboptimal screening (Kilmer et al., 2011). Several longitudinal studies support the hypothesis that prediabetes is a risk factor for diabetes and CVD (Coutinho et al., 1999; Meigs et al., 2002; DECODE study group, 1999; Gabir et al., 2000; Stern et al., 2002).

De Marco et a. (2011) evaluated whether prediabetes, as measured by IFG, is associated with cardiac alterations independently of major confounders in a population-based sample of adolescents and young adults. They found that prediabetic individuals had a significantly higher prevalence of LVH than participants with normal fasting glucose, reflecting important target organ damage already present at an early phase of alteration of glucose metabolism. Furthermore, the presence of geometry-related left ventricular functional alterations was found in prediabetic subjects. This fact is important, since there is a strong relation between LVH and adverse cardiovascular outcomes (Levy et al., 1990), recommending targeting prevention strategies in this subpopulation.

Postprandial, but not fasting, hyperglycemia is known to be a better predictor of major cardiovascular events or total mortality, but the impact of postprandial or fasting glucose levels on LV diastolic function has not been elucidated (Coutinho et al., 1999; DECODE Study Group, 2001). Recently, Shimabukuro et al. (2011) showed, in humans, that impaired glucose tolerance, but not impaired fasting glucose, underlies left ventricular diastolic dysfunction, and Wu et al. (2007) previously described epidemiological evidence of altered cardiac autonomic function in subjects with IGT but not isolated IFG, as previously suggested (Fujita et al., 2007). However, according to Diakakis et al. (2005), IGT patients have an increased HR and elevated cytokine levels, suggesting that these changes could serve as an index of the primary atherosclerotic process.

Two recent studies, the "ADVANCE" trial (Zoungas et al. 2009; Patel et al., 2008) and the "ACCORD" trial (Gerstein et al., 2008; ACCORD Study, 2010), reported no significant benefit from intensive HbA1c lowering in terms of cardiovascular outcomes in subjects with long-standing diabetes. Therefore, early interventions seem to be advisory. However, the state of myocardial performance in prediabetes has received little attention.

An ongoing debate is whether prediabetes deserves targeted identification and clinical intervention, as well as the search for feasible biomarkers for diagnosis and treatment (Tabák et al. 2012; Grundy, 2012; Ratner and Sathasivam, 2011; Pour et al., 2011; DeFronzo and Abdul-Ghani, 2011). The epidemiological relation between prediabetes and macrovascular disease can be confounded by clustering of vascular risk factors within individuals. Blood glucose in the prediabetic range is correlated with many risk factors, including general and central obesity, blood pressure, and triglyceride and lipoprotein concentrations (The Emerging Risk Factors Collaboration, 2010). Thus, well defined and characterized animal models could be important tools to study the mechanisms

underlying prediabetes evolution to diabetes and associated complications, including cardiomyopathy.

3. The high-sucrose diet as an experimental model of (pre)diabetes cardiomyopathy

Animal models have become a useful tool for investigating various genetic, environmental, behavioral and pharmacologic factors that play a role in metabolic abnormalities. Various animal models of diabetes have been used to study the causes and underlying cellular events of diabetic cardiomyopathy. However, one major impediment to progress in the knowledge of the appropriate animal models, and a one major difficulty is the presence of important factors, such as obesity and hypertension, which displays susceptibility to cardiomyopathy.

Since in the prediabetic state cardiac alterations may already be present, an understanding of the cellular effects of these metabolic disturbances on cardiomyocytes in the prediabetic state should be useful in predicting the structural and functional cardiac consequences. Experimental support is limited with most work focusing on chemically induced type 1 models of the disease that ultimately represent an extreme of the metabolic profile (rarely encountered in clinical practice) or type 2 models burdened with other element(s) of the metabolic syndrome and thus the evidence is scarce regarding the mechanisms involved in cardiomyopathy in this stage of disease. Therefore, animal models of prediabetes could help with the understanding of the events leading to the appearance of clinical symptoms. To elucidate whether the alteration at cardiac level are already present in prediabetic state and what the underlying mechanisms we pretend to use an animal model that presents with relatively minor metabolic abnormalities, consistent with

prediabetic insulin resistance in humans (Pagliassotti et al.,1996; Podolin et al., 1998), and that mimics early stages of type 2 diabetes, without other complicating factors that could lead to cardiac events.

Dietary factors are likely to be of importance in the etiology of the metabolic syndrome in humans. Several studies have demonstrated that the macronutrient composition of the diet is an important environmental determinant of the quality of insulin action (Storlien et al., 1988; Lombardo et al., 1996; Besesen, 2001). Most studies have examined the relationship between sucrose and insulin resistance. High sucrose (HSu) intakes have been shown to contribute to conditions such as hypertyglyceridemia, impaired glucose tolerance and hyperinsulinemia in rat models, mimicking the features of metabolic syndrome that precede the development of T2DM; however, the underlying mechanisms have not been elucidated.

Some of the controversy regarding the ability of sucrose to produce insulin resistance is probably due to different experimental conditions, including different models, dose of sucrose and/or duration of exposure to the nutrient.

Pagliassotti et al. (1995) demonstrated in the rat that the quantity of dietary sucrose influenced the time course of the development of insulin resistance in a tissue-specific manner, in that consumption of a diet high in sucrose (68% of kcal as sucrose) resulted in hepatic and peripheral insulin resistance within 8 weeks, whereas consumption of a diet lower in sucrose (18% of kcal as sucrose) required at least 16 weeks to elicit hepatic insulin resistance, with no change in peripheral insulin sensitivity.

Absorbed sucrose (a disaccharide comprising glucose and fructose in a 1:1 ratio) is degraded into glucose and fructose, and fructose has been found to be the moiety responsible for sucrose-induced insulin resistance (Thresher et al., 2000). For instance,

Thorburn et al. (2009) showed that a diet containing 35% fructose produced insulin resistance, whereas a diet containing 35% glucose did not. This fact could be explained explained based on fructose and glucose bioavailabilities: fructose is predominantly metabolized in the liver whereas the main part of an oral glucose load is metabolized in peripheral tissues; fructose bypasses the phosphofructokinase rate limiting step in glycolysis, potentially resulting in unrestrained production of glycolytic end-products and systemic dysregulation and, unlike glucose, fructose is transported into the cell insulin-independent manner (Laville and Nazare, 2009). Thus, follow-up studies demonstrated that fructose was the primary mediator of sucrose-induced impairments in insulin action and glucose intolerance in vivo (Pagliassotti, 1996; Thresher et al., 2000).

The addition of sucrose to either drinking water or rat chow has been shown to produce similar changes in the circulating insulin, triglycerides, cholesterol and glucose as well as in the body weight.

High sugars intake is linked to an increased risk of heart disease. Moreover, previous studies have shown that high-sucrose feeding in rats lead to the development of cardiac dysfunction. (Davidoff et al., 2004; Vasanji et al., 2006; Dutta et al., 2001). Mechanical properties of isolated ventricular myocytes from sucrose-fed rats demonstrated abnormalities in cardiac contractile performance and calcium hanlding, which are results of insulin resistance stage of this model (Vasanji et al., 2006). Sucrose feeding also increased systolic blood pressure in rats with increased LV mass but without cardiac fibrosis (Sharma et al., 2008). With these evidences, the high sucrose feeding rats may be considered an experimental DCM, with similar clinical manifestation of DCM in humans.

II. OBJECTIVES

The general goal of the study is to evaluate the impact of a high-sugar diet (sucrose consumption) on the cardiac tissue, as an attempt rat model of (pre)diabetic cardiomyopathy with insulin resistance, but lacking sustained/overt hyperglycemia. The mechanisms possibly involved in the alterations putatively encountered will be screened.

To accomplish the main purpose, the following specific aims will be pursued:

- This experimental model of (pre)diabetes and insulin resistance will be characterized in terms of body weight evolution, glycemic profile (fasting and fed glucose levels, HbA1c and GTT), insulinemia, insulin tolerance/resistance (ITT and HOMA-IR), as well as concerning basic lipid profile (serum Total-cholesterol and TGs levels). The expression of cardiac tissue IR-β, PI3K, MAPK and TRB3 will be assessed as a measure of possible alterations in some of the main insulin signaling proteins and resistance;
- The cardiac "(dys)function" and the underlying mechanisms will be assessed by measuring blood pressure and heart rate, heart and LV trophism indexes and protein and/or mRNA expression of markers of fibrosis, hypertrophy, proliferation, apoptosis, angiogenesis, endothelial function, inflammation and oxidative stress, including: TGF-β1, procollagen III , CTGF, TSP-1, BNP, Bax, Bcl-2, VEGF, TNF-α, IL-6, ICAM-1, VCAM-1, iNOS, SOD, HNE and RAGE. This cardiac tissue data will be accompanied with serum values of some markers of proliferation, inflammation and redox status: TGF-β, VEGF, IL-6, IFN-γ, PCR and MDA/TAS.

IV. MATERIAL AND METHODS

1. Animal model and diet

Experiments were performed on male adult (16 weeks-old) Wistar rats (Charles River Laboratories, Barcelona, Spain), weighting 332.9 ± 9.0g at the time of arrival. Animals were housed two per cage in the experimental vivarium of the Laboratory of Pharmacology and Experimental Therapeutics (IBILI, Faculty of Medicine, University of Coimbra) under controlled temperature (22-23ºC) and lighting (12:12-h light-dark cycle). On arrival, all rats were given standard chow and water *ad libitum*. After 1 week of acclimatization, animals were randomly divided into two groups (Figure 8): control group (n=7), rats continued to receive tap water for drinking; and high-sucrose diet group (HSu) (n=8), rats received 35% sucrose (S0389; Sigma-Aldrich) in drinking water for a period of 9 weeks. All animals were fed standard rat chow (containing 16.1% protein; 3.1% lipids; 3.9% fibers and 5.1% minerals, *(*AO4 Panlab, Barcelona, Espanha) *ad libitum* (with exception in the fasting periods). All experiments were conducted according to the National and European Directives on Animal Care.

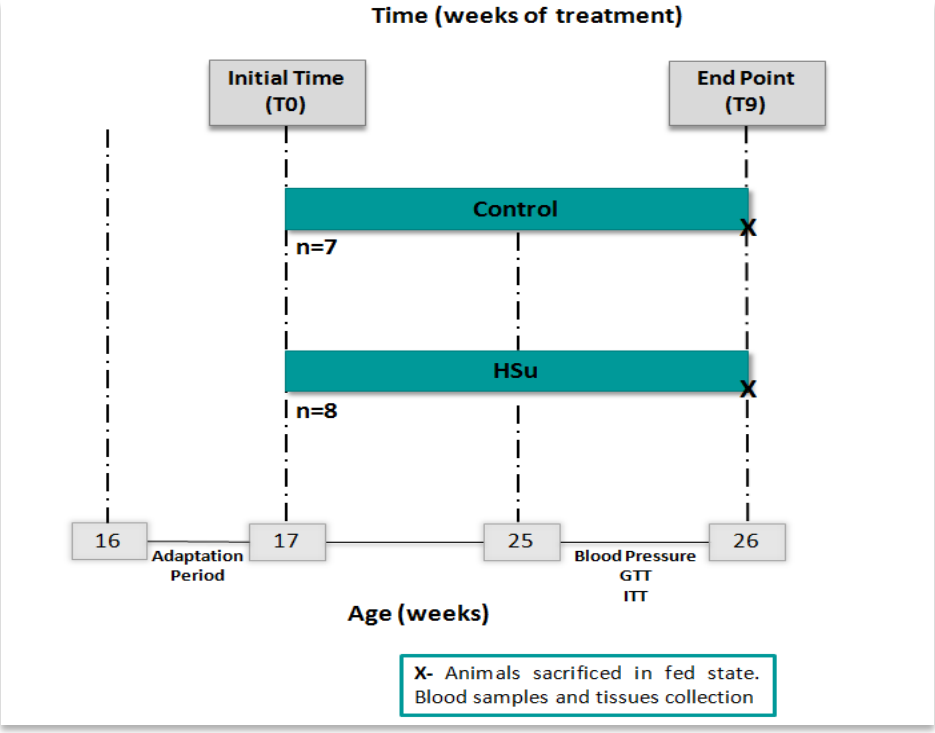


Figure 8. Schematic diagram of study design. GTT, Glucose Tolerance Test; ITT, Insulin Tolerance Test.

2. Monitoring of body weight

The body weight (BW) of each animal was recorded weekly during the experimental period, using an analytical balance (KERN CB 6 K1, Germany).

3. Blood pressure and heart rate assessment

Systolic blood pressure (SBP), diastolic blood pressure (DBP) and mean blood pressure (MBP) and heart rate (HR) values were obtained using a tail-cuff sphygmomanometer LE 5001 (Letica, Barcelona, Spain) in appropriate contention cages, and in conscious rats. Before the measurements, the animals were warmed for 10-20 min. at 25-30 °C in order to allow the detection of tail artery pulsations and to achieve the pulse level ready. BP and HR values were obtained by averaging 8-10 measurements. To minimize stress-induced fluctuations in BP, all the rats were adapted to appropriate cages and to measurements during at least 2 weeks before the beginning of data collection. The same person took the final values in the same peaceful environment between 14:00 h and 18:00 h.

4. Animal sacrifice and collection of blood and tissue samples

The day before the final time, after an overnight fasting period, rats were subjected to intraperitoneal anesthesia with a 2 mg/kg BW of a 50 mg/kg pentobarbital (Sigma-Aldrich, Portugal) solution and a blood sample was immediately collected by venipuncture from the jugular vein into syringes with Heparin-Lithium (Sarstedt, Monovette®) for plasma samples, in order to measure fasting glycemia and insulin levels.

At the final time (T9), animals were anhetized in the same conditions and blood samples were immediately collected by venipuncture from the jugular vein in needles without anticoagulant (for serum samples collection).

The rats were then sacrificed by cervical dislocation, and the heart was immediately removed, placed in ice-cold Krebs buffer, carefully cleaned of adherent fat and connective tissue, weighted and divided in left and right ventricle. Heart regions were frozen in liquid nitrogen and stored at -80ºC, for biochemical, gene expression or Western-blot analysis.

5. Determination of cardiac trophism indexes

The heart of each animal, collected as previously described, was weighed and further divided in left and ventricles in order to calculate the following ration as cardiac trophism indexes: heart weight (HW)/BW and left ventricle weight (LVW)/HW.

6. Glycemic and insulinemic profile

6.1. *In vivo* assays

6.1.1. Fasting glycemia

At the end of experimental period, 6-hour fasted rats were tested for estimation of blood glucose levels, which was measured in the tail vein using the portable device One Touch® UltraEasy® glucometer (Lifescan, Johnson and Johnson, Portugal).

6.1.2. Glucose tolerance test (GTT)

Rats submitted to a fasting period of 6-h were administered intraperitoneally (i.p.) with a glucose bolus of 2 g/kg BW and the tail vein blood glucose levels were measured in

samples taken immediately before the bolus and 30, 60, 90, and 120 min. after, using the portable device One Touch® UltraEasy® glucometer (Lifescan, Johnson and Johnson, Portugal). The area under the curve (AUC) for the GTT was calculated by using the trapezoidal method.

