AMERICAN UNIVERSITY OF BEIRUT

MESENCHEMAL STEM CELLS-CONDITIONED MEDIA AS A PROSPECTIVE THERAPY FOR CARDIOMYOPATHY IN TYPE 1 DIABETES

by
RASHA SHEHADE SLIKA

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RASHA SHEHDAE SLIKA

Approved by:

Dr. Assaad Eid, Associate Professor
Department of Anatomy, Cell Biology,
and Physiological Sciences
Advisor

Dr. Wassim Abou-Kheir, Associate Professor
Department of Anatomy, Cell Biology,
and Physiological Sciences
Member of Committee

Dr. Sami Azar, Professor
Department of Internal Medicine
Member of Committee

Dr. Bassam Badran, Professor
Dean of Faculty of Sciences, Lebanese University
Member of Committee

Date of thesis defense: September 16, 2020

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AN ABSTRACT OF THE THESIS OF

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Background: Diabetic cardiomyopathy is one of the major macrovascular complications that occur in patients with diabetes mellitus. It is characterized by morphological and functional alterations in the heart especially in the left ventricle (LV). In type 1 diabetes mellitus (T1DM), LV hypertrophy as well as systolic dysfunction occur, where the ejection fraction and fractional shortening values significantly decrease affecting the myocardial contractility. Several pathogenic mechanisms underlying T1DM contribute to collagen accumulation and glycosylated protein deposition, ultimately resulting in fibrosis and cardiac injury. Furthermore, an upregulation in the expression of alpha-smooth muscle actin (α-SMA), fibronectin, and NADPH oxidases have been reported in a diabetic milieu. An increasing body of evidence have shown that mesenchymal stem cells (MSCs) have the potential to enhance cardiac function and ameliorate diabetes-induced cardiac damage and myocardial fibrosis. More importantly, the cardio-protective mechanisms of MSCs are most likely to be attributed to their paracrine effect rather than their differentiation potential. Therefore, it is suggested that conditioned media derived from MSCs may attenuate diabetic cardiomyopathy.

Aim: The present study aims to assess the cardio-protective role of MSCs-conditioned media and to investigate the molecular mechanisms by which MSCs-conditioned media exert this protective role. We aim to determine the effect of MSCs conditioned media on cardiac function in Streptozotocin (STZ)-induced type 1 diabetic rats, particularly ejection fraction and fractional shortening. We also aim to investigate the molecular changes in cardiac markers of injury including α-SMA, fibronectin and NADPH oxidase 4, as well as the histological alterations mainly fibrosis.

Methods: Sprague-Dawley rats were divided into the following groups: a control group and a Streptozotocin-induced type 1 diabetic group. After 6 weeks from diabetes onset, rats were divided into the following groups: (1) Control; (2) Control with weekly injections of MSCs-conditioned media, (3) Diabetic; (4) Diabetic treated with weekly injections of MSCs-conditioned media. Echocardiography studies were performed at the 8^{th} and 12^{th} week of treatment. After 12 weeks of treatment from diabetes onset, functional, histological, biochemical, and molecular parameters of the heart were assessed.

Results: Treatment with MSCs-conditioned medium attenuated cardiac injury in diabetic rats. Protection against diabetic cardiomyopathy imparted by MSCs-conditioned media was denoted by decreased myocardial fibrosis and a decrease in collagen and glycosylated

proteins deposition in cardiomyocytes. Intriguingly, treatment with MSCs-conditioned media did not decrease blood glucose levels in type 1 diabetic rats, indicating that the cardio-protective role of MSCs-conditioned media is not attributed to the restoration of normal glucose levels. Furthermore, treatment with MSCs-conditioned media restored ejection fraction and fractional shortening to values similar to controls. Moreover, our results show that MSCs-conditioned media tend to decrease the expression of markers of cardiac injury including α-SMA, fibronectin, as well as nox4.

Conclusion: Our results suggest that MSCs-conditioned media may represent a therapeutic modality for diabetic cardiomyopathy. Further in-depth mechanistic investigations are needed to better understand the paracrine effect of MSCs on the heart and to further elucidate the potential molecular mechanisms of this cardioprotective role.

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LIST OF ABBREVIATIONS

DM: diabetes mellitus

LV: left ventricle

T1DM: type 1 diabetes mellitus

T2DM: type 2 diabetes mellitus

α-SMA: alpha smooth muscle actin

NADPH oxidase 4: nox4

MSCs: mesenchymal stem cells

CM: conditioned media

DC: diabetic cardiomyopathy

STZ: Streptozotocin

ROS: reactive oxidation species

GDM: gestational diabetes mellitus

ADA: American Diabetes Association

IDF: International Diabetes Federation

CV: cardiovascular

HF: heart failure

AGEs: advanced glycation end-products

RAAS: renin–angiotensin–aldosterone system

PARP: poly ADP ribose polymerase

TG: triglyceride

EV: extracellular vesicle

PDGF: platelet-derived growth factor

IL: interleukin

IV: intravenous

Exos: exosome

BM-MSC: bone marrow-derived MSC

EF: ejection fraction

FS: fractional shortening

DMEM: Dulbecco's Modified Eagle Medium

PBS: Phosphate-buffered saline

HPLC: high-performance liquid chromatography

CHAPTER I
INTRODUCTION

1. Diabetes Mellitus

A. Epidemiology

Diabetes mellitus (DM) is a metabolic, autoimmune, and chronic disease that remains to be a major health concern nowadays affecting children, adolescents, and adults. It has scored high rates of prevalence, deaths, and expenditure across the globe resulting in social, financial and health system burden [1-5]. The prevalence of diabetes has been dramatically increasing worldwide and is predicted to rise by 25% in 2030 and 51% in 2045 [3]. According to International Diabetes Federation (IDF), diabetes affects around 463 million individuals in 2019 and estimated to reach 700 million in 2045 [3]. Moreover, it was shown that large economic and health burdens are caused by diabetes, not only on the patient but also on his/her family, national health care system, and society as well. The American Diabetes Association (ADA) has reported $327 billion as an annual cost of diabetes in 2017 [5]. In Lebanon, results from a study performed on the financial burden of diabetes in 2015 show very high numbers of admissions, long duration of hospitalization and high cost for diabetes mellitus management [26]. Among non-Gulf Cooperation Council (GCC) countries, Lebanon shows the highest prevalence of diabetes in 2017 according to statistical analysis conducted between Arab countries [27]. It is worth mentioning that the challenge of controlling diabetes has been associated with psychological and social load causing stress, anxiety, and distress-related diabetes [2]. Individuals diagnosed with diabetes are more likely to attempt suicide than normal

population by three to four times [2]. All these facts mentioned above have affected the quality of life and made diabetes an interesting topic for researchers and clinicians.

B. Definition and Classification

Diabetes mellitus is a metabolic disorder characterized by impaired insulin secretion and/or action resulting in hyperglycemia. The chronic exposure to hyperglycemia is associated with long-term damage and organ failure specifically in eyes, kidneys, nerves, heart, and blood vessels. The pathogenic process of diabetes involves either the destruction of pancreatic beta cells with consequent insulin deficiency, or resistance to insulin action when the body tissues are no longer able to respond to insulin effectively. The two scenarios are defined as type 1 diabetes mellitus (T1DM) or type 2 diabetes mellitus (T2DM), respectively. They are considered the most prevalent forms of diabetes being associated with number of symptoms including polyuria, polydipsia, polyphagia, weight loss or gain, fatigue, and blurred vision. Patients with diabetes also sense tingling or numbness in their extremities, suffer from slow wound healing, and have higher susceptibility to infections [6]. Different symptoms are experienced by patients with T1DM and T2DM by which the diagnosis must be distinguished so that the therapy will be well determined. Other forms of diabetes also exist including gestational diabetes mellitus (GDM), type 3 diabetes - referring to Alzheimer's disease, and other specific types [6, 28, 29].

i. Type 1 Diabetes Mellitus

This form of diabetes is termed as Insulin-dependent diabetes mellitus (IDDM) or juvenile-onset diabetes accounting for about 10% of patients with diabetes [6]. It leads

to insulin deficiency which leave patients dependent on exogenous administration of insulin for their survival [30]. T1DM is the result of cellular-mediated autoimmune destruction of pancreatic beta cells and thus also known as immune-mediated diabetes [31]. It was previously shown that this destruction is mainly associated with multiple genetic predispositions; however, other environmental factors may also be related, yet, they remain to be poorly investigated [32]. The rate of beta cells destruction varies according to the individual's age; it occurs rapidly in infants and children and progresses slowly mainly in adults. Some patients, especially children and adolescents, develop ketoacidosis as an early manifestation. Others, particularly adults, may keep residual beta cell function enough to prevent ketoacidosis for many years [28]. It was reported that some forms of T1DM referred as idiopathic diabetes have no known etiologies. These patients exhibit permanent insulin deficiency and are susceptible to ketoacidosis with no proved autoimmunity [6]. Despite extensive research and studies, there is still no defined cure for T1DM, and patients are under life-threatening complications with social economic burdens.

ii. Type 2 Diabetes Mellitus

Type 2 diabetes mellitus (T2DM), previously referred to as Non–Insulin-Dependent Diabetes Mellitus (NIDDM) or adult-onset diabetes, is the most common form accounting for about 85% to 90% of all cases. Patients with T2DM are characterized by insulin resistance with relative, rather than absolute, insulin deficiency. It is suggested that insulin levels in these patients appear normal or elevated depending on the blood glucose levels. High blood glucose levels are shown to be associated with even higher insulin values [6]. Therefore, the defective insulin secretion in patients with T2DM is insufficient to compensate for their insulin resistance. Moreover, the progressive deterioration of

pancreatic beta cell function leads to sustained increase in blood glucose levels [33]. This deterioration may explain the reason behind of why patients with T2DM might develop into T1DM [34]. Although, the specific mechanism underlying the pathology of T2DM is still unknown, the destruction of beta cells does not take place. Thus patients diagnosed with T2DM do not require insulin treatment, at least initially, to survive. They remain undiagnosed for many years since at early stages hyperglycemia is not severe enough for the individual to notice the symptoms. However, they are considered at high risk of developing diabetes-induced complications. It is reported that genetic predisposition is a strong contributor for T2DM [35]. Nonetheless, its incidence increases with age, obesity, and lack of physical activity suggesting that environmental factors are also important mediators in the progression of the disease [35]. Several experimental studies show that T2DM occurs more frequently in patients with hypertension or dyslipidemia. It was also reported that visceral obesity is associated with insulin resistance [36].