6.1.3. Insulin tolerance test (ITT)

The ITT was performed through the i.p. administration of insulin (I9278, Sigma), 0.75 U/kg BW, in 6-h fasted rats. Blood glucose was monitored in the tail vein blood collected immediately before the insulin injection and 15, 30, 45, 60 and 120 min. after, using the portable device One Touch® UltraEasy® glucose monitoring system glucometer (Lifescan, Johnson and Johnson, Portugal).

6.2. *Ex vivo* assays

6.2.1. Postprandial (Fed) glycemia

Serum glucose levels were measured using an automatic analyzer (Hitachi 747 chemistry analyzer, Diamond Diagnostics Inc., Holliston, MA, EUA), standardized for clinical data. A glucose oxidase comercial kit (Sigma, St. Louis, Mo, USA) was used. This is based on a colorimetric based on a colorimetric enzymatic principle: in presence of atmospheric oxygen, glucose is oxidized by glucose oxidase (GOD) to gluconolactone and hydrogen peroxide (H_2O_2). The resulting H_2O_2 is oxidized in the presence of peroxidase (POD), and reacts with phenol and 4-aminophenazone, an oxygen acceptor, originating a red quinone-imine chromogen. The color intensity is directly proportional to the concentration of glucose present and can be photometrically measured.

6.2.2. HbA1c

HbA1c is the product of non-enzymatic glycation between glucose and free amino groups of hemoglobin. As the level of HbA1c is proportional to the level of glucose in the blood over a period of two months, it is used to characterize this concentration as an indicator of the mean daily blood glucose in recent months. To measure of HbA1c, the DCA Vantage latex immunoagglutination method (Siemens Healthcare Diagnostics, Barcelona, Spain) was used. This method is characterized by use of specific monoclonal antibody for HbA1c mouse.

For the measurement of total hemoglobin, potassium ferricyanide is used to oxidize hemoglobin in the sample to methemoglobin. The methemoglobin then complexes with thiocyanate to form thiocyan-methemoglobin, the coloured species measured. The extent of color development at 531 nm is proportional to the concentration of total hemoglobin in the sample.

For the measurement of specific HbA1c, an inhibition of latex agglutination assay is used. An agglutinator (synthetic polymer containing multiple copies of the immunoreactive portion of HbA1c) causes agglutination of latex coated with HbA1c specific mouse monoclonal antibody. This agglutination reaction causes increased scattering of light, which is measured as an increase in absorbance at 531 nm. HbA1c in whole blood specimens competes for the limited number of antibody-latex binding sites causing an inhibition of agglutination and a decreased scattering of light. The decreased scattering is measured as a decrease in absorbance at 531 nm. The HbA1c concentration is then quantified using a calibration curve of absorbance versus HbA1c concentration. The percent HbA1c in the sample is then calculated using the following equation:

$$\%\, HbA1c = \frac{[HbA1c]}{[Total\ Hemoglobin]} * 100$$

6.2.3. Serum insulin

Serum insulin levels were quantified by using a rat insulin ELISA (Enzyme-Linked Immuno-Sorbent Assay) kit from Mercodia (Uppsala, Sweden) which is a solid phase two-site enzyme immunoassay. This method is based on the direct sandwich technique, in which two monoclonal antibodies are directed against separate antigenic determinants on the insulin molecule. In incubation phase, molecules of insulin in sample react with peroxidase-conjugated anti-insulin antibodies and anti-insulin antibodies fixed in microtiter well. Unbound enzyme labeled antibody is removed in washing step. The bound conjugated enzyme is detected by reaction with 3,3',5,5'-tetramethylbenzidine. The final step of reaction occurs by adding stop solution acid, which forms a coloured compound that can be measured by spectrophotometry.

6.2.4. Insulin resistance index

The homeostasis model assessment of insulin resistance (HOMA-IR) index has been used as a measure of insulin resistance. This parameter was calculated using the Matthews *et al*. (1985) formula**:**

$$HOMA - IR = \frac{Glucose\ (mmol/L) * Insulin\ (\mu U/L)}{22{,}5}$$

The values used (insulin and glucose) were obtained after an overnight of food deprivation, as described in section 4.

7. Lipid profile

Blood was collected to syringes without anticoagulant and centrifuged (730 *g*, 15 min. at 4 °C). Serum total cholesterol (Total-c) and triglycerides (TGs) were analyzed on a Hitachi 717 automatic analyzer (Roche Diagnostics) using Cholesterol RTU® reagent (bioMérieux®, Lyon, France) and the triglycerides kit TG PAP 1000 (bioMérieux®, Lyon, France), respectively. These colorimetric methods are based on the application of some enzymes, whose final enzymatic chain compound staining intensity is directly proportional to the amount of triglycerides and cholesterol in the sample.

8. Serum markers of inflammatory,angiogenesis and proliferation

Serum levels of transforming growth factor β-1 (TGF-β1), vascular endothelial growth factor (VEGF), interferon-gamma (IFN-γ) and interleukin-6 (IL-6) were measured through micro-ELISA sandwich assay, using commercial ultrasensitive Quantikine® ELISA kits (R&D Systems,Minneapolis, USA). C-reactive protein (CRP) levels, in serum, were also measured by micro-Elisa method using a commercial kit (Alpha Diagnostic International, San Antonio, Texas, USA).

This assay employs the quantitative sandwich enzyme immunoassay technique and consists on use a mouse monoclonal antibody, specific for each of the elements to be determined, to coat onto the microplate. Standards, controls and samples are pipetted into the wells, and the substance to be assayed present in the serum bounds to immobilized antibody. In order to remove all unbound substances, washing the wells is carried out. Then, an enzyme-linked polyclonal antibody specific for each substance in analysis is added to the wells. Following a wash to remove any unbound antibody-enzime reagent, a substrate solution is added to the wells. The enzyme reaction yields a blue product that

turns yellow when the stop solution is added. The intensity of the colour measured is in proportion to the amount of substance to be determined. The sample values are then read off the standard curve.

9. Evaluation of oxidative stress

9.1. Serum and heart lipid peroxidation

The measurement of thiobarbituric acid reactive substances (TBARs) is a method to determine lipid peroxidation. Malondialdehyde (MDA) is one of the major aldehyde derive from the decomposition of lipid peroxides. When subjected to conditions of acidic pH (3.5) and high temperature (100 °C), this aldehyde reacts with thiobarbituric acid (TBA) originating the adduct (TBA-MDA), a red chromogen, that can be measured spectrophotometrically (532 nm) or by fluorescence (excitation λ of 534 nm and λ emission 551nm).

Two hundred micrograms of heart fraction was homogenized in 2 ml of phosphate buffer at pH=7.4 with 10 µl solution of antioxidant butylated hydroxytoluene (BHT) in acetonitrile, and then centrifuged at 10.000 rpm for 10 minutes at 4 °C, when the supernatant was collected to sonicate for 3 minutes. After another centrifugation at 4000rpm, 4 °C for 15 minutes, the supernatant was collected again, now ready for be quantified.

To quantify the concentration of MDA in the sample, 100 µl of serum, as weel as 100 µl of tissue, previously collected and homogenized, were used, and a calibration curve.

The calibration curve was established by preparing a comercial stock solution of malondialdehyde-bis-dimethylacetal at concentration of 83.5 mmol/l, from which the

standard solutions of known concentrations (0.42, 0.83, 1.67, 2.5, 4.17, 8.3, 16.7 mmol / l) were prepared, including the blank sample.

In a 10 ml tube, 100 µl of sample or distilled water (the blank) or solution (for standard samples) were placed. Regardless of the group, 100 µl of BHT dissolved in ethanol, 100 µl of catalytic iron trichloride hexahydrate ($FeCl_3.6H_2O$) dissolved in distilled water, 1.5 ml of glycine-HCl buffer at pH 3.5 and finally 1.5 ml of reagent chromogen, TBA were further added. All tubes were placed in the dark at a temperature of 5 °C for 60 min. The tubes "capped" with glass beads were the kept in water bath at 95-100 °C.

Thereafter, the tubes were cooled in an ice bath, where samples were extracted with 2.5 ml of a n-butanol-pyridine (15:1 v/v) solution and 0.5 ml of water distilled. Then, the tubes were centrifuged at 1000g for 10 minutes, after which the supernatant was collected and the absorbance read at 532 nm.

9.2. Quantification of serum total antioxidant status (TAS)

The serum total antioxidant status was quantified using a commercial kit Total Antioxidant Status (Randox Laboratories Ltd., UK) and measured on a Hitachi 704 analyzer.

This test is based on a colourimetric method, which is formed ferrylmyoglobin, which results from reaction of metmyoglobin, a peroxidase, with H_2O_2. In turn, the ferrylmyoglobin reacts with 2,2'-azinobis (3-ehylbenzothiazoline-6-sulfonate) (ABTS), resulting a cationic radical ($ABTS^{\bullet+}$), which, due to its blue-green colour, is detected spectrophotometrically at 600 nm. The antioxidants presents in the samples cause inhibition of this colour production to a degree that is proportional to its concentration.

10. Western bloting analysis

The extracts were obtained from the left ventricle and were homogenized in 1.5ml of RIPA lysis buffer (150 mM NaCl; 50 mM Tris-HCl pH=8.0; 5 mM EGTA; 1% Triton X-100; 0.5% DOC; 0.1% SDS), supplemented with a protease inhibitor cocktail (1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 1 µg/mL chymostatin, 1 µg/mL leupeptin, 1µg/mL antipain and 5 µg/mL pepstatin A; Sigma-Aldrich) and centrifuged 3 times (15500 x g, 15 min., 4ºC).

The resulting supernatant fraction (corresponding to total extract) was collected and total protein concentration was determined using the bicinchoninic acid assay (BCA assay) (Smith et al., 1985) and supernatants were stored at -80º C until further use.

Samples were denatured at 95ºC for 5 min in sample buffer (0.5M Tris-HCl 0.5M (pH=6.8), 10% (w/v) SDS, 30% (v/v) glycerol, 0.6M DTT, 0.01% (w/v) bromophenol blue), for 6 repetitions and proteins were separated by SDS-polyacrylamide gel electrophoresis (190 V), according to Laemmli procedure (Laemmli U., 1970). Ten % acrylamide gels were used for all proteins analyzed except for PI3K (≈80 kDa) and IR-β (90 kDa) and MAPK (≈38kDa), which were separated using a lower acrylamide percentage (7.5%).

For immunodetection analysis, 25 to 90 µg of total protein were loaded and separated by electrophoresis on sodium dodecyl sulfate polyacrylamide gel electrophoresis, electroblotted (110V, 90 min.) onto a polyvinylidene difluoride (PVDF) membranes (Immobilon PVDF transfer membranes 0.45µm, Millipore, Madrid, Spain) and blocked with 1% bovine serum albumin (BSA) in phosphate buffer saline with 0.1% Tween-20 (PBS-T) for 1 hour at room temperature. Membranes were then incubated with primary antibody, overnight at 4ºC The membranes were washed extensively in 0.1% PBS-T and

then incubated for one hour at room temperature with adequate alkaline phosphatase conjugated secondary antibodies (Table 1). After secondary antibody incubation, membranes were washed for one hour in 0.1% PBS-T. To confirm equal protein loading and sample transfer blots were reprobed with either rabbit anti-β-actin or mouse anti-GAPDH.

After reaction with enhanced chemifluorescence detection reagent (ECF, GE Healthcare), immunoreactive bands were revealed by scanning blots using a Fluorescent image analyzer Typhoon FLA 900 (GE Healthcare Bio-sciences) imaging system. Densitometric analyses were performed using the Image Quant 5.0 software (Molecular Dynamics). Results were normalized against β-actin or GAPDH and then expressed as percentage of control.

Table 1. Primary and secondary antibodies used for Western-blot analysis.

Antibody	Molecular Weight (kDa)	Loading (µg)	Dilution	Reference	Company
Rabbit anti-RAGE	≈55	25	1:1000	Ab-3611	Abcam
Rabbit anti-HNE	-	50	1:1000	393207	Calbiochem
Rabbit anti-IR-β (C-19)	90	90	1:1000	sc-711	Santa Cruz Biotechnology
Rabbit anti-p85 Pi3K	80	90	1:1000	4292	Cell Signaling Technology
Rabbit anti-p38 MPAK	38	90	1250	2507	Biolegend
Rabbit anti-TNF-α	≈26	75	1:600	Ab6671	Abcam
Rabbit anti-β-actin	42	-	1:5000	A 5441	Sigma life sciences
Mouse anti-GAPDH	38	-	1:5000	Ab9484	Abcam
Goat anti-mouse	-	-	1:5000	A 3562	Sigma life sciences
Goat anti-rabbit	-	-	1:5000	1317	GE Healthcare

11. Heart gene expression analysis

11.1. Total RNA isolation

The heart was isolated in autopsy and stored in RNA later™ solution (Ambion, Austin, TX, USA). Samples were removed from preservation solution and 1200 μL of RLT Lysis Buffer were added to proceed with disruption and homogenization for 2 min. at 30 Hz using TissueLyser (Qiagen, Hilden, Germany). Tissue lysate were processed according to the protocol from RNeasy® Mini Kit (Qiagen, Hilden, Germany). Total RNA was eluted in 50 μL of RNase-free water (without optional treatment with DNAse). In order to quantify the amount of total RNA extracted and verify RNA integrity (RIN, RNA Integrity Number), samples were analyzed using 6000 Nano Chip® kit, in Agilent 2100 bioanalyzer (Agilent Technologies, Walbronn, Germany) and 2100 expert software, following manufacturer instructions. The yield from isolation was from 0.5 to 3 μg; RIN values were 6.0–9.0 and purity (A_{260}/A_{280}) was 1.8-2.0.

11.2. Reverse transcription

RNA was reverse transcribed with SuperScript™ III first-strand synthesis system for RT-PCR (Invitrogen, California, USA). One microgram of total RNA was mixed with a 2× First-Strand Reaction Mix and a SuperScript™ III Enzyme Mix (Oligo(dT) plus Random hexamers). Reactions were carried out in a thermocycler Gene Amp PCR System 9600 (Perkin Elmer, Norwalk, CT, USA), 10 min. at 25°C, 50 min. at 50°C and 5 min. at 85°C. Reaction products were then digested with 1 μL RNase H for 20 min. at 37°C and, finally, cDNA eluted to a final volume of 100 μL and stored at -20°C.

11.3. Relative quantification of gene expression

Relative quantification of gene expression was performed using 7900 HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA). A normalization step preceded the gene expression quantification, using geNorm Housekeeping Gene Selection kit for *Rattus norvegicus* (Primer Design, Southampton, UK) and geNorm software (Ghent University Hospital, Center for Medical Genetics, Ghent, Belgium) to select optimal housekeeping genes for the study samples (Vandesompele et al., 2002). Real-time PCR reactions used specific QuantiTect Primer Assays (Qiagen, Hilden, Germany) with optimized primers (Table 2) for TGF-β1 as proliferative markers; BNP, CTGF, TSP-1 and Procollagen III as fibrotic/ hypertrophic; VEGF as an angiogenesis marker; ICAM-1, VCAM-1, and iNOS as indicator of endothelial lesion; IL-6 and TNF-α as inflammatory markers; and Bax and Bcl-2 as apoptotic indicators; Beta (β)-actin (QT00193473) was also used as endogenous control with QuantiTect SYBR Green PCR Kit Gene expression (Qiagen, Hilden, Germany) according to manufacturer's instructions. RT-qPCR reactions were carried out with 100 ng cDNA sample, primers (50–200 nM) and 1X QuantiTect SYBR Green PCR Master Mix. Nontemplate control reactions were performed for each gene, in order to assure no unspecific amplification. Reactions were performed with the following thermal profile: 10 min. at 95°C plus 40 cycles of 15 s at 95°C and 1 min. at 60°C. Real-time PCR results were analyzed with SDS 2.1 software (Applied Biosystems, Foster City, CA, USA) and quantification used the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Table 2. Primer sequences for real-time RT-PCR.

Gene	Primer sequence
BAX	Forward: 5'-CCAAGAAGCTGAGCGAGTGTCTC-3' Reverse: 5'-AGTTGCCATCAGCAAACATGTCA-3'
Bcl-2	Forward: 5'-GGAGCGTCAACAGGGAGATG-3' Reverse: 5'-GATGCCGGTTCAGGTACTCAG-3'
BNP	Forward: 5'-GGGCTGTGACGGGCTGAG GTT-3' Reverse: 5'-AGTTTGTGCTGGAAGTAAGA-3'
CTGF	Forward: 5'-CGTAGACGGTAAAGCAATGG-3' Reverse: 5'-AGTCAAAGAAGCAGCAAACAC-3'
ICAM-1	Forward: 5'-TTCAACCCGTGCCAGGC-3' Reverse: 5'-GTTCGTCTTTCATCCAGTTAGTCT-3'
IL-6	Forward: 5'-ACCACTTCACAAGTCGGAGG-3' Reverse: 5'-ACAGTGCATCATCGCTGTTC-3'
iNOS	Forward: 5'-CAGAAGCAGAATGTGACCATCAT-3' Reverse: 5'-CGGAGGGACCAGCCAAATC-3'
Procollagen typeIII	Forward: 5'-5'-GGTCACTTTCACTGGTTGACGA-3' Reverse: 5'-TTGAATATCAAACACGCAAGGC-3'
SOD	Forward: 5'-GACAAACCTGAGCCCTAACGG-3' Reverse: 5'-CTTCTTGCAAACTATG-3'
TGF-β1	Forward: 5'-ATACGCCTGAGTGGCTGTCT-3' Reverse: 5'-TGGGACTGATCCCATTGATT-3'
TNF-α	Forward :5'-CACGCTTTCTGTCTACTGA-3' Reverse 5'-GGACTCCGTGATGTCTAAGT-3'
TSP-1	Forward :5'-CTTTGCTGGTGCCAAGTGTA-3' Reverse: 5'-CGACGTCTTTGCACTGGATA-3'
TRB3	Forward:5'-TGATGCTGTCTGGATGACAA-3' Reverse: 5'-GTGAATGGGGACTTTGGTCT-3'
VCAM-1	Forward: 5'-GAAGCCGGTCATGGTCAAGT-3' Reverse: 5'-GACGGTCACCCTTGAACAGTTC-3'
VEGF	Forward: 5'-GAGAATTCGGCCCCAACCATGAACTTTCTGCT-3' Reverse: 5'-GAGCATGCCCTCCTGCCCGGCTCACCGC-3'

12. Statistical Analysis

Results were expressed as means ± standard errors of the mean (S.E.M.) and % of the Control, as indicated. Comparisons between groups were analysed by unpaired Student´s t-test, using GraphPad Prism software, Version 5.0. Differences were considered to be significant at $P < 0.05$.

V. RESULTS

1. Metabolic characterization of animal model

1.1. Evolution of body weight

Body weight (BW) was monitored during the 9 weeks of treatment. The HSu-treated rats showed a BW profile similar to that of the control animals (Figure 9).

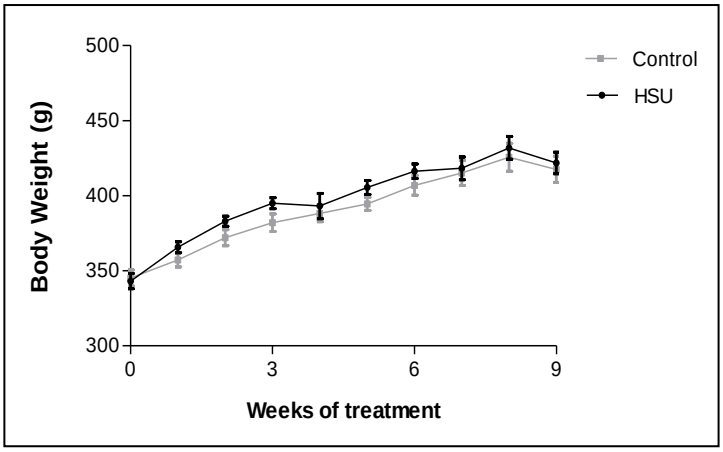


Figure 9. Body weight evolution of the Control and HSu groups throughout the 9 weeks of treatment. Values are expressed as mean ± SEM; n=7 for Control and n=8 for HSu.

1.2. Glycemic profile

1.2.1. Fasting and fed glycemia

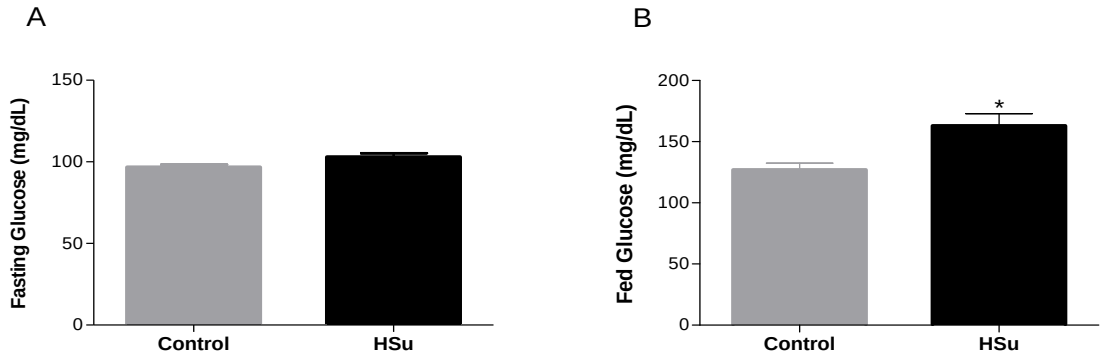


Figure 10. Fasting (A) and fed (B) glycemia values at the final time (9 weeks of treatment). Data are expressed as means ± SEM; n=7 per group. *p < 0.05 *vs* Control.

There was no significant difference in fasting blood glucose between the two groups (102.90 ± 6.98 *vs* 96.71 ± 4.49 mg/dL) (Figure 10A); however, in a fed state, glycemia was significant elevated for the HSu group compared to the Control one (162.90 ± 26.54 *vs* 126.80 ± 13.56 mg/dL; $p<0.05$) (Figure 10B).

1.2.2. Glycated hemoglobin (HbA1c)

Concerning HbA1c, a slight but significantly increased value was noted in the HSu group when compared with the Control (Figure 11).

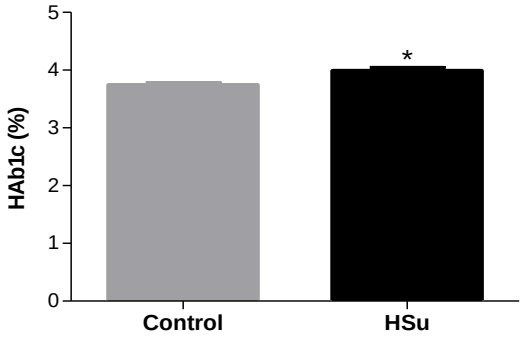


Figure 11. Glycated hemoglin (HbA1c) values in the Control group and HSu-treated rats. Data are expressed expressed as means ± SEM; n=7 for Control and n=8 for HSu. *p < 0.05 *vs* Control.

1.2.3. Glucose tolerance test (GTT)

Basal blood glucose concentrations were similar in Control and HSu groups. After 15 min of glucose injection, both groups achieved the maximum blood glucose level. However, 60 min. after of glucose stimulation, the HSu-treated group showed significantly increased blood glucose levels when compared with those of the Control group (215.50 ± 19.64 *vs* 159.00 ± 5.83 mg/dl; $p<0.05$). This difference persisted until the 120 min., when the blood glucose concentrations returned to the basal levels in the Control group, but remained significantly higher in the HSu-treated group (168.80 ± 10.64 *vs* 121.40 ± 3.69

mg/dL; p<0.01) (Figure 12A). Figure 12B represents the area under the curve (AUC) of GTT, which expresses blood glucose bioavailability, thus confirming the significantly (p<0.05) impaired blood glucose tolerance in the HSu group *vs* Control.

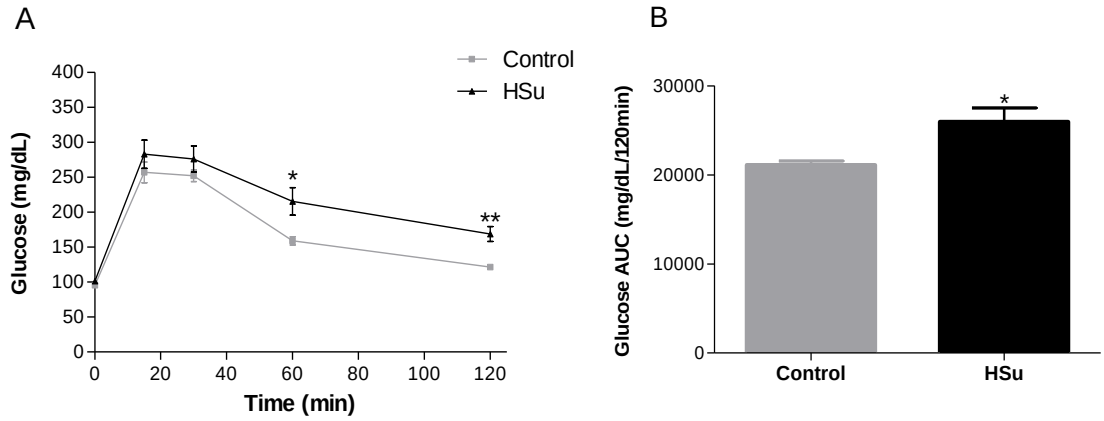


Figure 12. Glucose tolerance test (GTT) was performed during the last week of treatment. (A) Blood glucose levels at baseline and after i.p. injection of glucose (2 g/kg BW) at 15, 30, 60 and 120 min in Control and HSu groups. (B) Area under the curve (AUC) of total blood glucose after injection of glucose. Data are expressed as means ± SEM; n=6 per group. *p < 0.05; **p<0.01 *versus* Control.

1.3. Insulinemic profile and insulin tolerance/resistance

1.3.1. Fasting insulin levels

Serum insulin concentration in HSu-treated rats was significantly elevated when compared with the Control rats (10.83 ± 1.00 *vs* 3.74 ±1.84 µg/L; p<0.001) (Figure 13).

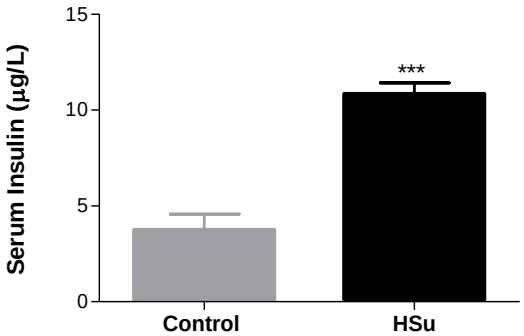


Figure 13. Serum insulin levels after 9 weeks of treatment. Data are expressed as mean ± SEM; n=5 for Control and n=3 for HSu. ***p < 0.001 versus Control.

1.3.2. Insulin tolerance test (ITT)

After sucrose consumption during 9 weeks, insulin tolerance was assessed by the ITT (Figure 14). The blood glucose levels 120 min. after insulin injection were significantly higher in the HSu group than in the Control one (63.86 ± 14.78 *vs* 37.17 ± 7.03 mg/dL; p<0.01).

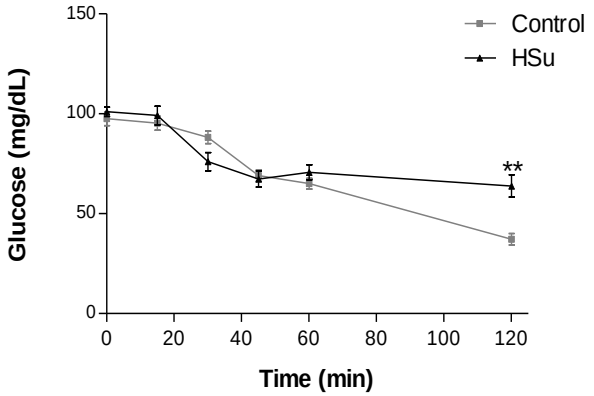


Figure 14. Insulin tolerance test (ITT) was performed during the last week of treatment. Blood glucose levels at baseline and after i.p. injection of insulin (0.75 U/kg BW) at 15, 30, 45, 60 and 120 min. in Control and HSu groups. Data are expressed as mean ± SEM; n=7 for Control and n=6 for HSu. **p<0.01 versus Control.

1.3.3. HOMA-IR index

HOMA-IR index was also calculated as an additional measure of insulin resistance (Figure 15), using glucose and insulin levels after a fasting state. Significantly higher (P<0.01) HOMA-IR values were observed in the HSu- treated group when compared to the Control.

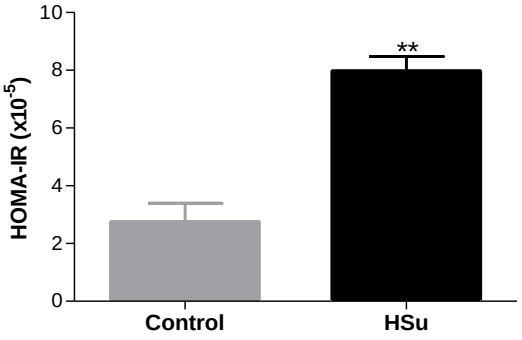


Figure 15. HOMA-IR index in Control and HSu groups. Data are expressed as mean ± SEM n=5 for Control and n=3 for HSu. **p<0.01 *vs* Control.

2. Heart insulin signaling/resistance

2.1. Cardiac IR-β expression

No differences were observed on cardiac IR-β expression after 9 weeks of sucrose consumption (Figure 16).