iii. Gestational Diabetes Mellitus

Gestational diabetes mellitus (GDM) is a major medical condition by which women experience high blood glucose levels during the second or third trimester of pregnancy. It affects approximately 7% of all pregnant women, ranging between 1% to 14% depending on the population studied, resulting in more than 200,000 cases annually [6]. In most cases, GDM resolve after delivery; however, it can impact the baby's health and, sometimes, increase the risk of developing T2DM later in life. This form of diabetes can be divided into two groups. Class A1 GDM may be managed by the woman's lifestyle through diet and exercise. Women with class A2 require exogenous insulin

treatment or other medications to survive. An appropriate follow-up strategy of women with GDM is essential to determine the diagnostic criteria used [37].

iv. Type 3 Diabetes

Type 3 diabetes involves the brain selectively and is known as 'brain diabetes'. Many studies attributed this description to the brain of an Alzheimer's patient. It was shown that Alzheimer's disease has molecular and biochemical characteristics that occur with both T1DM and T2DM [38, 39].

v. Other Specific Forms

Other forms of diabetes are associated with other diseases such as endocrinopathies (ex: Cushing's syndrome, Acromegaly, etc.), diseases of the pancreas (ex: pancreatitis, cystic fibrosis, etc.), or viral infections. Diabetes may also be caused by genetic defects in beta cell function or insulin action, or induced by drugs such as glucocorticoid, beta adrenergic agonist, thyroid hormone, etc. [6, 28]

C. Diagnostic Tests

According to (ADA), the diagnosis of diabetes is achieved based on plasma glucose levels through three main laboratory tests. These diagnostic tests are the fasting plasma glucose (FPG), 2-h plasma glucose (2-h PG) value during a 75-g oral glucose tolerance test (OGTT), and hemoglobin A1c (HbA1c). A patient is diagnosed with diabetes if he/she has FPG > or = to 110 mg/dL, OGGT > or = to 11.1 mmol/L or A1c > or = to 6.5 %. The simplest diagnostic test is the (FBG) that measures blood glucose after at least 8 hours of no caloric intake. The second widely used diagnostic test is the OGGT that assesses how fast glucose is cleared from the bloodstream. It is performed by giving

the patient 75g of anhydrous glucose dissolved in water to be ingested orally two hours before blood glucose measurement. The third diagnostic test is the HbA1c which is known to be the most accurate one. Hemoglobin is a protein found in the red blood cells and binds to glucose that is build up in the blood. Due to the fact that red blood cells have a life span of around 3 months, A1c is considered a widely marker of chronic hyperglycemia and is used to measure the average blood glucose levels over 2- to 3-months of time. Unfortunately, diabetes remains underdiagnosed. However, it is crucial to screen for diabetes using these three lab tests to have an early diagnosis and thus immediate medical intervention before the progression of diabetes and its complications [6, 28].

D. Complications

Maintaining normal glucose levels remains to be a substantial challenge for diabetic individuals. Unfortunately, patients with T1DM and T2DM are at high risk of developing diabetic complications due to the uncontrolled diabetes. Mechanisms underlying these complications are triggered by the chronic exposure to hyperglycemia. The degree of hyperglycemia may change over time according to the extent of the disease pathology which reflects its toxicity on several organs of the body mainly the heart, blood vessels, kidneys, eyes, and nerves. In fact, diabetic complications can be life-threatening and are classified into microvascular and macrovascular complications occurring due to damage in small and large blood vessels respectively. Microvascular complications, recently termed as triopathy which includes damage to eyes (retinopathy), to kidneys (nephropathy), and to nerves (neuropathy). Macrovascular complications affect the heart and large arteries leading to coronary artery disease, hypertension, heart failure,

atherosclerosis, peripheral vascular disease, strokes, as well as cerebrovascular disease. Large randomized-controlled trials show that the onset and progression of these complications may be delayed with a good metabolic control in T1DM and T2DM [7-9].

2. Cardiovascular diseases in diabetes

Diabetes has shown to be a powerful risk factor for cardiovascular (CV) diseases that occur at earlier ages in comparing with normal condition [40]. In a patient with diabetes, CV diseases extend to atherosclerosis, coronary artery disease (CAD), myocardial infarction (MI), heart failure (HF), arrhythmia, hypertension, and cardiomyopathies [12]. CV diseases are considered the leading cause of death in diabetic patients accounting for three quarters of the deaths among this population [10, 11]. The incidence of heart failure hospitalization is two times greater in diabetic than non-diabetic individuals, and higher heart failure case-fatality was shown to be associated with T1DM patients [41]. According to several epidemiologic studies including the Framingham study [42], United Kingdom Prospective Diabetic Study [43], Cardiovascular Health Study [44], and Euro Heart Failure Survey [45], the presence of diabetes increases the risk of developing heart failure and other cardiovascular complications.

Data from experimental and clinical studies have shown that diabetes causes cardiac changes at the structural, functional, and pathological levels. Structural abnormalities can be manifested by an increase in left ventricle (LV) mass, wall thickness, and arterial stiffness. Also diabetes has been shown to be associated with

concentric hypertrophy (defined by LV mass and wall thickness) which is linked to diastolic dysfunction [46]. Increasing evidence highlights impairment in diastolic function with increased LV end-diastolic pressure and a decreased LV end diastolic volume with a normal ejection fraction (EF) in diabetes mellitus [47-49]. In response to exercise, diabetic patients demonstrate a lower left ventricular ejection fraction (LVEF) which accounts for a reduction in cardiac reserve [50]. However, there is limited evidence describing the right ventricle diastolic dysfunction in patients with diabetes [51]. At the pathological level, results from diabetic heart biopsies reported an increase in cardiomyocyte protein glycosylation, myocardial interstitial fibrosis, as well as myocyte hypertrophy. Additional data demonstrate an increase in collagen deposition, advanced glycation end-products (AGEs) formation, and reactive oxygen species (ROS) production in diabetic cardiac tissues [52-54]

3. Diabetic cardiomyopathy

Rubler *et al*. was the first to introduce the concept of diabetic cardiomyopathy (DC) in 1972 after studying the pathology of four autopsy cases having diabetic glomerulosclerosis with no known cause of heart failure [55]. The definition of DC was known as diabetes-induced pathophysiological condition that causes myocardial structural and functional alterations or ventricular dysfunction independent of other factors. It can lead to heart failure in the absence of other events like coronary artery disease, hypertension, and valvular heart disease[13]. DC occur in progressive stages starting with metabolic and structural changes in the ventricle leading to ventricular hypertrophy and dysfunction resulting in heart failure [14]. It begins with a diminished

diastolic function, while the systolic function is preserved until the disease reaches a more advanced stage [49].

The first stage of DC is clinically asymptomatic being characterized by increased fibrosis and stiffness. There are also early manifestations of diastolic dysfunction including early defects in diastolic filling, increased atrial filling and enlargement, prolonged isovolumetric relaxation, as well as elevated LV end-diastolic pressure [15, 56]. There are several systemic contributors behind these pathological events including hyperglycemia, hyperinsulinemia, insulin resistance, oxidative stress, activation of the renin–angiotensin–aldosterone system (RAAS), and inflammation [15, 16]. Also local perturbations are involved such as myocardial lipotoxicity and glucotoxicity, mitochondrial dysfunction, altered substrate utilization, and impaired calcium homeostasis [16].

In the second stage of DC, LV hypertrophy, reduced LV compliance, and cardiac remodeling are observed. As such diastolic dysfunction becomes more advanced. Consequently, heart failure with normal ejection fraction occurs. At late stages of the disease, diastolic dysfunction may coexist with systolic dysfunction and eventually ejection fraction is reduced, pre-ejection performance is prolonged, ejection period is shortened, and the filling resistance and pressure become higher [15].

A. Mechanisms underlying diabetic cardiomyopathy

The pathogenesis of DC is complex and multifactorial. It is essential to understand the underlying mechanisms as it facilitates the development of possible new therapeutic strategies. Hyperglycemia and its outcomes are considered the main

instigators associated with DC. In diabetes, the chronic exposure to high glucose levels in the blood can cause damages through an increase in ROS and AGE.

ROS consist of both free radicals (superoxide) and chemicals that generate free radicals (hydrogen peroxide). In hyperglycemia environment, the production of ROS in the mitochondrial respiratory chain exceeds their degradation by antioxidant defense leading to oxidative stress. This in turn reduces myocardial contractility and eventually causes myocardial fibrosis [17]. Moreover, oxidative stress induces cardiomyocyte apoptosis and cellular DNA damage which activates poly (ADP-ribose) polymerase (PARP) as a reparative enzyme [17, 57]. This enzyme cause cardiac damage through activation of nuclear factor kappa (NF-κB) which may contribute to the switch from α-myosin heavy chain (α-MHC) to β-myosin heavy chain (β-MHC) isoform expression in diabetic hearts [58, 59]. An elevation in transverse growth factor β (TGF-β) is observed in DC cases due to the activation of PARP which are also involved in inter-myofibril and perivascular fibrosis [60]. Furthermore, PARP enzyme can redirects glucose metabolism from its usual glycolytic pathway into alternative biochemical pathways by inhibiting glyceraldehyde phosphate dehydrogenase (GADPH). This diversion is shown to be the inducer of hyperglycemia-mediated cellular injury. Examples of such injuries include increased formation of AGE and activation of protein kinase C (PKC) [57, 58]. PKC impaired calcium handling and cardiac contractility in myocytes as it phosphorylates proteins that are directly participating in the cardiac excitation-contraction coupling [61]. Importantly, the increased production of AGE can alter structural proteins producing crosslinks within or between proteins. The crosslinks involve collagen and elastin; long-lived extracellular proteins, affecting the ability of collagen to be degraded and thus being

accumulated resulting in fibrosis. This also progresses to increased myocardial stiffness and impaired cardiac relaxation [62, 63]. AGE acts through its receptors (RAGE) that are found on cardiomyocyte and activated by oxidative stress in the diabetic myocardium [64]. Upon RAGE activation, NF-κB signaling pathway is also activated triggering a cascade of events, which finally switch (MHC) gene expression [59].

Moreover, a decrease in the activity of calcium (Ca^{2+}) pump is considered another player in the development of DC. It was found that in DC, the impairment of Ca^{2+} handling increases action potential duration and prolongs diastolic relaxation time [65]. Moreover, the reduction of sarcoplasmic reticulum Ca^{2+} pumping and the impaired SR Ca^{2+} reuptake leads to abnormal cytosolic Ca^{2+} transient and increased SR Ca^{2+} leakage [66, 67]. These events are considered to play a key role in cardiac diastolic dysfunction of an early DC.

Several investigations from human and animal experiments support the role of activation of RAAS in development of DC. This hyperglycemia-induced activation result in high levels of intracellular angiotensin II (AGT II) which has a direct effect on cell signaling via its receptor [68]. It leads to cardiomyocyte hypertrophy, upregulates the activity of NADPH oxidase, and increases ROS production [69]. This will eventually result in oxidative damage to cardiomyocytes and endothelial cell apoptosis and necrosis [70]. It was also shown that the elevated amount of ROS in DC not only increases expression of AGE receptors but also inhibits endothelial nitric oxide synthase (eNOS) and prostacyclin synthase activity [71]. Thus, the increase in fibronectin and collagen levels in the myocardium and myocardial interstitial characterize the fibrotic pattern of DC [18].

Therefore, further investigations are crucial to precisely understand the mechanisms implicated in initiation and progression of DC, facilitating the generation of novel therapies.

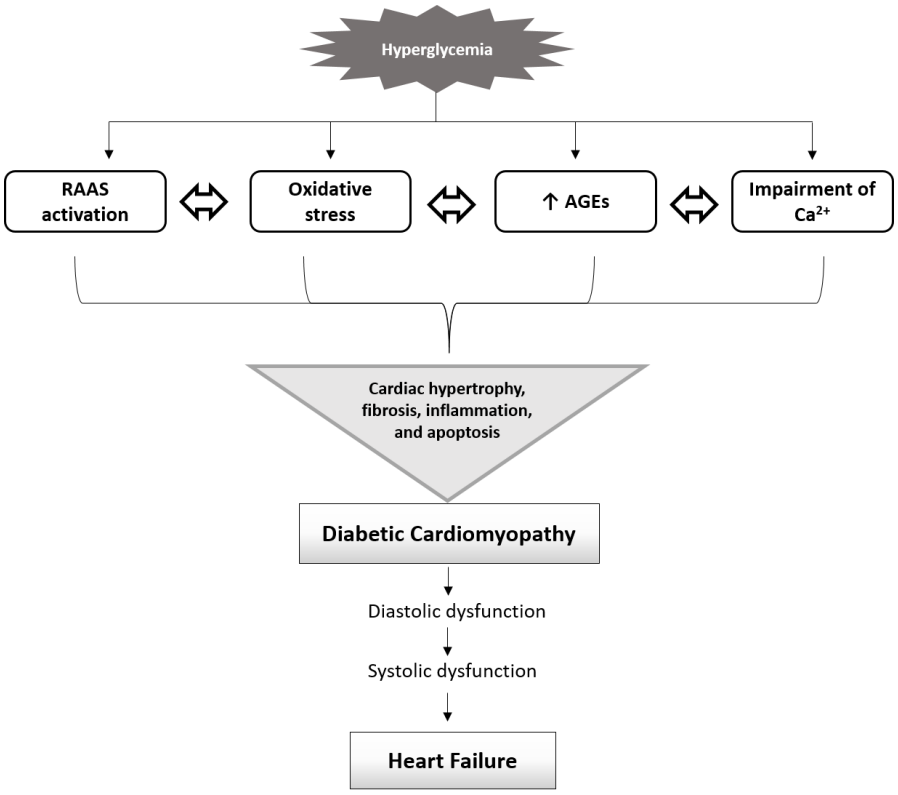


Figure 1 : General pathophysiological mechanisms of diabetic cardiomyopathy. Schematic representation of the general potential mechanisms that have been implicated in the pathogenesis of diabetic cardiomyopathy resulting in heart failure. The chronic exposure of hyperglycemia trigger activation of RAAS, oxidative stress, AGEs formation, and impairment of Ca^{2+} handling. These hyperglycemia-induced mechanisms can induce cardiac hypertrophy, fibrosis, inflammation, and apoptosis. This will eventually lead to diabetic cardiomyopathy, and thus diastolic dysfunction, systolic dysfunction in more advanced stage, resulting in heart failure.

B. Diagnosis of diabetic cardiomyopathy

Currently, there are several diagnostic strategies for DC that can assess functional, structural, metabolic, and/or hemodynamic changes [72, 73]. One of the best approaches is the echocardiography; Tissue Doppler Imaging (TDI), that can measure

myocardial tissue velocities during the cardiac cycle and quantitatively assess regional and global diastolic and systolic myocardial functions. It provides the percentage of ejection fraction and fractional shortening that are found to be reduced in DC below 70% and 35% respectively. The magnetic resonance imaging (MRI) is another diagnostic modality that can identify myocardial steatosis and diastolic dysfunction via late gadolium-enhancement technique. In addition to the magnetic resonance spectroscopy (MRS) that can measure myocardial Triglycerides (TG), phosphocreatine (PCr), and ATP levels in the myocardium. It demonstrates an increase in TG content and a reduced PCr/ATP ratio in DC. Moreover, the coronary angiography and cardiac catheterization are considered an important diagnostic tool to assess hemodynamic events within the heart. They can invasively determine the mean pulmonary capillary wedge pressure > 12 mmHg or the LV end-diastolic pressure > 16 mmHg as important diagnostic parameters for diastolic dysfunction. Several serum/plasma cardiac biomarkers could be also used in diagnosis of DC. They are shown to be elevated in DC. These parameters may include matrix metalloproteinase (MMP) for myocardial fibrosis, brain natriuretic peptide (BNP) (> 90 pg/mL) and Troponin for LV dysfunction.

4. Stem Cells

A. General Overview

Stem cells are unspecialized and undifferentiated cells with the ability to proliferate and regenerate. They also have paracrine secretion function, as well as the potential to differentiate into almost any cell type of the body. Any improper step in differentiation or proliferation of stem cells can lead to serious medical conditions such

as birth defects or cancer. Recently, stem cell topic has developed a great interest for researchers and scientists. It can help in better understanding of several body functions and mechanisms in both physiological and pathophysiological states [74-76]. Moreover, stem cell research put a promising hope in medicine as it may bring new ways of treating several chronic and incurable diseases. In fact, stem cells are characterized by antifibrotic, pro-angiogenic and anti-apoptotic properties that may enhance treatment of diseases for which pharmacological or surgical therapies are lacking. Currently, stem cell-based therapies provide a therapeutic approach for spinal cord injury, heart failure [77], retinal and macular degeneration [78], tendon ruptures, and T1DM [79]. However, there are several challenges in the clinical development of stem cell therapy including dosage and route of administration, risk of tumorigenesis, autologous and allogeneic grafting, duration and degree of cell expansion, as well as their unclear underlying mechanisms [80-84]. Therefore, further in-vivo and in-vitro applications are necessary in order to provide a long-term, efficient, and safe stem cell therapy in human clinical trials. Interestingly, emerging studies describe that the protective mechanisms underlying stem cell therapy are most likely attributed to stem cells paracrine secretions rather than their differentiation potential [85]. It is strongly proved that this stem cell secretome, also termed as MSC-conditioned media (MSC-CM), can strictly regulate vital cellular functions including proliferation, differentiation, communication and migration [86]. Fortunately, it was shown that stem cell secretome offers numerous advantages over stem cell-based strategies with similar therapeutic effects [87]. Using stem cells and their secretome, as a treatment tool, could be a turning point in modern medicine.

B. Types

Stem cells can be divided into embryonic, cord blood, and adult stem cells which are further divided into several other types [75, 76].

i. Embryonic Stem Cells

Stem cells that exist in developing embryos are found at different stages. Early embryonic stem cells at the morula stage are the least differentiated cells and can give rise to embryonic and extra embryonic structures. Embryonic stem cells at the blastocyst stage are found in its inner cell mass and can differentiate into all specialized embryonic tissues to develop into a human body. They are less specific than adult stem cells and can differentiate into more cell types, thus called pluripotent [75, 76].

ii. Cord Blood Stem Cells

The cord blood stem cells are harvested from the umbilical cord after childbirth. These cells can be frozen in cell banks for later use in the future to treat children with blood cancers or certain genetic blood disorder [75, 76].

iii. Adults Stem Cells

Adult stem cells are found in different types of tissues and stay throughout life so that the body can use them once needed. These cells remain unspecific and non-dividing until they are required to do specific purposes. They act as a repair and maintenance system for the body; dividing regularly, renewing its tissues constantly and replenishing specialized cells definitely. Scientists have recently found how to genetically reprogram adult stem cells to behave like embryonic stem cells, and they are termed as induced pluripotent stem cells (iPSC). Adult stem cells can be further classified into 4 types; hematopoietic, neuronal, epithelial, and mesenchymal stem cells [75, 76].

5. Mesenchymal stem cells

A. General overview

Mesenchymal stem cells (MSCs) are first described by Friedenstein in 1974 as a unique subset of adult stem cells [88]. They possess a broad differentiation potential into endodermal, mesodermal and ectodermal lineages, unlike tissue-specific adult stem cells [89]. MSCs are traditionally derived from the bone marrow stroma but have also been isolated from other tissues including muscle, adipose, skin, cartilage, bone, fallopian tissue, umbilical cord blood and menstrual blood [90]. The multilineage differentiation of MSCs is comparable to those of embryonic stem cells; however, MSCs have overcome the ethical restrictions as well as the risk of tumorigenesis and maldifferentiation. Moreover, MSCs has avoided the issue of allograft rejection due to the fact that they can be easily obtained from the bone marrow of each patient [91]. In the last few decades, MSC's properties and mechanisms of action have highlighted its use in tissue engineering and regenerative medicine. In fact, their therapeutic regenerative effects are not related solely or directly to their differentiation ability. However, they are known to be exerted in a paracrine manner through the secretion of soluble factors [21, 92, 93]. These findings have highlighted the role of MSCs, their secretome, and their crucial role in clinical interventions.