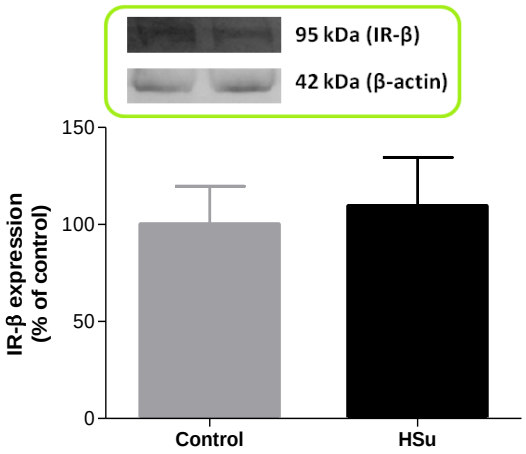


Figure 16. Cardiac IR-β expression in the Control and HSu-treated rats. Representative Western-blot bands are shown on top of bars. β-actin was used as the internal control. Results are expressed as percentage of the control and presented as means ± SEM. n=3 for Control and n=4 for HSu.

2.2. Cardiac PI3K and MAPK expression

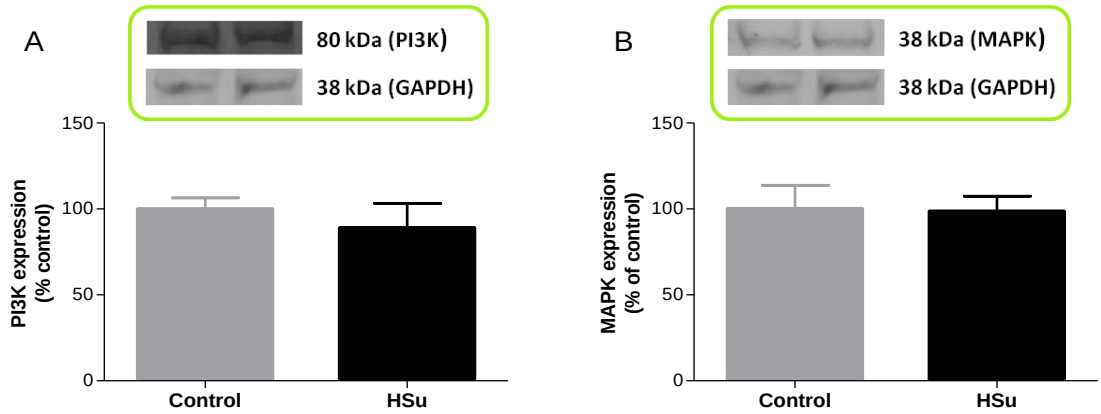


Figure 17. Cardiac PI3K and MAPK expression in the Control and HSu-treated rats. Representative Western-blot bands are shown on top of bars. GAPDH was used as the internal control. Results are expressed as percentage of the control and expressed as means ± SEM. n=3 for Control and n=4 for HSu.

The PI3K and MAPK contents, proteins involved in the insulin signaling cascade, were also analyzed. The Control and HSu-treated rats presented identical PI3K and MAPK expression in the heart tissue (Figure 17A and B).

2.3. Cardiac TRB3 mRNA expression

TRB3 has been implicated in heart insulin resistance and the mRNA expression in the cardiac tissue was assessed as an additional measure of heart insulin resistance. There was no significant difference on the heart TRB3 mRNA expression between groups (Figure 18).

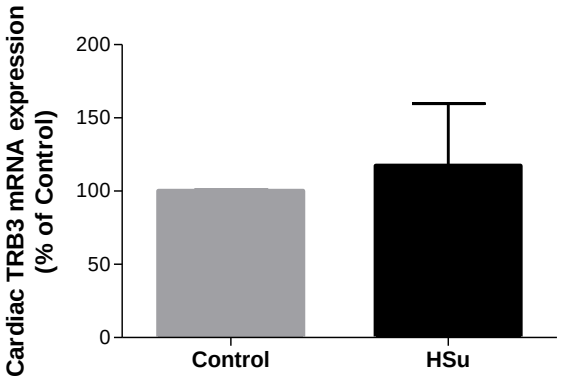


Figure 18. Cardiac TRB3 mRNA expression in the Control and HSu-treated rats. β-actin was used as the internal control. Results are expressed as percentage of the control and means ± SEM; n= 5 per group.

3. Lipid profile

3.1. Serum triglyceride and total-cholesterol levels

The HSu group showed elevated serum triglyceride contents compared to the Control (Figure 19A), while no significant difference was observed on total-cholesterol concentration between the two groups (Figure 19B).

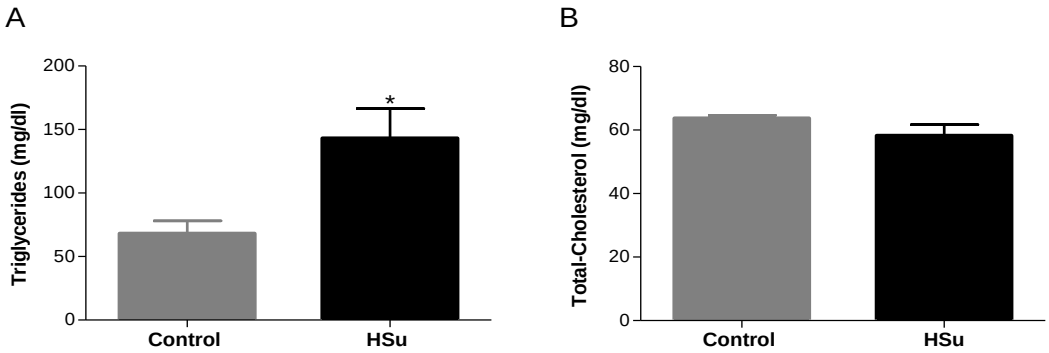


Figure 19. Serum triglycerides (A) and total-cholesterol (B) levels in Control group and HSu-treated groups after 9 weeks of treatment. Data are expressed as mean ± SEM, n=7 per group. *p<0.05 *vs* Control.

4. Cardiac "(dys)function"

4.1. Blood pressure and heart rate

No significant differences were found in systolic, diastolic and mean blood pressure, as well as in heart rate, between the two groups at the final time (Table 3).

Table 3. Blood pressure and heart rate values in Control and HSu rats after 9 weeks of experiment.

Parameter	Control (n=7)	HSu (n=8)
SBP (mmHg)	122.0 ± 1.8	121.6 ± 1.1
DBP (mmHg)	100.1 ± 1.5	98.4± 1.3
MBP (mmHg)	108.8 ± 1.3	109.8 ± 1.2
Heart Rate (beats/min)	356.7 ± 1.0	368.9 ± 8.1

Data are expressed as mean ± SEM.
SBP, systolic blood pressure; DBP, diastolic blood pressure; MBP, mean blood pressure.

4.2. Heart and LV trophism

The parameters of cardiac trophism were evaluated at the final time of experiment. Despite an elevated value of heart weight in the HSu-treated rats *versus* Control, similar values between groups were encountered. However, LV weight and LVW/BW were significantly higher in the HSu-treated rats versus the Control ones ($p<0.01$ and $p<0.001$, respectively) (Table 4).

Table 4. Parameters of cardiac trophism in Control and HSu rats at the final time of study (9 weeks).

Parameter	Control	HSu
Heart (g)	0.89 ± 0.09	1.10 ± 0.02*
LV (g)	0.45 ± 0.00	0.53 ± 0.02***
Heart / BW (g/kg)	2.14 ± 0.23	2.62 ± 0.07
LV / BW (g/kg)	1.07 ± 0.02	1.28 ± 0.04**

Data are expressed as mean ± SEM, *P<0.05; ** P<0.01; ***P<0.001 versus Control.
LV, left ventricular mass, BW, body weight.

5. Markers of fibrosis and hypertrophy/proliferation

5.1. Serum TGF-β1 levels

There was no significant difference in serum TGF-β1 levels between the two groups at the final time of experiment (Figure 20).

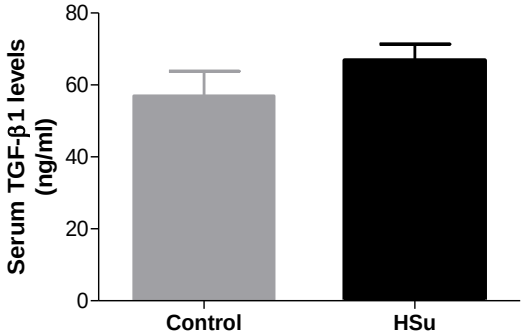


Figure 20. Serum TGF-β1 levels in the Control and HSu-treated rats. Data are expressed as mean ± SEM; n=7 for Control and n=8 for HSu group.

5.2. Cardiac mRNA expression

To assess the influence of sucrose consumption on heart proliferation, hypertrophy and fibrosis, the following proteins were evaluated: TGF-β1, TSP-1, pro-collagen III, CTGF, BNP. No significant changes were encountered concerning the cardiac mRNA expression of TGF-β1, TSP-1, pro-collagen III and CTFG (Figure 21A and B). However, in the heart tissue, significantly BNP gene overexpression was found in HSu-treated animals ($p<0.01$) (Figure 21C).

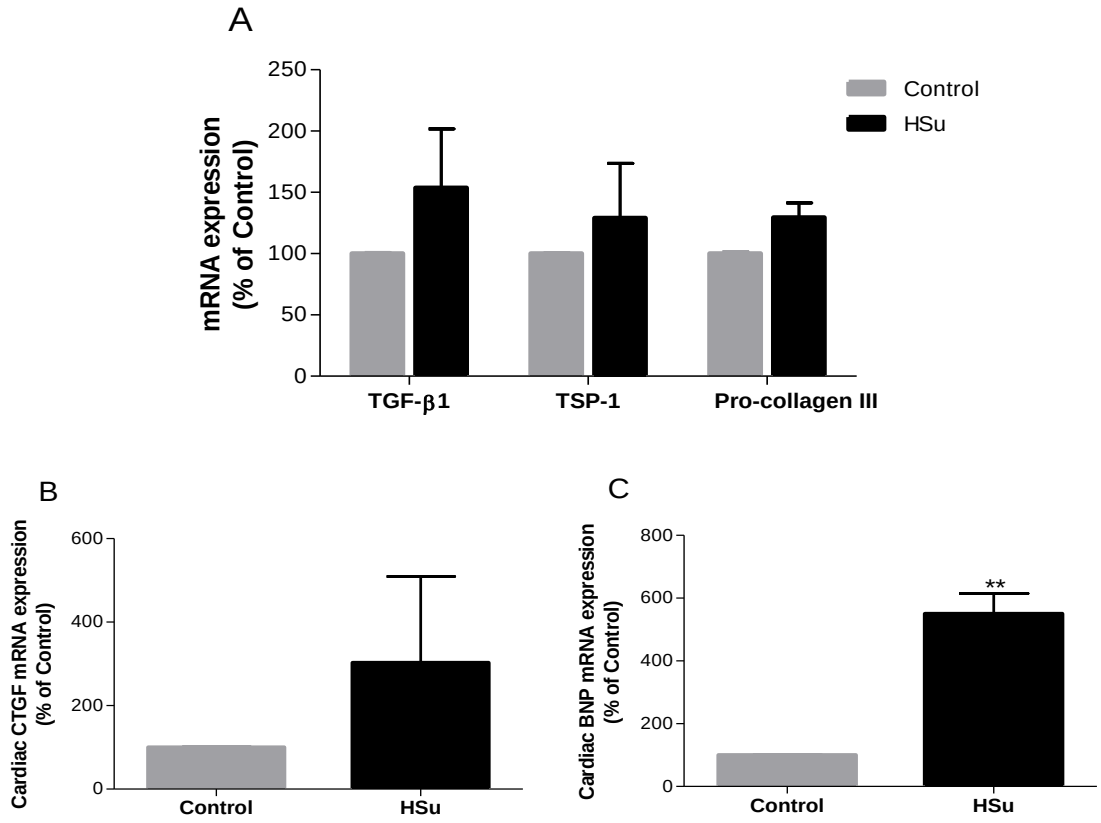


Figure 21. Cardiac TGF-β, TSP-1, Pro-collagen III (A), CTGF (B) and BNP (C) mRNA expression at the final time (9 weeks) in the Control and HSu-treated rats. B-actin was used as the internal control. Data are expressed as percentage of the control and expressed as means ± SEM; n=5 per group. **P<0.01 versus Control.

6. Markers of apoptotic machinery

Concerning the apoptotic machinery markers, no changes were encountered in the heart tissue for BAX and Bcl-2 mRNA expression in the HSu rats *vs* the Control ones (Figure 22).

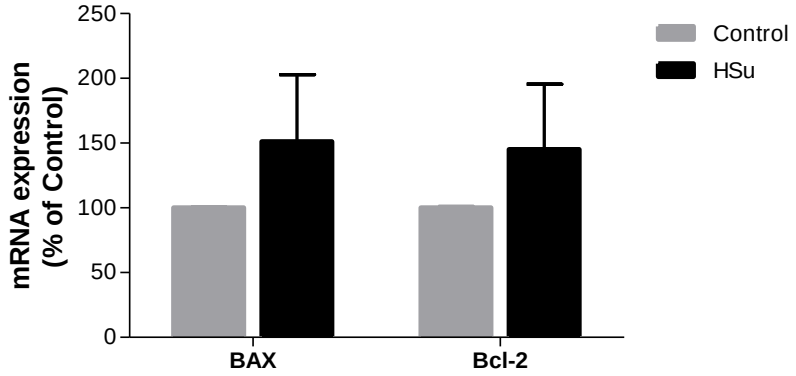


Figure 22. Cardiac BAX and Bcl-2 mRNA expression at the final time (9weeks) in the Control and HSu-treated rats. β -actin was used as the internal control. Results are expressed as percentage of the control and expressed as means ± SEM; 5 per group.

7. Marker of angiogenesis and endothelial lesion

7.1. Serum VEGF concentrations

The HSu-treated rats showed significantly ($p<0.05$) elevated values of serum VEGF *vs* the Control group (Figure 23).

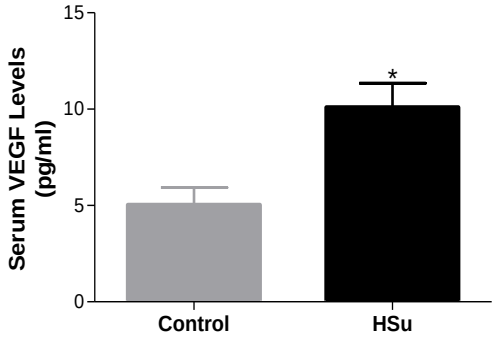


Figure 23. Serum vascular endothelial growth factor (VEGF) in the Control and HSu-treated rats. Data are expressed as mean ± SEM; n=7 for Control and n=8 for HSu group. *P<0.05 vs Control.

7.2. Cardiac mRNA expression

VEGF, iNOS, ICAM-1 and VCAM-1 gene expression was assessed as markers of putative changes on angiogenesis and endothelial lesion. Unchanged values were found for VEGF (Figura 24A), iNOS (Figura 24B), ICAM-1 and VCAM-1 (Figure 24C) mRNA expression, despite a trend to higher values for all the genes vs Control.