B. The use of MSCs as cell-based and cell-free therapeutic agents

In a growing body of studies, MSCs injections into diseased tissues like heart and brain show functional enhancement with little differentiation of the surrounding cell types [94, 95]. These studies have shown that MSCs enhance wound healing, angiogenesis, and regeneration in the surrounding tissues. The regenerative benefits of

MSCs have appeared through modulation of local inflammatory responses, attenuation of fibrosis and apoptosis, and secretion of trophic factors for growth and survival [93, 96, 97]. Moreover, other studies revealed that MSCs are attracted and seem to migrate to pathological regions in the brain and heart such as ischemic areas and facilitate repair [98, 99]. However, MSCs-based therapies are limited by the risk of unwanted differentiation and immune responses. This was bypassed by MSCs-derived secretome as a cell-free therapeutic approach and considered to be an analog manner for conventional pharmaceutical agents. Interestingly, MSCs-derived secretome has been immediately available and massively produced from cell lines. In contrast, MSCs transportation require an invasive collection procedure and cell culture expansion to reach an optimal cell number [23]. Taken together, MSCs-secretome provides a safer, cheaper, and more efficient therapeutic approach. It avoids the risk of tumorigenicity, immunoreactivity and maldifferentiation associated with cell-based therapy as well as the expenses of cellular expansion and maintenance [87]. It was found that MSCs-secretome is now used as a treatment for fulminant hepatitis, cerebral ischemia, myocardial infarction, and other inflammatory and degenerative diseases. Their protective role was observed through attenuating fibrosis, inflammation, oxidative stress, and apoptosis [100-104].

C. Mechanism of action of MSCs

MSCs migrate from bone marrow to their target tissue through the circulatory system by a gradient of cytokines. The process by which MSCs can localize to damaged organs and/or injured tissues is defined as homing. It occurs in response to chemical signals released by the injured cells and involves interaction between the chemokine

ligand and chemokine receptor present on the surface of MSCs [105, 106]. MSCs are found to express a set of surface markers such as C-C chemokine receptor type 1 (CCR1), CCR7, C-X-C chemokine receptor type 4 (CXCR4), CXCR5, CXCR6, TGF-β receptor 2, TNF receptor superfamily member 1A, PDGF receptor A and B. Also, a number of cytokines has been discovered to control MSCs homing and migration including platelet-derived growth factor (PDGF), transforming growth factor-β (TGFβ), tumor necrosis factor (TNF), and stromal-derived factor 1 (SDF1) [107, 108]. For example, SDF1 that is also known as CXCL12 was shown to be expressed on endothelial cells in case of injury and interact with MSCs via its surface receptor (CXCR4) [109]. After MSCs reach their destination in the blood, they have to get into their target tissue. So, they must transmigrate through endothelial cell layers in a process that will involve selectin and integrin as adhesion molecules [108]. Once they reach their target, MSCs are assumed to act through several mechanisms. They might transdifferentiate into different cell types or engraft into the site of injury and eventually restore the function of the injured tissue [110]. This hypothesis was reinforced by several studies showing that MSCs will take residency in the injured tissues after the treatment [111]. Conversely, other studies demonstrate that the therapeutic effects of the injected MSCs can be exerted even if the MSCs are distant from their target organ or even after they disappear or become limited in number. This second hypothesis suggests that MSCs can function through local or systemic secretion of bioactive paracrine factors [21, 22]. This paracrine hypothesis is strongly supported by investigations that study the therapeutic effect of MSCs secretome. A study on a rat model receiving intravenous MSCs injections after 1 week of middle cerebral artery occlusion reveals a neurological improvement and decreased fibrosis, with

only a small number of MSCs present in the brain parenchyma [112]. Another study on hamsters with congestive heart failure shows that the injected MSCs enhance cardiac function and attenuate fibrosis. This study has reported that the MSCs did not travel outside of the hamstring and the level of trophic factors secreted from MSCs was high in circulation [21]. In addition, MSCs possess a powerful immunomodulatory effect by which they can inhibit proliferation, activation, and function of T cells, B cells, and natural killer cells [113, 114]. Taken together, MSCs exert its therapeutic effects through pro-angiogenic, immunosuppressive, antifibrotic, and anti-apoptotic benefits that, in turn, favor the regeneration of injured tissues.

D. Secretome of MSCs

MSCs-derived secretome is defined as the complex array of all factors that are derived from MSCs and secreted to the extracellular space, either as soluble bioactive molecules or as MSC-derived encapsulated extracellular vesicle (EV). These soluble components can consist of genetic materials (DNA, RNA fragments, microRNAs (miRNAs), enzymes, signaling and signal transduction proteins, immunomodulatory and growth factors, cytokines, hormones, and lipid mediators. This set of soluble molecules creates a proper microenvironment for regeneration [23-25]. A number of studies show that packaging the soluble molecules in EV allow better remote communication and targeting [115, 116]. MSC–derived EV (MSC-EV) is enveloped by a lipid bilayer enriched with proteins that promote adhesion, trafficking, and endocrine function. They can be divided into three types including apoptotic bodies, microvesicles and exosomes that are distinguished by size and origin. It is found that MSC-EV can travel distant sites

via biological fluids to exert their biological effects in an endocrine manner [23]. They interact with the target cell in various mechanisms. They can fuse with the plasma membrane to deliver their content directly into the cytosol of the target cell. Alternatively, they can bind to membrane-bound receptor, internalize their content, and trigger an intracellular signaling pathway in the target cell. It is found that MSC-EV can influence the function of immune cells, endothelial cells, pericytes and other tissue-resident cells [25]. Importantly, MSC-derived conditioned medium (MSC-CM) encompasses the whole set of soluble factors and vesicular component that are sourced from MSCs. A number of experimental animals demonstrate several biological outcomes upon administration of MSC-EV and MSC-CM. Collectively, MSC secretome mediates angiogenesis, modulates inflammation and immunity, inhibits fibrosis and apoptosis, and eventually enhances function and repair [102]. Interestingly, MSC secretome has bypassed several limitations due to their biological and logistical advantages over MSC-based therapy [23]. Thus, the administration of MSC-derived secretome has been highlighted and emphasized as a powerful therapeutic approach in inflammatory and degenerative diseases [87, 100, 117, 118].

i. Immunosuppressive and anti-inflammatory properties

In certain pathological cases, our immune system can cause harm and damage rather than defense and repair. In such situations, the immune system should be suppressed to ensure survival. Importantly, MSC-secretome contain a set of immunomodulatory factors that exert an immunosuppressive effect and regulate the immune system [25]. These factors include transforming growth factor-β (TGF-β), hepatic growth factor (HGF), indoleamine 2,3-dioxygenase-1 (IDO-1), interleukin (IL)-

10, IL-1 receptor antagonist (IL-1Ra) and prostaglandin E2 (PGE2), and nitric oxide (NO) [119, 120]. These factors lead to cascade of events that result in inhibiting the activation of natural killer cells as well as maturation and function of dendritic cells [121, 122]. They also have a role in switching the activated macrophages to an anti-inflammatory type [120]. Moreover, these factors can create a balance between pro-inflammatory T cells (T helper 1 (T_H1) cell and/or T_H17 cell) and anti-inflammatory T cells (Regulatory T (Tregs) cell and/or T_H2 cell) [123]. For example, a balance between Tregs and T_H17 cell was maintained by sphingosine 1-phosphate (S1P) contained in MSC secretome. When SIP is delivered from MSC secretome to CD4+T cells, IL-10-producing Tregs were generated but IL-17-producing Th17 cell expansion was decreased in peripheral blood of aplastic anemia patient [124]. Interestingly, several findings show that MSCs can change their secretory profile and immunomodulatory properties according to their local microenvironment. In the presence of high inflammatory milieu, MSCs obtain anti-inflammatory phenotype and produce immunosuppressive factors. In contrast, if MSCs are exposed to low concentration of inflammatory cytokines, MSCs possess a pro-inflammatory phenotype and generate large amounts of inflammatory factors [123, 125].

ii. Anti-fibrotic properties

In general, human body responds to injuries by fibrosis (the process of scar formation) rather than by regeneration to heal wounds and injured tissues. However, some tissues such as liver, epidermis, or uterine may have the potential to regenerate in case stem cells are residing in these tissues [126, 127]. It is thought that MSCs secretome can possess regenerative potential instead of fibrotic role during tissue healing process after an injury or surgery. Studies show that animals treated with MSC-conditioned

media (MSC-CM) present an anti-fibrotic effect which could be the cause of a reduced scar formation and an enhanced wound healing process [128]. Moreover, MSC-CM exert their anti-fibrotic effect through trophic and anti-inflammatory cytokines which allow keratinocyte and endothelial cells to migrate to injured sites [129]. In fact, some fibrotic responses are induced by inflammation. For example, MSCs treatment for chronic kidney disease (CKD) rat model results in attenuation of fibrosis and glomerulosclerosis. This is attributed to a downregulation of pro-inflammatory genes encoding IL-6 and TNF, as well as an upregulation of the expression of genes that encode anti-inflammatory cytokines, such as haemoxygenase 1 (HO1) and hepatocyte growth factor (HGF) [130, 131].

iii. Anti-apoptotic properties

Several findings describe the ability of MSC-secretome to regulate apoptosis in pathological and physiological states. They demonstrate that MSC-derived bioactive factors can inhibit apoptosis and induce cytoprotective effects, directly or indirectly. They can either cause direct prevention of apoptosis or exert their anti-apoptotic effect via their immunomodulatory and angiogenic actions. A number of detected cytokines in the MSC-CM play a role in suppressing apoptosis such as PDGF and insulin-like growth factor 1 (IGF1), as well as VEGF and IL-1β. Significantly, MSC-CM downregulate the expression of pro-apoptotic BAX and caspase-3 but upregulate the expression of BCL-2 which is an anti-apoptotic protein in alveolar macrophage [23, 132]. Interestingly, a complete opposite action was observed when tumor cells were treated with MSC-CM. It attenuates cancerous cells growth and allows the survival of tumor bearing mice. This powerful effect was noticed using MSC-CM derived from uterine cervix and not from adipose tissue [133]. These findings indicate that the origin of MSCs can influence the

content of MSC-CM and eventually its effects. Thus, a precise assessment of the components of MSC-CM is necessary before clinical applications.

iv. Pro-angiogenic properties

Angiogenesis is defined as the process of formation of new blood vessels from already existing one, in order to provide oxygen and nutrients to injured tissues. It was shown that a number of pro-angiogenic cytokines are found in MSC-CM including vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), placental growth factor (PGF), platelet-derived growth factor (PDGF) and monocyte chemoattractant protein 1 (MCP1; also known as CCL2). In *in vitro* studies, MSC-CM can promote proliferation of endothelial cells through these angiogenic cytokines [22]. This was also replicated when MSC-CM was injected in a mouse model with ischemic disease. The beneficial effects of MSC-CM can enhance blood flow and promote collateral formation in paracrine mechanisms [134]. Moreover, a recovery of renal function after acute kidney injury (AKI) was attributed to the vasculogenic characteristics of MSC secretome [135]. It is important to mention that the concentration of pro-angiogenic and anti-angiogenic cytokines are affected by the conditions in which MSCs are cultured. In a hypoxic microenvironment, MSCs derived HIF-1α (hypoxia-inducible factor 1 alpha) is mainly responsible to increase generation of VEGF [136]. However, an inflammatory condition can promote the anti-angiogenic effect of MSCs secretome [137]. Unfortunately, cancer cells might harness the beneficial properties of MSCs secretome to grow and proliferate [138]. Indeed, proper culture conditions of MSCs and their secretome should be maintained to prevent any unexpected outcomes.

6. MSCs applications in diabetes and diabetic complications

In diabetes mellitus, MSCs therapy was shown to help patients in reducing their dependency on medications and in enhancing their quality of life. In some cases, MSC therapy was described to reverse diabetic damages and symptoms [79, 139]. MSCs can protect diabetic tissues from inflammation-induced injuries including oxidative stress and pancreatic beta cells damage and apoptosis. This was proved by a study revealing that IL-1Ra (Interleukin-1Ra) expressed by MSCs can effectively binds to IL-1 receptor, which is responsible for various pro-inflammatory events leading to its inhibition. These data suggest that MSC may provide a potential therapeutic approach for T1DM and T2DM and their complications [140]. In STZ rodent model with diabetic retinopathy, IV injection of adipose-derived MSCs significantly decreased blood glucose levels and preserved the integrity of blood-retinal barrier [141]. In another study using the same model, intravitreal injection of placental derived MSCs significantly reduced retinal apoptosis and increased the concentrations of neuroprotective growth factors in the retina and vitreous chamber [142]. Furthermore, MSCs transplantation in preclinical studies was shown to be a promising potential therapy to treat diabetic kidney disease [143]. Enhancement in renal and pancreatic function was observed in diabetic mice upon transplantation with MSCs. This was detected by reduction in mesangial thickening and macrophage infiltration, increase in pancreatic islets and beta cells producing mouse insulin, as well as enhanced repairing of renal glomeruli [144]. Both proteinuria and podocyte damage were prevented upon intra-arterial administration of MSCs in Type 1 diabetic rat models with nephropathy. This approach attenuates the loss of podocytes, foot process effacement and widening, as well as the loss of nephrin and podocin [145-

147]. In addition, MSCs and their secretome can offer a novel therapy to treat diabetic neuropathy. In diabetic rodent model, intramuscular injections of MSCs ameliorate symptoms of diabetic neuropathy in paracrine manner [148]. It was also found that bone marrow MSCs (BM-MSCs) transplantation in diabetic mice models decrease sciatic nerve blood flow, improves conduction velocity in the motor nerve, and switches pro-inflammatory macrophages to anti-inflammatory macrophages [149].

7. MSCs applications in cardiovascular diseases

An emerging body of evidence assessed the role of MSCs and their secretome in cardiovascular diseases including diabetic cardiomyopathy describing their cardioprotective effects. In vivo and in vitro studies demonstrate the beneficial effects of MSC secretome particularly MSC-Exosomes (Exos) in Rodent model with regional myocardial ischemia/infarction. MSC-Exos significantly restore cardiac contractile function and reduce the infarct size. It also contributes to a reduction in cardiomyocyte apoptosis and an increase in their survival [104]. Similarly, in an ischemic/reperfused heart, MSC-Exos reduce infarct size and enhance the myocardial viability. This occurs through activation of PI3K/Akt signaling pathway by which it also reduces oxidative stress and improves cardiac structure and function [20]. More experimental studies resulted in same cardioprotective outcomes via other signaling pathways such as AMPK/mTOR and Akt/mTOR [150]. Moreover, MSC secretome can rely on their anti-inflammatory effects to attenuate cardiac dilation and reduce cardiomyocyte apoptosis in cardiomyopathy heart [151]. In addition, the beneficial effects of MSC-secretome may be observed on cardiac stem cells (CSC). Administration of MSC-Exos significantly

improves survival, regenerative potency, and angiogenic ability of CSC. This function improvement is attributed to specific microRNAs (miR-15, miR-21, miR-22, miR-126, miR-146a, miR-210) that are delivered from MSC-Exos to CSC [152, 153]. Furthermore, endothelial cells of the heart blood vessels can benefit from MSC secretome via delivery of (SDF-1) and (miR-132) resulting in neo-angiogenesis and neovascularization respectively [154, 155]. Based on preclinical studies, the effect of MSC transplantation was assessed in clinical trials on myocardial infarction and ischemic cardiomyopathy. Three months after intravenous administration of autologous BM-MSCs to 34 patients, LV ejection fraction was improved, and ventricular tachycardia was reduced. This study provides a safe administration procedure of MSCs to human population [156].

8. MSC applications in diabetic cardiomyopathy

In diabetic cardiomyopathy, MSC treatment has attracted widespread attention in preclinical and clinical studies. Cardioprotection and cardioregeneration was shown in experimental studies using type 1 diabetic rats. IV administration of BM-MSCs resulted in cardiomyocyte differentiation, angiogenesis improvement, as well we reduction in collagen deposition in the myocardium [157]. Similarly, intramyocardial administration of MSCs into diabetic rat lead to increased secretion of angiogenic and anti-apoptotic factors and reduction in myocardial fibrosis. Also, the injected MSCs were differentiated into cardiomyocytes, vascular endothelial cells, and smooth muscle cells [158]. Interestingly, it was found that anoxic pre-conditioning of MSCs enhances their protective effect resulting in an increased fractional shortening and attenuation of fibrosis and apoptosis [19]. Moreover, beneficial cardiac effects were seen in STZ-induced type 1

diabetic mice. However, in mice with obesity-induced diabetic cardiomyopathy, no cardio-protective effects were observed following MSC administration. The contradiction in these two studies could be attributed to the difference in the route, time, dose, and culture conditions of the administrated MSCs [159, 160]. In another study, adipose-derived MSC were administered intravenously to T2DM rat model with cardiomyopathy. This resulted in attenuation of myocardial fibrosis and cardiac dysfunction through secretion of prostaglandin E2 cytokine [161]. Importantly, MSCs cardioprotective effects were seen in doxorubicin (DOX)-induced cardiomyopathy animal model, not through direct differentiation but more likely via paracrine secretions, anti-inflammatory and anti-oxidative actions [162].

Taken all together, MSC and their secretome could possess a promising potential therapy to treat diabetic cardiomyopathy, but challenges remain in terms of all factors that can influence their outcome.

CHAPTER II
AIM OF THE STUDY

Despite the availability of several tools to tackle diabetes including effective drug therapies, advanced technologies, and preventive strategies, the struggle to protect people from diabetes and diabetic cardiomyopathy remains a challenge. In pharmacological therapy, several drugs have been recommended for patients with diabetic cardiomyopathy including anti-diabetic, vasoactive, and lipid lowering medications. However, tight glycemic control remains hard to achieve and is always burdened with complications. Therefore, scientists nowadays aim to investigate new therapeutic strategies based on regenerative medicine for diabetic cardiomyopathy. In this context, stem cells-based therapy has attracted widespread attention in recent years, particularly the use of mesenchymal stem cells. Several studies have demonstrated the beneficial cardioprotective effect of MSC and MSC-derived secretome through attenuating diabetes-induced cardiac damage.

In our study, we aim to assess the therapeutic effect of MSCs-conditioned media (MSC-CM) and to investigate the molecular mechanisms by which MSC-CM exert this protective role. We also aim to determine the effect of MSC-CM on cardiac function in Streptozotocin (STZ)-induced type 1 diabetic rats, particularly ejection fraction and fractional shortening. We intend to investigate the molecular changes in cardiac markers of injury including α-SMA and fibronectin and assess the histological alterations mainly fibrosis. Furthermore, we aim to assess the effect of MSC-CM on diabetic

cardiomyopathy-associated oxidative stress, studying the activity of NADPH oxidase 4 (Nox4).

In that spirit, we hypothesized that MSC-CM may enhance cardiac function through restoration of fractional shortening and ejection fraction. We suggest that MSC-CM could ameliorate myocardial fibrosis by decreasing collagen deposition and glycosylated proteins in the left ventricle tissue. We also hypothesized that MSC-CM can decrease ROS production and promote molecular changes by downregulating the expression of α-SMA, fibronectin and Nox4, thus attenuating cardiac hypertrophy, fibrosis, oxidative stress, and other diabetes-induced cardiac injuries.

CHAPTER III
MATERIALS AND METHODS

1. Treatment preparation

The treatment given to our rat models is conditioned media (CM) derived from rat bone-marrow mesenchymal stem cells (MSCs) that is prepared in the cell culture under the hood. The cells are incubated in a T75 flask in Dulbecco's modified Eagle's medium (DMEM)-low glucose under standard conditions at 37ºC in a 5% CO2 incubator. Once the cells reach 80% confluency, the media is removed and 6ml serum starved DMEM-low glucose media is added to the T75 flask. The cells are then incubated for 48 hours under standard conditions at 37ºC in a 5% CO2 incubator so that the cells secrete its secretome in the flask. The media is then collected as MSC-conditioned media (MSC-CM). MSC-CM is transferred to a 15 ml conical and centrifuged at 1200g for 5 minutes. The supernatant is transferred into another conical. At this step, the treatment is either injected intravenously in the rat or stored at -80ºC for further use. The dose of injection is 2 ml of CM per rat.