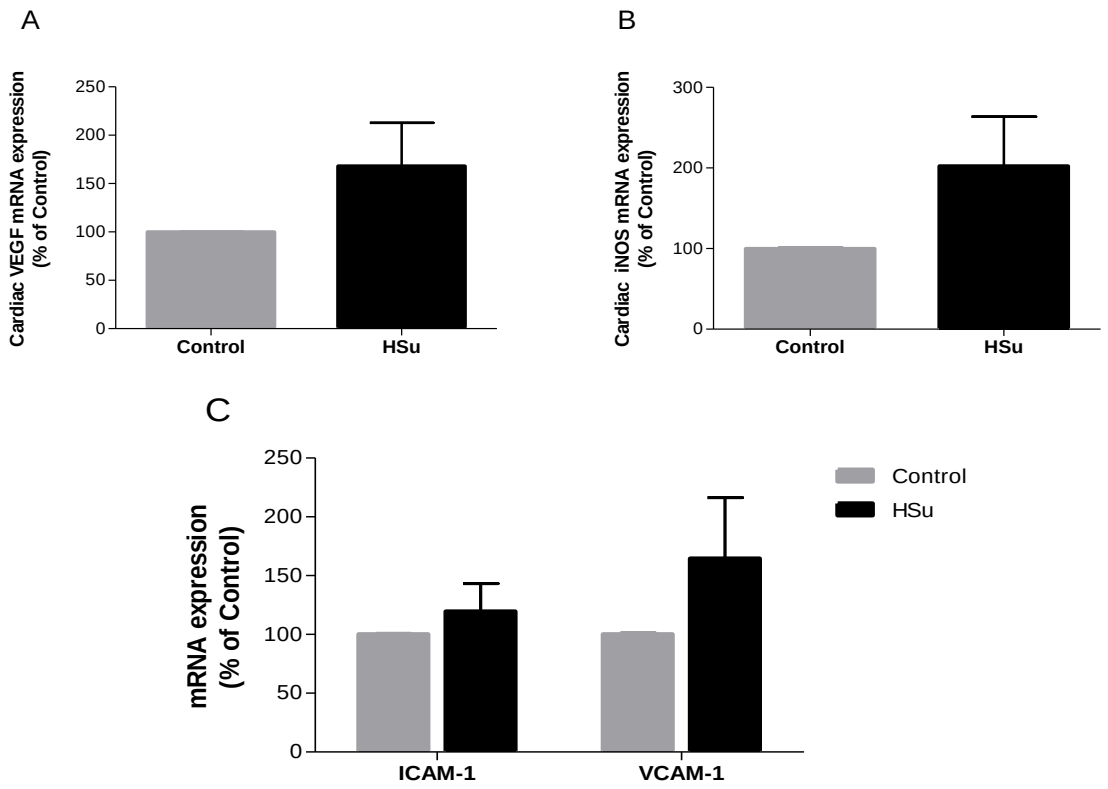


Figure 24. Cardiac VEGF (A), iNOS (B), ICAM-1 and VCAM-1 (C) mRNA expression at the final time (9 weeks) in the Control and HSu-treated rats. β-actin was used as the internal control. Results are expressed as percentage of the control and expressed as means ± SEM; 5 per group.

8. Inflammatory mediators

8.1. Serum concentrations

There were no differences in serum IL-6 and IFN-γ values between the two groups at the final time (Figure 25A and B). Concerning the serum CRP levels, a statistically significant decrease ($p<0.01$) was observed in the HSu group when compared with the Control (Figure 25C).

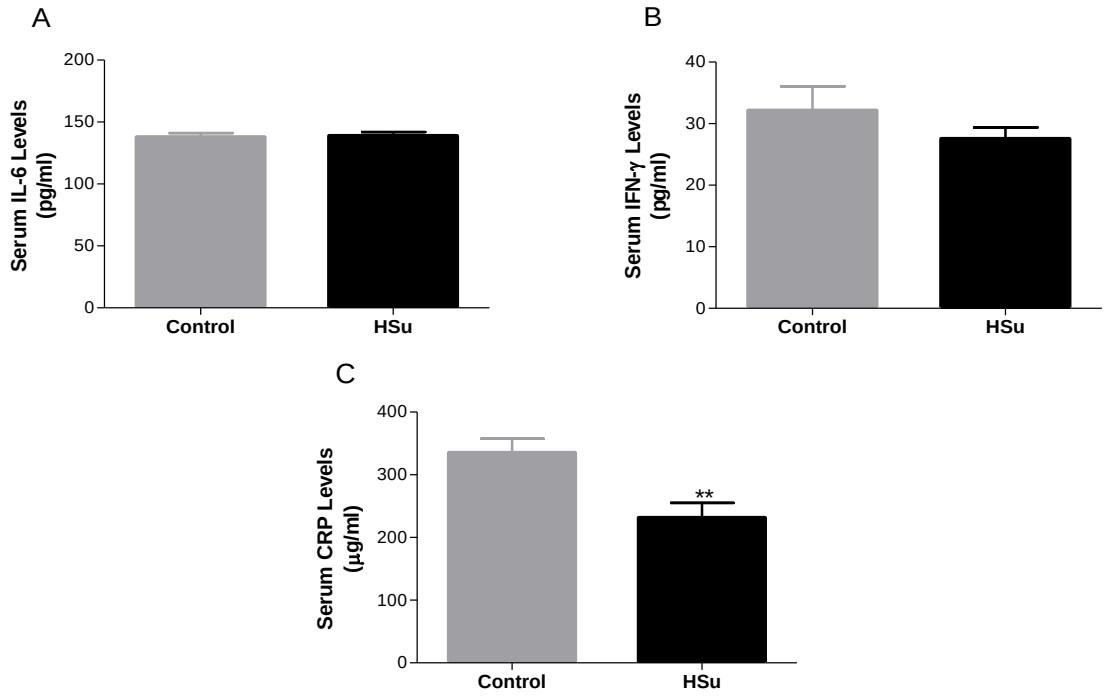


Figure 25. Serum IL-6 (A), IFN-γ (B) and CRP (C) levels in the Control and HSu-treated rats. Data are expressed as mean ± SEM; n=7 for Control and n=8 for HSu group. **P<0.01 versus Control.

8.2. Cardiac IL-6 and TNF-α mRNA expression

The influence of sucrose on the heart IL-6 and TNF-α was also assessed. Concerning the sucrose-treated rats, a trend to decreased TNF-α mRNA expression was observed but the values did not read statistical significance (Figure 26).

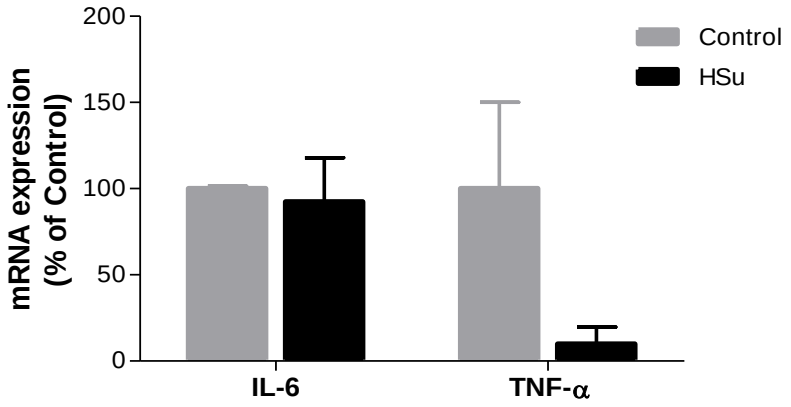


Figure 26. Cardiac mRNA expression of inflammatory marker at the final time (9 weeks) in the Control and HSu-treated rats. β-actin was used as the internal control. Results are expressed as percentage of the control and expressed as means ± SEM; n= 5 per group.

8.3. Cardiac TNF-α protein expression

TNF-α adducts were detected in a broad range of different molecular weight. Although no significant differences were observed for each adduct between groups, the TNF-α content showed a trend to increase in HSu-treated rats (Figure 27).

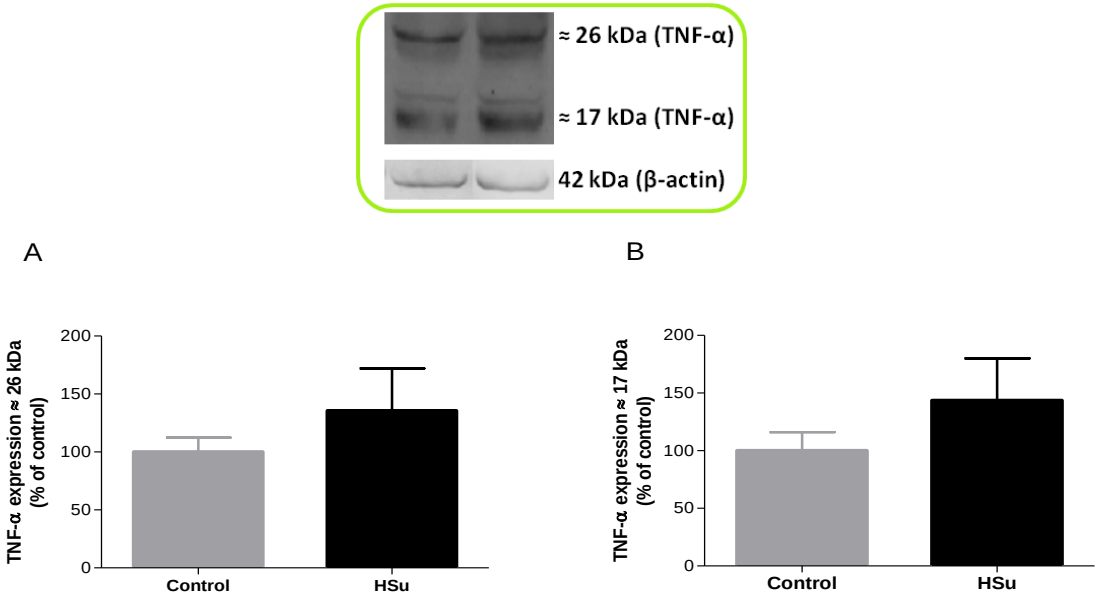


Figure 27. Cardiac TNF-α expression in the Control and Hsu-treated rats. Representative Western-blot bands are shown on top of bars. β-actin was used as the internal control. Results are expressed as percentage of the control and expressed as means ± SEM. n=3 for Control and n=5 for Hsu.

9. Redox status

9.1. Serum and heart lipid peroxidation (TBARS)

Malondialdehyde concentrations were evaluated in serum and cardiac tissue at the end of experiment. No differences were found between groups; however, both in serum and cardiac tissue MDA levels showed the same profile, with a trend trend to increased values in the HSu-treated rats *vs* the Control ones (Figure 28A and B).

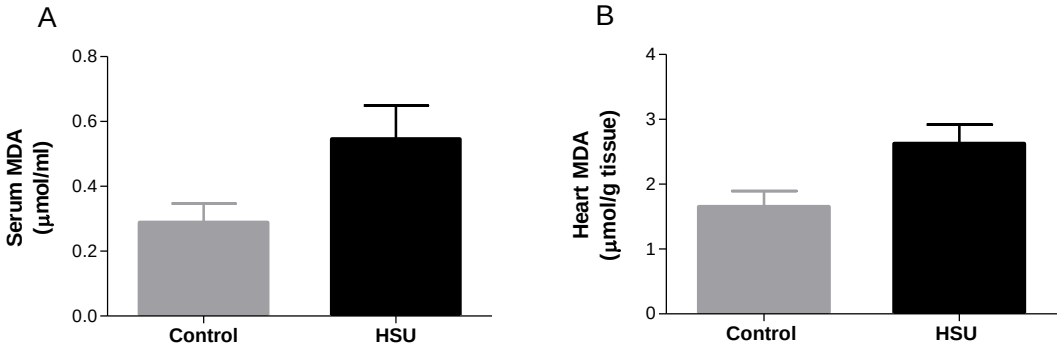


Figure 28. Malondialdehyde (MDA) concentrations in serum (A) and in the cardiac tissue (B). Data are expressed as mean ± SEM, n=7 for Control and n=8 for HSu group.

9.2. Serum total antioxidant status (TAS)

The value of total antioxidant capacity (TAS) was significantly higher ($P<0.05$**)** in the HSu group when compared with the Control (Figure 29).

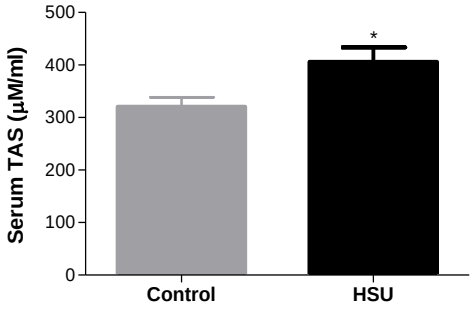


Figure 29. Serum total antioxidant status (TAS) in the Control and HSu-treated rats. Data are expressed as mean ± SEM; n=7 for Control and n=8 for HSu group. *P<0.05 versus Control.

9.3. Serum TBARS/TAS ratio

No significant differences were encountered for MDA/TAS between the groups (Figure 30).

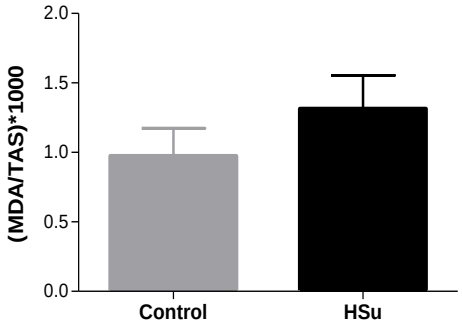


Figure 30. MDA/TAS ratio in the Control and HSu-treated rats. Data are expressed as mean ± SEM; n=7 for Control and n=8 for HSu group. *P<0.05 versus Control.

9.4. Cardiac HNE expression

HNE adducts were detected in a broad range of different molecular weights. Only HNE-adduct weighing ≈30 kDa was significantly ($p<0.05$) decrease in the HSu group (Figure 31C). However, although no significant difference were observed in HNE adducts with weight ≈70 kDa and ≈ 40 kDa between Control and HSu rats, both showed an identical profile to that found for the HNE- adduct ≈30 kDa, with a trend to decrease in the HSu-treated group (Figure 31A and B).