2. Experimental animal

Male Sprague-Dawley rats were kept in a temperature-controlled room and on a 12/12 dark/light cycle and had free food and water access. Body weight and blood glucose level of all rats were measured every week throughout the whole project using a

glucometer (Contour Plus). All protocols were approved by the Institutional Animal Care and Use Committee of the American University of Beirut.

At 12 weeks of age; rats were injected intravenously via the tail vein with one single high dose of 60 mg/kg of Streptozotocin (STZ) dissolved in sodium citrate buffer (0.01 M, pH 4.5) to induce T1DM. Aged-matched control rats were injected with an equivalent amount of sodium citrate buffer alone. After 2 days, rats with blood glucose levels greater than 250mg/dL were considered as diabetic.

At 18 weeks old; control and diabetic rats were injected intravenously with 2 ml of CM (prepared as previously mentioned) once every week. At the same time, other control and diabetic rats were injected weekly with 2 ml serum starved media.

As such, we have 4 groups of rats: (1) Control with weekly injection of serum starved media; (2) Control with weekly injections of MSCs-conditioned media, (3) Diabetic with weekly injection of serum starved media; (4) Diabetic treated with weekly injections of MSCs-conditioned media.

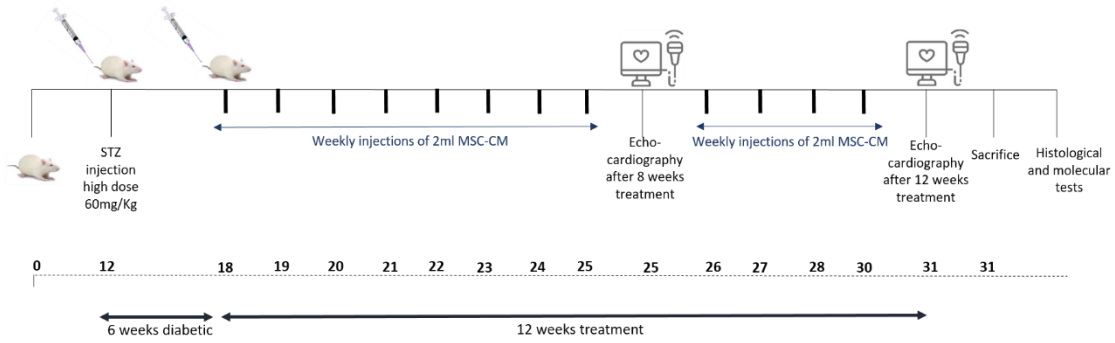


Figure 2: Experimental design and treatment plan. After 6 weeks of STZ-induced diabetes. Rats were injected intravenously via tail with 2 ml MSC-CM for 12 weeks.

Echocardiography measurements were done after 8 and 12 weeks of treatment. Then rats are sacrificed and other histological and molecular tests are performed.

3. Echocardiography

Rats underwent echocardiography at 8^{th} week of treatment and 12^{th} week of treatment before sacrifice using 2D electrocardiography. The rats were weighed and anesthetized intraperitoneal using ketamine 250 mg/5ml (40-80 mg/Kg) and xylazine 20mg/ml (5-10mg/Kg). Initially, 70% of the dose is administered. If after few minutes the rats weren't anesthetized, the remaining 30% of the dose is given. The anesthesia should be maintained throughout the echocardiographic examination. After shaving the animals from the anterior chest, rats were placed in supine position and transducer was placed directly on the shaved area. M mode and two-dimensional echocardiography images were obtained and analyzed. Echocardiographic parameters are measured including LV end diastolic diameter (LVEDD), LV end systolic diameter (LVESD), LV end diastolic volume (LVEDV), and LV end systolic volume (LVESV). Other parameters such as percentage of LV fractional shortening and LV ejection fraction are derived by using the following equations: FS= (LVEDD-LVESD) *100/LVEDD and EF= (LVEDV-LVESV) *100/LVEDV.

4. Histological Staining

Rats were euthanized, and the heart was removed and weighed. Cuts of the left and the right ventricle tissues were flash frozen. A cut of the left ventricle was fixed in 4% formaldehyde and then tissues were embedded in paraffin blocks. Blood was collected into

EDTA tubes for HbA1c test. Functional, histological, biochemical, and molecular parameters of the heart were assessed. From the left ventricle of each animal, two cross sectional slices of 4-5μm thickness each were cut and mounted on a glass slide.

Tissues were stained with Masson's trichrome stain to detect collagen deposition with green color, and periodic acid Schiff (PAS) that stains glycosylated depositions in blue. Tissues were examined under a light microscope and several pictures were taken per section at 20X, 40X, and 100X. Analysis was performed on 40X images using Image J software. Figures are represented at 100X.

5. Western Blot Analysis

Cuts from the left ventricle tissue were homogenized in 200 μL RIPA lysis buffer (0.1% sodium dodecyl sulfate (SDS), 0.5% sodium deoxylate, 300mM sodium chloride (NaCl), 100 mM EDTA, 100mM Tris-HCl pH 8, 1% Tergitol (NP-40), 1mM phenyl-methane-sulfonyl-fluoride (PMSF), protease inhibitor cocktail, and phosphatase inhibitor cocktail). Homogenates were placed in TissueLyser machine with P8 memory, frequency 30 1/sec, and for 3 minutes. Then, the homogenates were incubated overnight on the rotator at 4°C and centrifuged the next day at 13,500 rpm for 30 minutes at 4°C. The supernatant containing the proteins was collected and stored at -20°C. Protein concentration was measured using Lowry Protein Assay.

For immunoblotting, 40 μg of proteins of left ventricular homogenates were separated on 8-12% polyacrylamide gel electrophoresis according to their molecular weights (BioRad Laboratory, CA, USA). The gel was then transferred to a PVDF

membrane (BioRad Laboratory, CA, USA) that was activated in methanol for 30 seconds and then placed in transfer buffer for 2 to 3 minutes. The membranes were then blocked with 5% Bovine Serum Albumin (BSA) for one hour. Afterwards, membranes were incubated with one of the following primary antibodies overnight: rabbit polyclonal anti-αSMA (1:1000, Abcam 5694), rabbit polyclonal anti-Fibronectin (1:1000, Abcam 2413), rabbit polyclonal anti-NOX4 (1:000, Abcam 133303).

The primary antibodies were detected using the appropriate horseradish peroxidase conjugated IgG (1:10,000 for mouse, BioRad). Bands were visualized by enhanced chemi-luminescence ECL (BioRad), and they were semi-quantified by densitometric analysis using Image J software.

6. Detection of intracellular superoxide in LV tissue using HPLC

Cellular superoxide production in podocytes was assessed by High-performance liquid chromatography (HPLC) analysis of Dihydroethidium (DHE)-derived oxidation products, as described previously (S. Eid et al., 2016). The HPLC-based assay allows separation of superoxide-specific 2-hydroxyethidium (EOH) from the nonspecific ethidium, as previously described. Briefly, The LV homogenate was dried under vacuum and analyzed by HPLC with fluorescence detectors. Quantification of DHE, EOH, and ethidium concentrations was performed by comparison of integrated peak areas between the obtained and standard curves of each product under chromatographic conditions identical to those described above. EOH and ethidium were monitored by fluorescence detection with excitation at 510 nm and emission at 595 nm, whereas DHE was

monitored by ultraviolet absorption at 370 nm. The results are expressed as the amount of EOH produced (nmol) normalized for the amount of DHE consumed (i.e., initial minus remaining DHE in the sample; μmol).

7. Statistical Analysis

Results are represented as Mean ± Standard Error of Mean (SE) for n=3. Statistical significance is determined using one-way ANOVA, followed by Fisher's LSD test. Statistical Significance was determined as a probability (P value) of less than 0.05. All statistical analyses were performed with Prism 8 Software (GraphPad Software).

CHAPTER IV
RESULTS

1. Metabolic parameters

The therapeutic effect of MSCs-conditioned media was assessed in STZ induced type 1 diabetic rat model. Blood collection at the end of the study was performed for HbA1c analysis. High HbA1c levels was observed in both diabetic (8.833 ± 0.5487) and diabetic treated with MSC-CM (8.667 ± 0.2603) when compared to controls (5.4 ± 0.2646) and control with MSC-CM (4.633 ± 0.1764). Our finding indicates that treatment with MSC-CM didn't restore glycated hemoglobin levels to normal. This indicate that our results weren't based on changes in glucose levels. **(Figure 3) (Table 1)**.

Also, heart weight was assessed. It shows a significant decrease in diabetic groups (1.39 ± 0.15) compared to controls (2.03 ± 0.10), which may suggest a heart atrophy in diabetic rats. Moreover, heart weight showed a trend to increase in diabetic treated groups (1.617 ± 0.04) compare to diabetic, but wasn't restored to normal. This may suggest a little enhancement in the heart weight upon treatment with MC-CM **(Table 1)**.

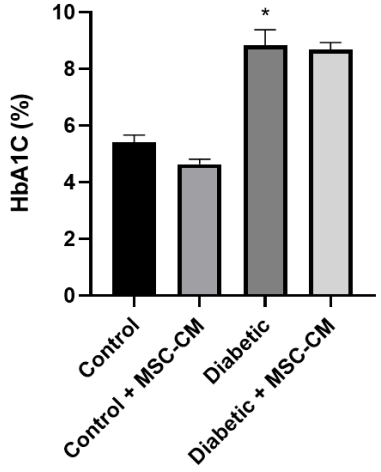


Figure 3: Glycated hemoglobin levels in (%) by HbA1c tests. HbA1c done by HPLC using the variant II Hemoglobin Testing System (BIORAD) on blood samples collected at sacrifice. Measurements were performed for the following groups: Control, Control + MSC-CM, Diabetic, and Diabetic + MSC-CM. Results were expressed for n=3 and values are mean ± SE
* is used for $p<0.05$ vs control

	Control	**Control + MSC-CM**	**Diabetic**	**Diabetic + MSC-CM**
HbA1c (%)	5.4 ± 0.2646	4.633 ± 0.1764	8.833 ± 0.5487*	8.667 ± 0.2603
Heart Weight (g)	2.03 ± 0.1054	1.77 ± 0.2364	1.39 ± 0.1562 *	1.617 ± 0.04163

Table 1: Metabolic parameters: HbA1c (%) and Heart weight (g) of the following groups: Control, Control + MSC-CM, Diabetic, Diabetic + MSC-CM.
Results were expressed for n=3 and values are mean ± SE
* is used for $p<0.05$ vs control

2. Treatment with MSC-CM enhances cardiac function

In both echocardiography assessments (after 8 and 12 weeks of treatment), EF% and FS% decreased significantly in the untreated diabetic rats when compared to their control littermates **(Figure 5-6)**. At 8th week of treatment, EF% and FS% didn't show a significant increase, yet, a tendency to be corrected in diabetic rats treated with MSC-CM was observed **(Figure 5)**. Importantly, after 12 weeks of treatment with MSC-CM, the EF% and FS% significantly increased in diabetic group treated with MSC-CM to values similar to that of the control group **(Figure 6)**.

Our echocardiography results suggest that MSC-CM significantly enhances the cardiac function in STZ-induced type 1 diabetic rats after 12 weeks of treatment. This enhancement was manifested in the left ventricle function mainly by EF% and FS%.

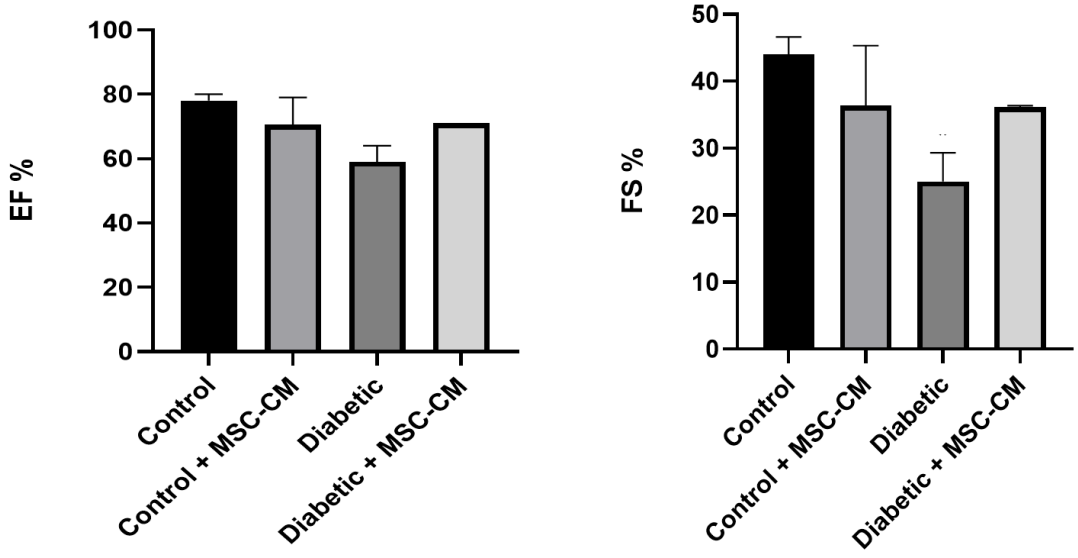


Figure 4: Left Ventricular Ejection Fraction and Left Ventricular Fractional Shortening Indicators of Cardiac Function. Rats were anesthetized at 8th week of

treatment and 2D echocardiography was performed on the following groups: Control, Control + MSC-CM, Diabetic, Diabetic + MSC-CM.
Results were expressed for n=3 and values are mean ± SE
* is used for p<0.05 vs control

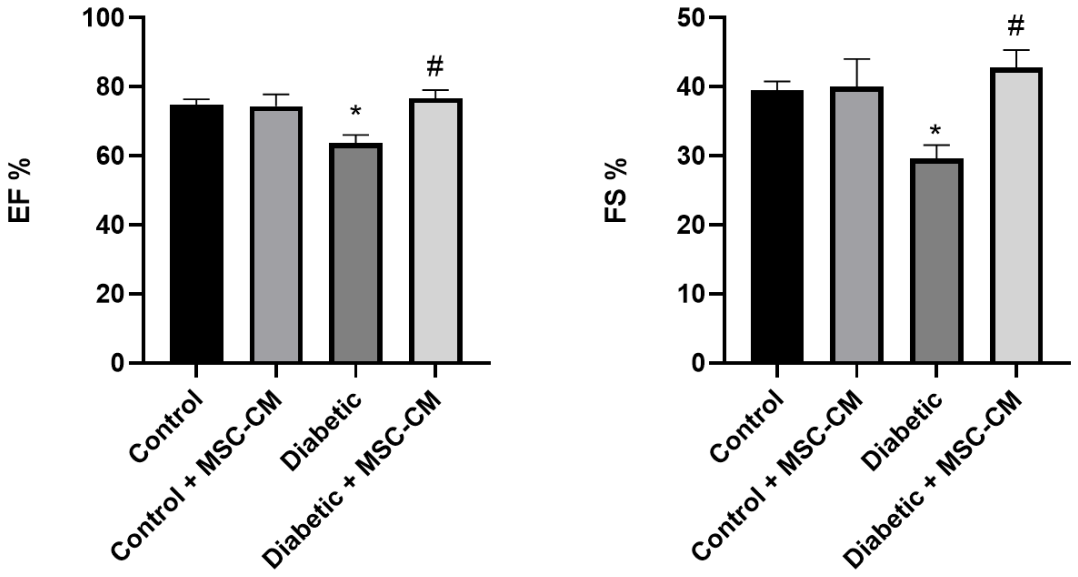


Figure 5: Left Ventricular Ejection Fraction and Left Ventricular Fractional Shortening Indicators of Cardiac Function. Rats were anesthetized at 12th week of treatment and 2D echocardiography was performed on the following groups: Control, Control + MSC-CM, Diabetic, Diabetic + MSC-CM.
Results were expressed for n=3 and values are mean ± SE
* is used for p<0.05 vs control
# is used for P<0.05 vs diabetic

3. Treatment with MSC-CM significantly decreases collagen deposition and glycosylation

Masson's Trichrome is a stain that detects collagen deposition which is considered a major contributor to myocardial fibrosis. Our results show that hyperglycemia induces collagen deposition in the left ventricle tissues of untreated

diabetic rats when compared to that of the control group. Interestingly, rats treated with MSC-CM show a significant decrease in collagen deposition compared to the untreated diabetic group **(Figure 7-A, 7-B)**.

PAS is a stain used to detect glycosylated proteins within the tissue which is a marker of fibrosis. Our results show that in the left ventricular tissue of the untreated diabetic rats, there was a significant increase in protein glycosylation when compared to control group. This glycosylation was significantly decreased in diabetic rats treated with MSCs-CM **(Figure 8-A, 8-B)**.

Taken together, our immuno-histological results indicate that MSC-CM attenuates myocardial fibrosis in STZ-induced type 1 diabetic rats as it decreases collagen accumulation and protein glycosylation in diabetic myocardial tissue.

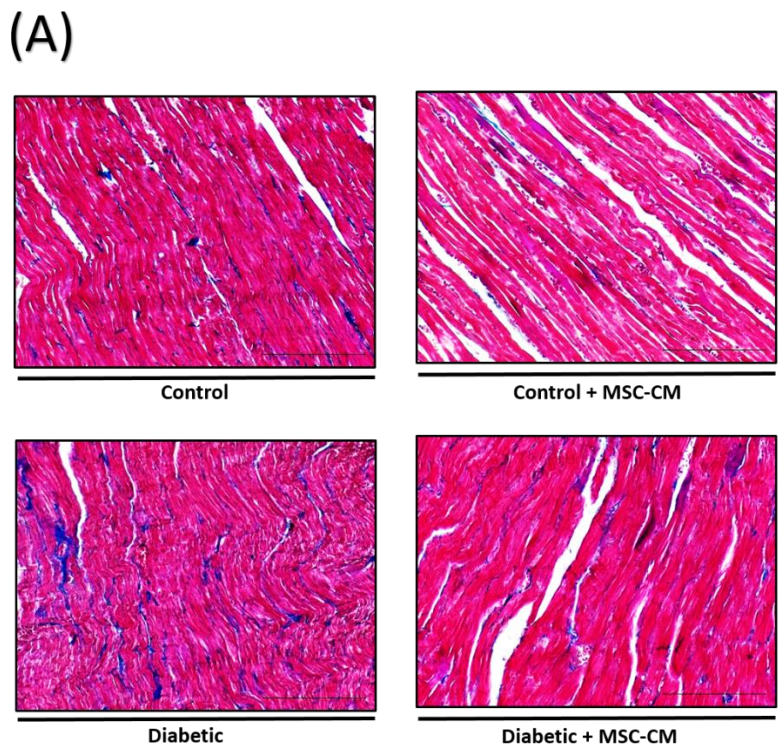
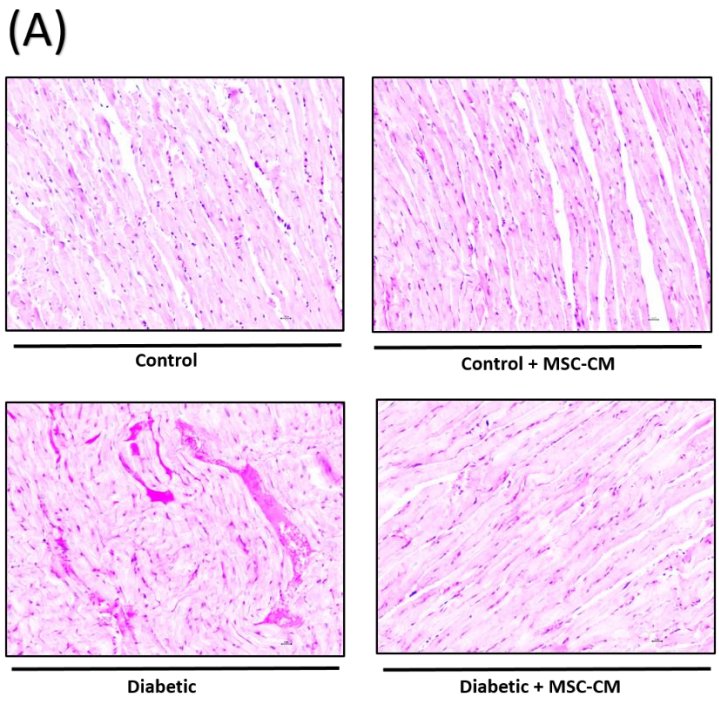