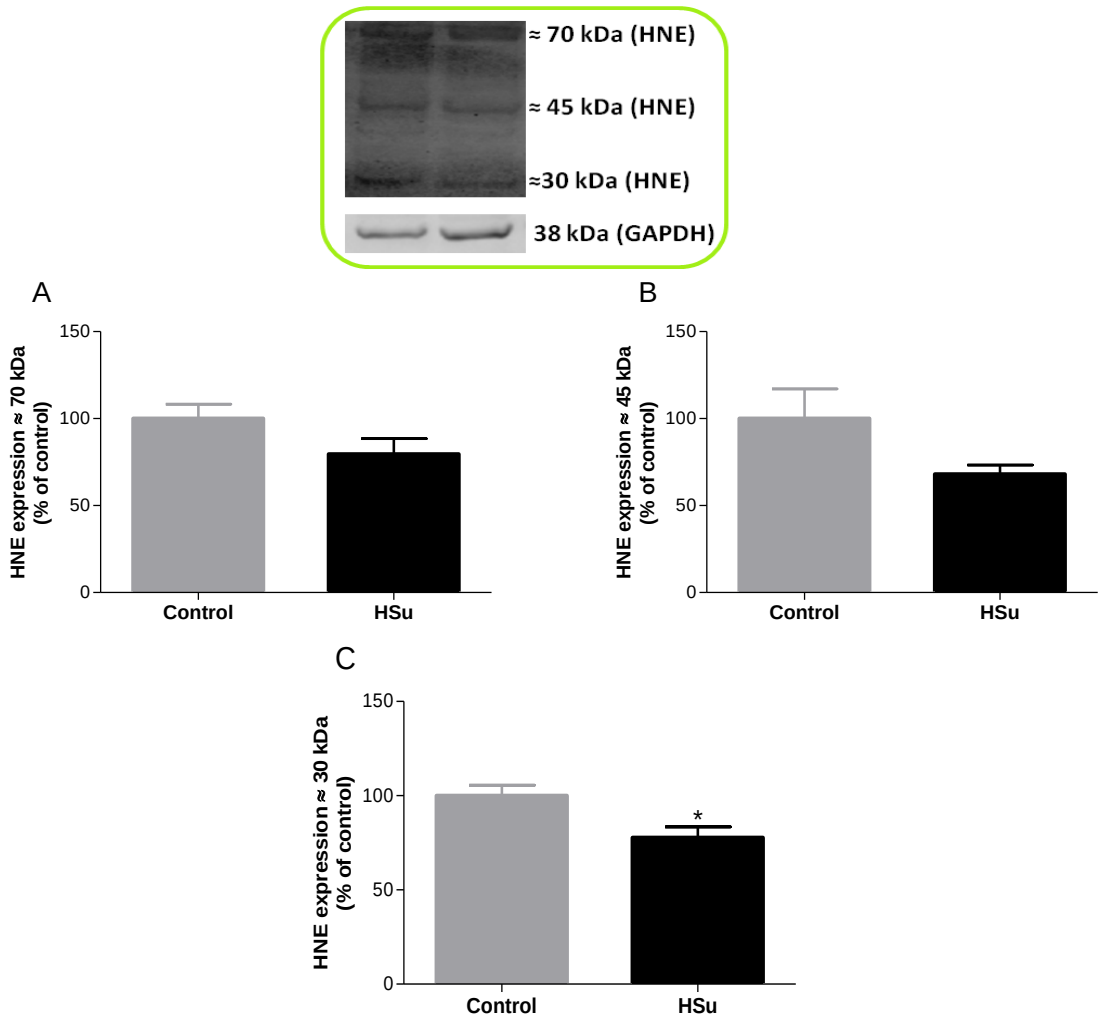


Figure 31. Cardiac HNE expression in the Control and HSu-treated rats. Representative Western-blot bands are shown on top of bars. GAPDH was used as the internal control. Results are expressed as percentage of the control and expressed as means ± SEM. n=3 for Control and n=4 for Hsu. *P<0.05 versus Control.

9.5. Cardiac RAGE expression

A significant decrease ($p<0.01$) of RAGE expression was observed 9 weeks after sucrose consumption *vs* the untreated Control rats (Figure 32).

.

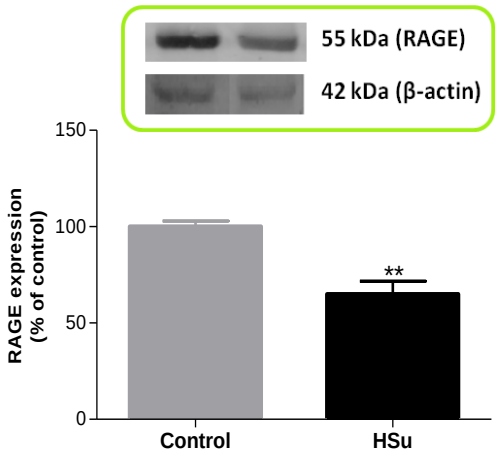


Figure 32. Cardiac RAGE expression in the Control and HSu-treated rats. Representative Western-blot bands are shown on top of bars. β-actin was used as the internal control. Results are expressed as percentage of the control and expressed as means ± SEM. n=3 for Control and n=5 for HSu. **P<0.01 versus Control.

9.6. Cardiac SOD mRNA expression

Concerning the sucrose treatment, a remarkable increment ($p<0.05$) in cardiac SOD mRNA expression was observed (Figure 33).

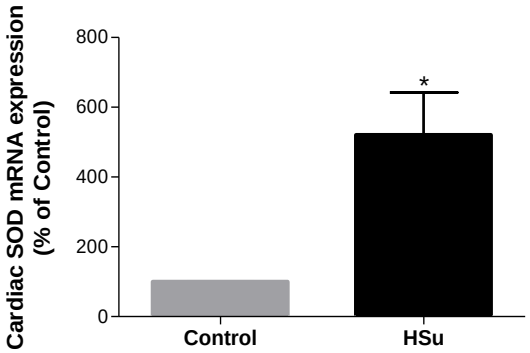


Figure 33. Cardiac SOD mRNA expression at the final time (9weeks) in the Control and HSu-treated rats. GAPDH was used as the internal control. Results are expressed as percentage of the control and expressed as means ± SEM. n=3 for Control and n=5 for HSu. *P<0.05 versus Control.

VI. DISCUSSION

The global prevalence of DM is increasing very fast; currently the number of diabetic people is over 300 million and expected to reach close to 500 million within 20 years (Silink, 2009; Acar et al., 2011). Cardiovascular diseases are responsible for three quarters of the deaths among this population (Khavandi et al., 2009). Although CAD is very common, HF is also a major cause of mortality and morbidity in patients with DM (Cohen-Solal et al., 2008). In addition, diabetic individuals are under increased risk for HF development after adjusting concomitant risk factors, such as hypertension and CAD (Murarka and Moahed, 2010; Bertoni et al., 2003, Boundina and Abel, 2010). The Framingham study, United Kingdom Prospective Diabetic Study and Euro Heart Failure Survey all suggested that the presence of diabetes may independently increase the risk of developing HF. Up to 75% of patients with unexplained idiopathic dilated cardiomyopathy were found to be diabetic (Bertoni et al., 2003).

The natural history of prediabetes predicts that a significant number of persons with the condition progress to diabetes in the long run (Colagiuri, 2011). The most common definition of prediabetes refers to IGT and/or IFG, which were conceived to define categories of glycemia associated with an increased risk of developing diabetes (Buysschaert and Bergman, 2011). In the earlier stage, i.e. in prediabetic state, T2DM is almost or even totally asymptomatic, complicating its diagnosis. However, many of complications associated with the disease, specifically the cardiovascular ones, appear in that phase. Furthermore, late diagnosis is associated with a worsening of the disease and, consequently, evolution of its complications. Thus, early screening of patients with subclinical cardiac alterations is useful to prevent the development of diabetic cardiomyopathy and, therefore, reduce heart disease progression. This need is even more important when considering that the prevalence of children and adolescent diabetes is also increasing, particularly due to premature obesity.

An understanding of the cellular effects of these metabolic disturbances on cardiomyocytes in the prediabetic state should be useful in predicting the structural and functional cardiac consequences. The problem lies in our ability to dissect away confounding variables such as concomitant obesity, dyslipidemia, hypertension, and proinflammatory and prothrombic states. All of the confounding variables listed, which are components of metabolic syndrome, are commonly present in persons with prediabetes (Ferrannini et al., 2011; Grundy, 2012).

Furthermore, the discussion of whether the glucose or the insulin resistance is per se, or both, the driving forces for the cardiovascular and the cardiac changes starting before the diagnosis, is also relevant. Insulin exerts multiple effects in the heart that collectively have a beneficial effect on myocardial substrate metabolism and cardiac function. Myocardial insulin resistance accompanies cardiac dysfunction in diabetes-related heart disease (Ouwens and Diamant, 2007). Interventions aimed at ameliorating myocardial insulin sensitivity have indicated beneficial effects on insulin sensitivity and cardiac function in rodent models (Abel, 2005) and may constitute a major therapeutic target in the battle against diabetes-related heart disease. Thus, in a state of normoglycemia and insulin resistance with hyperinsulinemia, the relative role of glucose and of insulin remains to be determined. Insulin resistance is a risk factor of left ventricular dysfunction and HF, and is one of the hallmarks of type 2 diabetes (Doehner et al., 2005; Dinh et al, 2011; Stahrenberg et al., 2010). However, despite whole body hyperinsulinemia and hyperglycemia, the diabetic heart relies almost entirely on FA utilization at the expense of glucose (Carley and Severson, 2005). This change in substrate utilization has been described in rodent models as well as in humans and can be largely attributed to nutrition of excessive fat containing food (Dirkx et al., 2011). Rodents can be made diabetic by prolonged periods of exposure to a high fat diet (Bugger and Abel, 2011).

To elucidate whether the alteration at cardiac level are already present in prediabetic stage, and the underlying mechanisms, we must chose an animal model that presents with relatively minor metabolic abnormalities, consistent with prediabetic insulin resistance in humans (Pagliassotti et al., 1996; Podolin et al., 1998), and that mimics early stages of type 2 diabetes, without other complicating factors that could lead to cardiac events (e.g. obesity and hypertension). Experimental support is limited with most work focusing on chemically induced type 1 models of the disease that ultimately represent an extreme of the metabolic profile (rarely encountered in clinical practice) or type 2 models burdened with other element(s) of the metabolic syndrome and, thus, the evidence is scarce regarding the mechanisms involved in cardiomyopathy in this stage of disease. Therefore, animal models of prediabetes could help in the understanding of the events leading to the appearance of clinical symptoms.

The high-sucrose diet rat as a model of prediabetes/insulin resistance

In agreement to the previous topics, we decided to choose the high-sucrose diet rat as a model of prediabetes to evaluate the insulin resistance induced-cardiac dysfunction, consisting of 9 weeks of 35% sucrose diet, according to previous studies of Ribeiro et al. (2005) and Conde et al. (2011). The insulin resistance described in this animal model is associated with glucose intolerance, increased serum insulin concentration (hyperinsulinemia) and decreased insulin sensitivity, as described by other authors (Kanazawa et al., 2003; Ribeiro et al., 2005; Conde et al., 2011; Sumiyoshi et al., 2011).

First of all, concerning the metabolic effects of this diet, we must clarify that we supplemented a standard non purified diet with a sucrose solution that replaces water in the diet. Although the main aim was to assess the effect of adding sucrose to the diet, if the rest

of the diet remains constant, the percentage of total energy contributed by components other than the added sugar decreases proportionately and there may be an increase in total energy intake. Thus, higher energy intakes may also have contributed to differences between the HSu and Control groups other than sucrose *per se*. Animals were given *ad libitum* access to food and drink, so they may consume different amounts of energy, which may also confound and influence the response to the diet.

Our data demonstrate that sucrose consumption induced no significant changes on body weight, which was unchanged compared to the Control rats. These results indicate that insulin resistance produced in this model is, at least initially, independent of the overt obesity, which is consistent with previous studies (Kanazawa et al., 2003). On the other hand, Song et al. (2007), showed higher body weight and greater body weight gain in HSu rats compared with Control during 16 week of exposure.

It is important to note the fact sucrose is a disaccharide that consists of 50:50 of glucose and fructose that undergo different metabolic pathways after absorption from the small intestine. The higher postprandial/fed glycemia values can be explained by the glucose availability resultant from both sucrose and diet. On the other hand, the rise in glucose level can partly explain the large increase in postprandial insulin level on this diet. However, we were unable to evaluate serum insulin levels in a postprandial/fed situation. After absorption from the small intestine, fructose is primarily taken up by the liver, whereas glucose is metabolized by all cells in the body. Although fructose is absorbed more slowly than glucose, it is more readily metabolized because cellular uptake and the early steps of fructose metabolism differ markedly from those of glucose. Unlike glucose, fructose has only a modest effect on insulin action and does not require the presence of insulin to access to the intracellular compartment. Once inside the cell, fructose is very rapidly converted to fructose-1-phosphate. This phosphorylation bypasses the early rate-

limiting step of insulin-stimulated transport, which glucose must undergo. Thus, fructose is more lipogenic than glucose and it could potentially modify fatty acids balance in very LDL (VLDL) and has harmful effects like hiperlipidemia or insulin resistance. By this way, several evidences suggest that fructose is the primary nutrient mediator of sucrose-induced hypertriglyceridemia, insulin resistance and glucose intolerance (Thresher *et al*., 2000; Gadja *et al*., 2007).

Because of the preferential entry of fructose into lipogenesis, HSu shown increased TG levels, which would increase its deposition in adipose tissue, liver and muscle, eventually resulting in impaired insulin signaling and dyslipidemia, increasingly associated with proatherogenic conditions. Other factors can contribute to reduced insulin sensitivity such as decreased mitochondrial fatty acid oxidation and the accumulation of hepatic DAG, which in turn inhibited insulin signaling via activation of PKC-ζ (Dekker *et al*., 2010). On the other hand, dietary glucose increases serum glucose and insulin concentrations in the postprandial state (Shaeffer *et al*., 2009).

Concerning the effects on glycemic profile, our results demonstrate that no differences on plasma glucose in the fasted state, but a significant increase in a postprandial state in the HSu-treated rats *versus* Control ones. These finding have also been reported by others in rats and humans (Hulman and Falkner 1994; Daly et al., 1998). Furthermore, some authors stated that diet-induced insulin resistance is characterized by fasting normoglycemia (Thresher et al., 2000; Wright et al., 1999). The higher postprandial glucose response found in the HSu group might be explained by the large amount of available glucose from both sucrose and starch on this diet.

Previous studied demonstrated that sucrose consumption produces insulin resistance in rats (Santuré et al., 2002; Pagliassotti et al., 1996; Podolin, 1998). After 9 weeks of sucrose consumption, insulin resistance was confirmed by GTT and ITT. Therefore, the

GTT showed significantly increase blood glucose levels in the HSu animals at 60 min. after glucose stimulation, indicating the impaired glucose tolerance. This profile was confirmed by the higher AUC value of GTT found in the HSu-treated rats. Similarly, ITT revealed impaired insulin sensitivity since 120 min. after insulin injection the HSu rats had significantly higher blood glucose levels than those found in the Control animals. The profile of insulin resistance was reinforced by the elevated HOMA-IR.

In this model, a hyperinsulinemia state was observed (increased levels of serum insulin). Thus, HSu-evoked glucose tolerance might be attributed to peripheral insulin resistance, as a compensatory hyperinsulinemia is observed in this group compared to the Control. In fact, T2DM is recognized as a progressive disorder, which is associated with diminishing pancreatic function over time. However, in an early stage, as may occur after 9 weeks of high sucrose intake, the presence of insulin resistance must be compensated by hyperinsulinemia to maintain normoglycemia. It has also been observed that in those individuals who develop diabetes, a progressive loss of the insulin secretory capacity of β-cells appears to begin years before the clinical diagnosis (Cefalu, 2006). Furthermore, a considerable proportion of whole-body insulin-stimulated glucose uptake is dependent upon the hepatic insulin-sensitizing substance (HISS) in a pathway mediated by the hepatic parasympathetic nerves (HPNs) and, according to Ribeiro et al. (2005), a high-sucrose diet leads to insulin resistance by rapid impairment of such pathway.

Elevated serum triglycerides content were observed in HSu-treated rats, which is consistent with many other reports (Kanazawa et al., 2003; Cao et al., 2007; Sumiyoshi et al., 2006) and may be a result from of *de novo* lipogenesis, the enhanced rate of hepatic triglyceride synthesis and the decrease in peripheral clearance (Suzuki et al., 2004). In this study, sucrose consumption do not induce higher serum cholesterol, which is in agreement

with Sumiyoshi et al. (2006) that found no increment in total cholesterol in mice fed high sucrose diet for 55 weeks.

Thus, our animal model, consisting of a 35% sucrose diet during 9 weeks of treatment, is characterized by a fasting normoglycemia, impaired glucose tolerance, accompanied by hyperinsulinemia and impaired insulin tolerance (or insulin resistance), together with hypertriglyceridemia, but without obesity.

Several studies have investigated alteration in insulin signaling in T2DM subjects and in animal model of insulin resistance. As was above reviewed, impaired cardiac insulin signaling may be a key mechanism underlying the myocardial dysmetabolic profile. Furthermore, previous studies have shown that cardiac dysfunction induced by high-fat diet is associated with altered myocardial insulin signaling (Ouwens et al., 2005). However, data about insulin-mediated cardiac effect or concerning the development of insulin insensitivity, as well related with the molecular aspects of cardiac insulin signaling are lacking in our model.

Insulin resistance in the heart is characterized by impaired PI3K/Akt signaling. Hence, we looked to the basal expression levels of proteins involved in insulin signaling and found no differences between groups on cardiac insulin receptor β, PI3K and MAPK. Concerning the insulin receptor, this result is consistent with Pagliassotti et al. (2002) that found that sucrose feeding did not lead to reduction of insulin receptor protein levels in liver. However, insulin stimulation of tyrosine phosphorylation of IRS proteins, association of IRS proteins with p85 subunit of PI3K and PI3k activity were reduced in HSD versus STD. One of the major limitations of our study concerning this topic is the lack of evaluation of the activity and phosphorylation of each protein involved in insulin signaling, which would require an exogenous insulin stimulation prior to sacrifice. Further studies should complete the information now obtained.

We also analyzed the heart MAPK expression and TRB3 mRNA expression. TRB3 is a member of Mammalian tribbles homologs (TRB) protein family which, recently, was considered to be a critical mediator for insulin resistance which is implicated in DCM, thus, emerged our interest in the gene expression evaluation. Our result showed no differences in MAPK expression as well in TRB3 mRNA expression between HSu and Control group. These results may suggest that TRB3 is not involved in sucrose-induced prediabetic state.

Concerning other aspects related with the cardiac performance, there was no significant difference in blood pressure and heart rate at the end of experiment between the high-sucrose diet rats and the control ones, indicating that the ingestion of sucrose during 9 weeks, at least at this concentration (35%), was not sufficient to cause impact on this aspect. This appears to contradict previous studies in which inducing insulin resistance with a high-sugar diet led to a rise in blood pressure in different rats strains (Hwang et al., 1987; Reaven and Ho, 1991), which seem to be mediated by the activation of the sympathetic nervous and rennin-angiotensin systems (Freitas et al., 2007). Other investigators have also failed to show any hypertension when blood pressure was directly recorded from an intra-arterial catheter in fructose-fed rats not acutely restrained (Brands et al., 1994). Therefore, these findings suggest that insulin resistance can be induced by a high-sucrose diet in rats without causing hypertension or significant alterations in heart rate.

As summary, our model is a model of prediabetes/insulin resistance, characterized by a fasting normoglycemia, impaired glucose tolerance, hyperinsulinemia, insulin resistance, together with hypertriglyceridemia, which could be a useful tool to evaluate the impact on

the cardiac tissue and the existence of cardiomyopathy, independently of other confounding factors, such as hypertension and obesity.

Cardiac impact of prediabetes/insulin resistance using a HSu-diet rat model

Diabetic Cardiomyopathy is globally defined as myocardial structural or functional abnormalities in the absence of hypertension, CAD and valvular heart disease. DCM is frequently seen in the asymptomatic diabetic patients and, thus, screening the disease at the earliest stage of development is important for long-term prognosis and prevention of the progression to congestive HF. DCM has been a poorly understood disease and the underlying mechanisms are not completely elucidated. Development of DCM includes complex and multifactorial pathophysiological mechanisms. Common pathological changes of diabetic heart are myocyte hypertrophy, interstitial fibrosis and increase in contractile protein glycosylation. As a result of these changes, diastolic compliance decrease, ventricle hypertrophies and, in advanced stages, systolic functions may worsen (Murarka and Movahed, 2010; Turan et al., 2011; Voulgari et al., 2010; Khullar et al., 2010; Dobrin et al., 2010; Tarquini et al., 2011; Boudina and Abel, 2010).

The existence of earlier cardiac changes in prediabetes has been suggested, but the human studies, and even the animal models, are usually limited because of the coexistence of other perturbations. In our model of prediabetes/insulin resistance, the existence of earlier cardiac changes was not previously reported and, thus, a larger evaluation of the mechanisms putatively involved was chosen. Considering the amount of different pathways suggested as contributors for the pathophysiological mechanisms of human DCM, we choose to screen some of the aspects putatively implicated, evaluating some

serum markers and some cardiac tissues proteins and genes, since the early modifications could be all and none in a model previously tested, but notoriously with other features than the most common HSu-diet model.

Cardiac fibrosis and hypertrophy are the two major structural changes associated with cardiac dysfunction inherent to DCM, and may occur independently of hypertension. Cardiomyocyte hypertrophy as well as subsequent apoptosis and focal myocardial fibrosis are structural hallmarks of DCM and functionally manifest as defective cardiac contractility (Chen et al., 2000). Previous studies have shown that cardiomyocyte hypertrophy in diabetes is regulated at the transcriptional level (Hileeto et al., 2002; Chen et al., 2003; Farhangkhoee et al., 2003; Feng et al., 2008). Thus, it is important to examine some molecular biomarkers of the putative cardiac alterations. In this study, we evaluated cardiac mRNA levels of TGF-β1, TSP-1, pro-collagen III, CTGF and brain BNP, which have been associated with cardiac hypertrophy and/or fibrosis.

It has been shown that TGF-β1 plays a role in cell growth, differentiation, apoptosis, inflammatory processes, and gene expression (Lawrence, 1996; Bujak and Frangogiannis, 2007). TGF-β1 activation has consistent implications in diabetic cardiomyopathy. It is becoming increasingly apparent that TGF-β1 represents a key mediator of cardiac adaptation to a dysmetabolic environment and/or hemodynamic overload. TGF-β1 has pro-fibrotic properties and induces the expression of many matrix proteins, including collagens. The composition of extracellular matrix, a complex network of structural proteins, mainly collagen type I and III in the myocardium provides architectural support for the myocardium and plays an important role in myocardial function (Pauschinger et al., 1999). In the hearts of patients with diabetes, collagen remodeling mainly as a result of an increase in collagen type III occurs in the perimysium and perivascular region (Shimizu et

al., 1993). Several studies have demonstrated an accumulation of collagens including collagen type III in diabetic cardiomyopathy, which has been related to left-ventricular diastolic and systolic dysfunction (Mizushige et al., 2000; Westermann et al., 2007).TSP-1 is a matricellular glycoprotein that influences cellular phenotype and the structure of the extracellular matrix (Lawler, 2002). TSP-1 is a trimer of disulfide-linked 180 kD subunits, which consists of three identical polypeptide chains linked by disulfide bonds. Each chain is composed of six distinct structural and functional domains. It has been reported that high glucose stimulated TGF-β1 bioactivation by fibroblasts through upregulation of TSP-1. Recently, Tang et al. (2011) showed that high ambient glucose leads to upregulation of collagen III, which is mediated by TGF-β1. Indeed the TSP-1 is involved in this process through up-regulating the production of TGF-β1. CTGF is another potent profibrotic protein and play important roles in tissue and organ fibrosis. CTGF is increasingly being implicated in structural and functional abnormality of the heart in diabetes, and has diverse biological actions including the regulation of fibroblast proliferation, angiogenesis collagen synthesis, extracellular matrix deposition and cellular apoptosis. The expression of CTGF is increased in the myocardium of rats with STZ-induced diabetes (Leask et al., 2002; Way et al., 2002). Upregulation of CTGF can be mediated by hyperglycemia, ROS, Ang II, VEGF, PKC, AGEs and also by TGF-β1. Finally, BNP is among the most relevant molecular markers of cardiac hypertrophy. BNP is a peptide hormone released from the cardiac ventricles in response to pressure and volume overload, which may trigger cardiac hypertrophy in HSu-treated rats.

In our study, we found no statistically significant difference in cardiac mRNA expression of TGF-β1, TSP-1, pro-collagen III and CTGF between the HSu and the Control rats, despite a trend to higher values in the HSu animals for all the markers, accompanied by a similar profile in serum TGF-β1 concentration. Moreover, BNP mRNA

levels were significantly higher in the HSu group *versus* the Control. As previously commented, the heart and LV weight of the HSu-treated rats showed higher values than those found for the Control animals, being statistically significant the changes of heart weight and LVW/BW. Even considering that LV weights could be influenced by the variability of the cut, whole heart weight/body weight are feasible measures, and showed a trend to higher values in the HSu-treated rats. Thus, collectively, this data indicate that 9 weeks of sucrose diet might be a short period to promote pronounced changes on cardiac trophism, but the initial changes might be already in course, being BNP the earlier biomarker of those changes. A longer time of diet exposure might most probably confirm the effects of high-sucrose diet on cardiac hypertrophy and fibrosis, but after 9 weeks the earlier changes has already started to begin. Other studies have already shown that patients with T2DM have an increase in LVH, independent of other confounding factors, including hypertension (Devereux et al., 2000). However, LVH in the diabetic patient generally represents a more advanced stage of the disease and may manifest after a longer period.

Other studies have previously tried to evaluate whether prediabetes, as measured by IFG, is associated with cardiac alterations. De Marco et a. (2011) found, evaluating a population-based sample of adolescents and young adults, that prediabetic individuals had a significantly higher prevalence of LVH than participants with normal fasting glucose, reflecting important target organ damage already present at an early phase of alteration of glucose metabolism. This fact is important, since there is a strong relation between LVH and adverse cardiovascular outcomes (Levy et al., 1990), recommending targeting prevention strategies in this subpopulation. Postprandial, but not fasting, hyperglycemia is known to be a better predictor of major cardiovascular events or total mortality, but the impact of postprandial or fasting glucose levels on LV diastolic function has not been elucidated (Coutinho et al., 1999; DECODE Study Group, 2001). Shimabukuro et al.

(2011) showed, in humans, that impaired glucose tolerance, but not impaired fasting glucose, underlies left ventricular diastolic dysfunction, and Wu et al. (2007) previously described epidemiological evidence of altered cardiac autonomic function in subjects with IGT but not isolated IFG, as previously suggested (Fujita et al., 2007). Our data seem to be in agreement with this idea that IGT is associated with changes of the left ventricular. However, protein levels (measured by western blot or immunohistochemistry) of the markers analyzed, as well as other putatively relevant molecular markers could complete the information concerning the effects of HSu on cardiac hypertrophy and fibrosis in future works.

Cardiac hypertrophy and fibrosis is usually associated with apoptosis. Physiological hypertrophy, such as exercise-induced cardiac hypertrophy, is a favorable adaptive change in the heart that accommodates it to increases in bodily demand and does not lead to heart failure. In contrast, pathological hypertrophy, such as pressure overload-induced hypertrophy, is a maladaptive response to pathological stimuli, and, if not ameliorated, usually leads to HF. Moreover, pathological cardiac hypertrophy may progress to HF because the cardiomyocytes are more susceptible to apoptosis (Kan et al., 2003). In our study, the pair in Bax and Bcl-2 was evaluated as a measure of impairment of apoptotic machinery. No significant changes in Bax and Bcl-2 cardiac mRNA expression were found. Whether these are or not related with the effect of insulin in the absence of hyperglycemia, deserve further evaluation. Therefore, it is known that insulin have anti-apoptotic effects (Aikawa et al., 2000), which seem to be mediated by activation of different pathways, including the PI3K/Akt and MAPK/ERKs pathway, which are independently activated, and may exert their protective action in a cooperative fashion, by phosphorylating different serine residues of Bad, thus protecting cardiac myocyte from oxidative-stress-induced apoptosis. Although Bax and Bcl-2 are important molecules in the

apoptotic machinery, as among the most presented in the studies available on the literature, the lack of significant changes do not exclude the existence of perturbations in other important mediators of the process.

Endothelial dysfunction is one of the mechanisms involved in the pathogenesis of DCM. Generically, endothelial dysfunction is characterized by impaired endothelium-dependent vasodilation and usually reflects a reduced bioavailability of NO, which is an important vasodilator. Most of studies have been suggested that diabetes is associated with decreased expression of eNOS and consequently, a decreased production of NO, which is linked with impaired vasodilation; however, the source of NO in such setting is not clear. It is possible that NO from other sources, such as inducible NO synthase (iNOS), contribute to increased amounts of NO in diabetes. iNOS is an enzyme pathway with recognized implications in ROS production, particularly under inflammatory conditions and it is also involved in insulin resistance. Furthermore, Nagareddy et al. (2005) suggest that increased iNOS activity may contribute to an increased NO pool and depressed MAP and HR and endothelial dysfunction in 3 weeks STZ-induced diabetic rats.

Activation of iNOS contributes to the pathogenesis of cardiovascular disease, probably because of excessive release of NO and generation of ROS. The expression of iNOS is upregulated by most, if not all, inducers of insulin resistance, including proinflammatory cytokines, obesity, FFAs, hyperglycemia, endotoxins and oxidative stress (Elizalde et al., 2000; Shimabukuro et al., 1998; Sharma et al., 1995; Xie et al., 1994, Haidara et al., 2010). Perreault and Marette (2001) demonstrated that elevated expression of iNOS was associated with development of muscle insulin resistance in high-fat diet-fed mice. Futhermore, iNOS mRNA is highly expressed in heart of obese Zucker diabetic fatty rats. In addition, iNOS deficiency protects from high-fat diet-induced insulin resistance in

the obese Zucker rat model (Zhou et al., 2000). These clearly demonstrate the important role of iNOs in insulin resistance.

In our study, no significant change of cardiac iNOS mRNA levels was found between Hsu-treated rats and the Control ones, despite a trend to higher values in the insulin resistant animals. Lee et al. (2009) suggest that upregulation of iNOS may be a protective mechanism against excessive contraction, abnormal signaling resulting from oxidative stress and due to enhanced inflammation in the diabetic vasculature. Whether this hypothetic protection is present or nor in our model, deserves additional research. Therefore, future work should confirm the trend encountered, which might be complemented with supplementary measures. In fact, it would be interesting to measure eNOS mRNA expression and/or eNOS activity in these experimental conditions, in order to estimate NO availability in these conditions of insulin resistance, as well as the contribution of endothelial dysfunction to changes in early stages of DCM.

Endothelial dysfunction is also assessed by measurement of elevated levels of cellular adhesion molecules (CAMs), including ICAM-1 and VCAM-1. Elevated levels of CAMs have been a consistent finding in cross-sectional studies of patients with type 2 diabetes (Albertini et al., 1998; Caballero et al., 1999) and nondiabetic subjects at increased risk of diabetes, including subjects with impaired glucose tolerance, and in nondiabetic first-degree relatives of patients with type 2 diabetes (Caballero et al., 1999; Vehkavaara et al., 1999 Balletshofer et al., 2000). Elevated levels of CAMs predict the incident development of CVD events independent of standard CVD risk factors (Hwang et al., 1997). Our results showed no differences in ICAM-1 and VCAM-1 cardiac mRNA expression between the two groups, suggesting that this could be later markers of the changes, if implicated.

Angiogenesis is a key component of organ development, tissue repair and disease. Increased vascular permeability is one of the first steps in new vessel creation. VEGF, through vascular fenestration and tight junction modulation, mediates angiogenesis by increasing vascular permeability to water and large molecular weight proteins, assisting normal tissue repair and extracellular remodeling (Akhavani et al., 2007; Bates and Harper, 2002). However, increased vascular permeability associated with pathological angiogenesis contributes to organ pathology and vascular disease (Bates and Harper, 2002; Simo´ et al., 2006). For more than a decade, the role of vascular endothelial growth factor (VEGF) in the regulation of angiogenesis has been the object of intense investigation (Ferrara, 2004). Recent evidence indicates that new vessel growth and maturation are highly complex and coordinated processes, requiring the sequential activation of a series of receptors by numerous ligands in endothelial and mural cells (Carmeliet, 2003; Jain, 2003). However, VEGF signaling often represents a critical rate-limiting step in physiological angiogenesis and is one the most important growth factors, which affects all aspects of angiogenesis including matrix degradation and endothelial cell migration and proliferation (Neufeld et al., 1999). Our results showed a significant increment in serum VEGF levels in HSu compared with Control. These data may be explained by the fact that in insulin resistant-state, insulin is no longer capable of antagonizing VEGF level (Wan et al., 2004). Although non-significant, a trend to higher levels of cardiac tissue VEGF mRNA levels in the HSu-related rats was found, which deserve future confirmation, eventually in rats treated for longer periods with high-sucrose diet, as the changes suggested by our data are in opposition to those found in other studies. Therefore, VEGF overexpression was found in cardiac myocytes and arteriolar smooth muscle cells following MI in non-diabetic patients (Shinohara et al., 1996). However, the expression of VEGF protein and mRNA, as well as its receptors, are significantly decreased in the myocardium of both diabetic and

insulin-resistant non-diabetic rats (Chou et al., 2002), suggesting that, in diabetic patients, the normal molecular processes which regulate angiogenesis may be impaired (downregulated).

Accumulating evidences suggest that chronic low-grade inflammation is involved not only in obesity and T2DM pathophysiology, but also in DCM development. Elevated inflammatory cytokines have been found in circulation and in the diabetic hearts o T2DM patients (Yu et al., 2011; Mano et al., 2011). Furthermore, inflammatory mediators, such as IL1-β and TNF-α, have been implicated in diabetic cardiomyopathy, increasing epicardial thickness and promoting myocyte contractile dysfunction, thus depressing myocardial function, contributing to HF (Westermann et al., 2006). Additionally, cardiac overexpression of TNF-α has been associated with cardiac hypertrophy and fibrosis, as well with left ventricular dysfunction (Yokoyama et al., 1997; Sun et al., 2004) and IL-6 has been also described as an inducer of myocardial damage by promoting LV dysfunction and cardiac hypertrophy under acute myocardial infarction (Younce et al., 2010). In our study, however, no significant changes were found concerning serum levels of IL-6 and IFN-γ and cardiac mRNA expression of IL-6 and TNF-α, which was accompanied by a reduction of serum CRP contents in the HSu-treated rats. Thus, according to our data, which might be further completed with additional markers (such as other interleukins and the NF-kB, which is an important mediator of the inflammatory process), inflammation is not the key factor of early changes related to cardiomyopathy.

One of the major mechanisms associated with DCM is the generation of ROS and oxidative stress. Increased ROS and reduced antioxidant defenses have been shown in T2DM patients, which might be due to hyperglycemia, glucose autoxidation, and elevated levels of FFAs (Davı et al. 2005; Jay et al. 2006). Over-generation of mitochondrial ROS has been also shown in several tissues exposed to hyperglycemia (Camici et al. 2007).

Hyperglycemia-induced overproduction of ROS, such as superoxide, by the mitochondrial electron transport chain could be one of the first and key episodes in the activation of all other pathways involved in the pathogenesis of diabetic complications (Piconi et al. 2003; Rolo and Palmeira 2006). Increased mitochondrial protein nitration, hydrogen peroxide production, and reduced glutathione levels have been observed to occur in diabetic hearts (Nishio et al. 2004). In hyperglycemia associated with diabetes mellitus, there is activation of the polyol pathway, hexosamine pathway, protein kinase C, and increased AGEs (Khullar et al., 2010). Increased ROS production was also shown to be associated with increased apoptosis in hearts of ob/ob and db/db mice (Barouch et al. 2003). It has been proposed that ROS-mediated cell death could promote abnormal cardiac remodeling, which ultimately may contribute to the characteristic morphological and functional abnormalities that are associated with diabetic cardiomyopathy (Boudina and Abel, 2007). Under diabetic conditions, there is an inflammatory response by oxidative mechanisms, which may contribute to the development of diabetic cardiomyopathy (Khansari et al. 2009). Hyperglycemia has been observed to be associated with greater systemic inflammation that may be an important contributor to cardiovascular risk among diabetic patients. Although the mechanisms behind this association are not clearly understood, hyperglycemia has been shown to increase the binding of inflammatory cells (such as the T cells and mononuclear phagocytes) to the endothelium and to increase the inflammatory cytokine production by monocytes (Basta et al. 2004; Rolo and Palmeira, 2006). However, in a state of hyperglycemia and insulin resistance, without hyperglycemia, as occurs in early stages of diabetes (prediabetes), the contribution of ROS and consequent oxidative stress to the cardiac changes remain to be elucidated.

We have measure serum and cardiac tissues markers of redox status (lipid peroxidation and antioxidants), in order to evaluate the contribution of a putative

impairment of ROS/antioxidants equilibrium in this state of insulin resistance without hyperglycemia. We found a trend to increased MDA (a marker of lipid peroxidation) levels in both the serum and the heart tissue of Hsu-treated rats vs the control. However, a significantly increased amount of serum TAS (a measure of total antioxidant capacity) was also encountered in the HSu rats, which seem to reflect a compensatory response against the eventual increment of ROS. The serum results were accompanied by increased cardiac SOD mRNA expression, together with decreased HNE (also a lipid peroxidation marker) and RAGE mRNA expression.

The results of our study are not consistent with previous studies. It has been shown that rats fed a high sucrose diet had reduced heart antioxidant enzyme activity (Cu-Zn-SOD), greater hear susceptibility to peroxidation and/or decreased protection from non-enzymatic or enzymatic antioxidants (Bussereoles et al., 2002). In the heart tissues of diabetic rats, upregulation of RAGE has been found and it is strongly associated with impaired cardiac function (Simm et al., 2004). Activation of RAGE is known to influence myocardial calcium homeostasis and contribute to interstitial fibrinogenesis in the diabetic heart ((Petrova et al., 2002, Candido et al., 2003). Indeed, increased gene expression of RAGE was observed in high-fat feed animals and inhibition of prevention of RAGE activation in knockout animals able to prevent the induction of inflammation, oxidative stress and mitochondrial dysfunction the heart associated with high fat feeding (Tikellis et al., 2008).

We must note that our model present hyperinsulinemia, which is still able to normalize blood glucose levels, at least viewed by the fast glycemia, despite impaired glucose tolerance. Thus, in a model like this, it seems that the putative (since the data is only a trend to increased MDA) generation of reactive species is yet compensated by

additional generation of antioxidants, viewed by increased serum TAS and cardiac mRNA expression of SOD. Furthermore, RAGE mRNA downexpression in the cardiac tissue might eventually be also viewed as a compensatory mechanism, in order to protect the cardiac tissue.

Oxidative stress has been indicated as one of the main pathophysiological implicated in the development of insulin resistance and further cardiovascular toxicity. In this model of prediabetic insulin-resistance induced by sucrose consumption, if the trend to increased values of MDA in serum and heart are representative of an initial increased generation of ROS, then it is an earlier phase of the damaging process, in which there is yet a compensatory antioxidant defense. However, we cannot exclude the possibility that longer exposure times of sucrose may lead to reduction of these antioxidant defence mechanisms and, consequently, to toxic effects induced by oxidative stress aggravating cardiac function.

Hyperglycemia has been indicated as one of the major causes of DCM, due to activation of several mechanisms, leading to an increase in oxidative stress by several pathways, such as increased activation of the polyol and hexosamine pathways, increased formation of AGEs and activation of classical isoforms of PKC, which induce increased production of mitochondrial ROS, nonenzymatic glycation of proteins and glucose auto-oxidation. Activation of these pathways corresponds to an increased glucotoxicity, which may cause cellular injury. If sustained hyperglycemia, is a pivotal factor for the cardiac changes that evolutes further to DCM, then the lack of substantial cardiac impairment in this model could be explained by the limited glucose dysfunction (impaired glucose tolerance) of this prediabetes/insulin resistance state. However, it will be crucial to better evaluate this putative earlier oxidative stress, using additional markers, in order to further

assess if it is present and if has an effective impact on the cardiac tissue, since some other markers, mainly of hypertrophy and fibrosis, seem to be already affected.

Figure 34 summarizes the main results obtained concerning the animal model characterization as well as the impact of high-sucrose diet on the cardiac tissue.

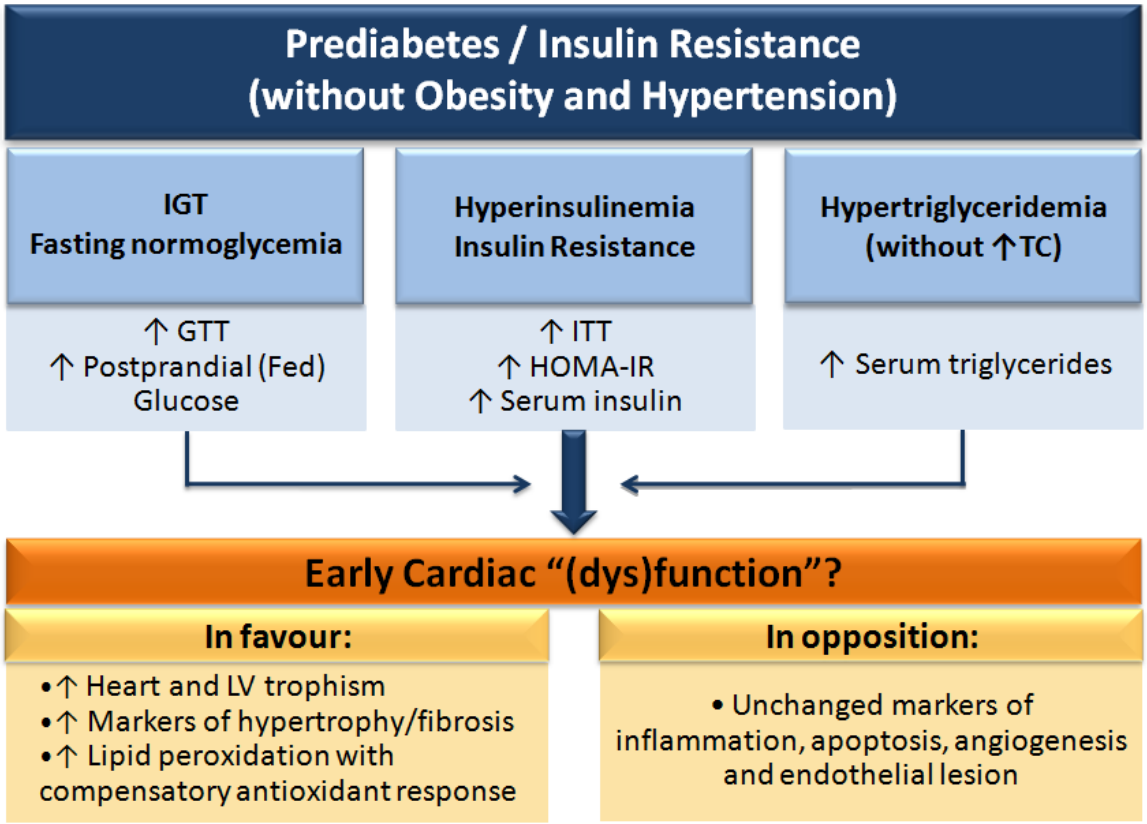


Figure 34. Summary scheme of the obtained results in this study.

VII. CONCLUSIONS

1. The animal model chosen to mimic a situation of prediabetes/ insulin resistance, consisting of 35% sucrose consumption during 9 weeks, presented as main metabolic features: a) Fasting normoglycemia; b) Impaired glucose tolerance; c) Hyperinsulinemia; d) Hypertryglyceridemia and e) Absence of obesity and hypertension.

2. This animal model presented some alterations suggestive of hypertrophy/fibrosis, namely in the left ventricle. Therefore, apart from the trend to increased values of several markers of hypertrophy/fibrosis, there was a significant increment in the BNP mRNA expression in the HSu group, which was accompanied by increased heart and left ventricle mass.

3. Despite some variations in the values encountered for markers endothelial lesion, angiogenesis and inflammation in the HSu-treated rats, the differences were not statistically significant and, thus, these pathways seem to be less relevant at this stage of dysmetabolism concerning an impact on the cardiac tissue.

4. Some of the data encountered is suggestive that the oxidative stress, and compensated antioxidant response, are the more pronounced change at this stage. Therefore, increased serum total antioxidant capacity and cardiac mRNA expression of SOD, together with decreased HNE (a lipid peroxidation marker) and RAGE (receptor of AGEs) mRNA expression are eventual indicator of a compensatory antioxidant response. This response might have been produced as a result for an increased ROS, as the variations of serum and heart MDA suggest, although not yert totaly confirmed.

As summary conclusion, this animal model of prediabetes/insulin resistance, induced by high sucrose consumption during 9 weeks, could be an important tool to evaluate the impact of dysmetabolism (mainly hyperinsulinemia and impaired glucose tolerance) on cardiac function/performance, without confounding factors such as obesity and hypertension. However, if hyperglycemia is the major driving force for the cardiac changes underlying cardiomyopathy, most probably due to induction of oxidative stress, has is suggested, then the lack of substantial cardiac impairment in this model could be explained by the limited glucose impairment of this prediabetes/insulin resistance state. However additional studies are needed to better understand the underlying molecular and cellular mechanisms responsible for the effects of HSu on this earlier phase of DCM, namely evaluation other durations of diet exposure, as well as other relevant markers of cardiac impairment.

VIII. BIBLIOGRAPHY

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