Figure 6: **Masson Trichrome staining of the collagen fibers in the left ventricle.** At the end of the study rats were euthanized, and a cut of the left ventricle was fixed in 4% formaldehyde and then tissues were embedded in paraffin blocks. Masson Trichrome

staining was used to stain collagen fibers in the LV. It was performed for the following groups Control, Control + MSC-CM, Diabetic, Diabetic + MSC-CM. A) Representative Immuno-Histological stains at 100x magnification. B) Histogram reflecting the quantification of trichrome staining at 40x magnification using ImageJ software. Results were expressed for n=3 and values are mean ± SE
* is used for p<0.05 vs control
# is used for P<0.05 vs diabetic



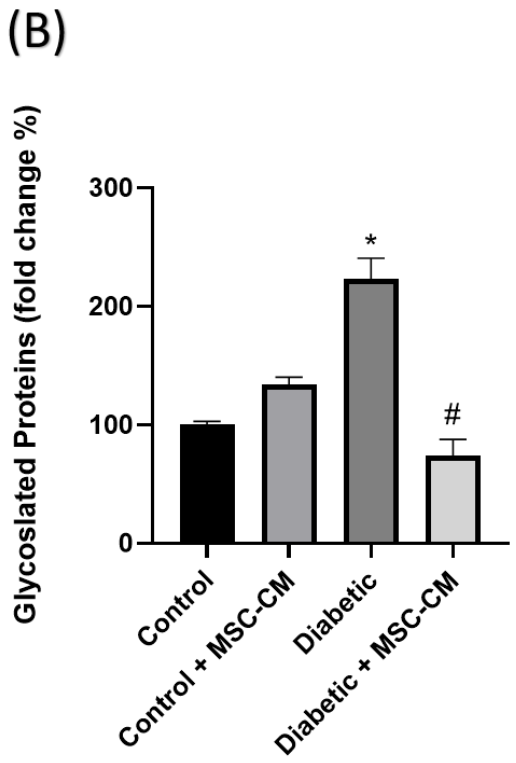


Figure 7: **Periodic Acid-Schiff (PAS) staining of the glycosylated proteins in the left ventricle.** At the end of the study rats were euthanized, and a cut of the left ventricle was fixed in 4% formaldehyde and then tissues were embedded in paraffin blocks. PAS staining was used to stain glycosylated proteins in the LV. It was performed for the following groups Control, Control + MSC-CM, Diabetic, Diabetic + MSC-CM. A) Representative Immuno-Histological stains at 100x magnification. B) Histogram reflecting the quantification of PAS staining at 40x magnification using ImageJ software. Results were expressed for n=3 and values are mean ± SE
* is used for $p<0.05$ vs control
# is used for $P<0.05$ vs diabetic

4. Treatment with MSC-CM decreases α-SMA protein expression

Cardiac changes in hypertrophy and fibrogenesis are assessed by measuring the levels of alpha smooth muscle actin (α-SMA) protein; a marker for cardiac myofibroblasts in a hypertrophic and fibrotic heart. Levels of α-SMA protein were analyzed by western blot at first between two groups: control and control + MSC-

CM. The results show no difference in expression of α-SMA between the two conditions **(Figure 9)**. Thus, the subsequent western blot assessments exclude the control + MSC-CM group and include only the three following groups: control, diabetic, diabetic + MSC-CM.

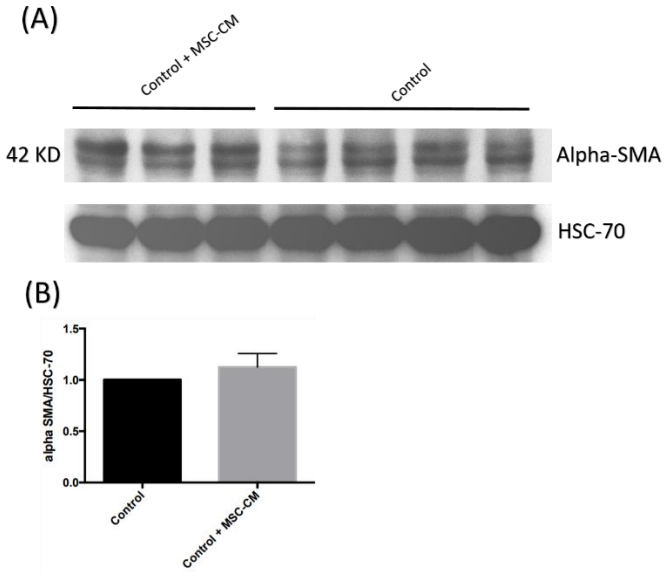


Figure 8: Molecular studies: Expression of α-SMA protein in the left ventricle was assessed using western blot analysis for the two following groups: Control and Control +MSC-CM. A) Western blot representing the expression of α-SMA and HSC-70. B) Histogram showing α-SMA protein level relative to HSC-70 protein level for Control and Control +MSC-CM groups.
Values are mean ± SE

Protein expression levels of α-SMA increased in untreated diabetic rats compared to controls. Interestingly, treatment with MSC-CM significantly decreased the levels of α-SMA in diabetic treated rats compared to the untreated diabetic rats **(Figure 10)**. These results imply that treatment with MSC-CM may reduce fibrosis in the left ventricle of diabetic heart.

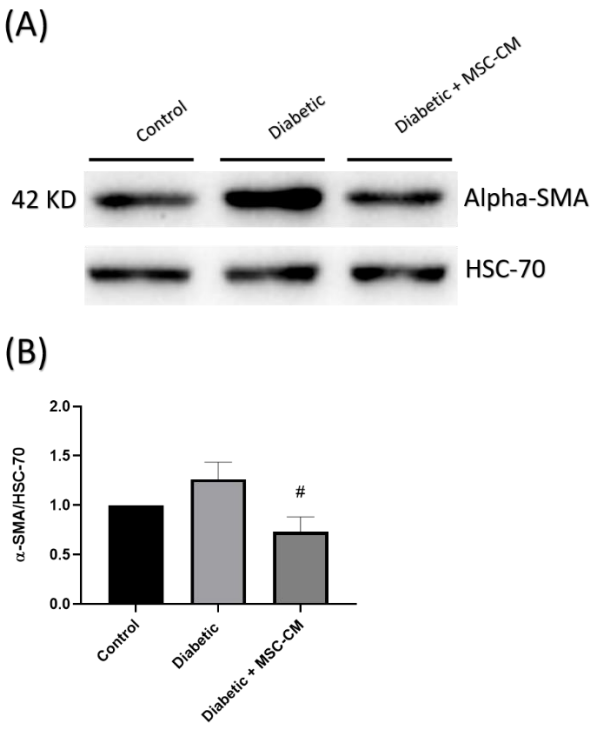


Figure 9: Expression of α-SMA protein levels in the left ventricle by western blot analysis. A) Representative Western blot of α-SMA and HSC-70. B) Histogram showing the quantification of α-SMA protein level relative to HSC-70 protein level for the following groups: Control, Diabetic, Diabetic + MSC-CM.
Results were expressed for n=3 and values are mean ± SE
is used for $P<0.05$ vs diabetic

5. Treatment with MSC-CM downregulates levels of fibronectin

Levels of fibronectin, which is a marker of cardiac injury, were assessed by western blot on homogenates extracted from the left ventricular tissue. Our results show that fibronectin protein expression levels increased in untreated diabetic rats when compared to the controls and treatment with MSC-CM slightly decreased the levels to values similar to controls. However, these results show only a trend with no significance which might be due to a small sample size **(Figure 11)**. These findings further suggest that the treatment with MSC-CM tends to attenuate cardiac injury in diabetic rats.

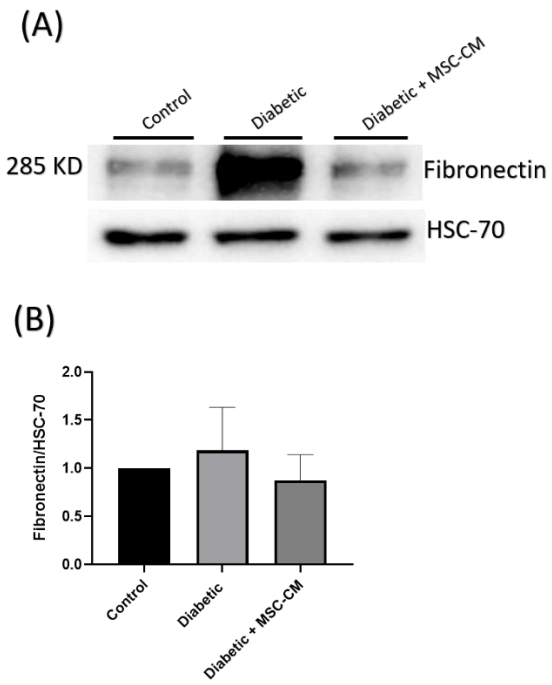


Figure 10: Expression of fibronectin protein levels in the left ventricle using western blot analysis. A) Representative Western blot of fibronectin and HSC-70. B) Histogram showing the quantification of fibronectin protein level relative to HSC-70 protein level for the following groups: Control, Diabetic, Diabetic + MSC-CM.
Results were expressed for n=3 and values are mean ± SE

6. Treatment with MSC-CM attenuates oxidative stress

Our HPLC results show that ROS production was significantly increased in the diabetic non-treated rats when compared to the control rats. Of interest, MSC-CM treatment significantly reduces ROS production in STZ-induced type 1 diabetic rats **(Figure 12)**. These results indicate that MSC-CM can attenuate hyperglycemia-induced oxidative stress through a decrease in ROS production.

Expression of Nox4 protein levels showed a trend to increase in untreated diabetic rats compared to their control littermates. Also Nox4 expression is slightly

decreased upon treatment with MSC-CM compared to untreated diabetic group **(Figure 13)**. A trend is clear; yet more animals are required to get significant results. This could also suggest that other ROS sources might be involved.

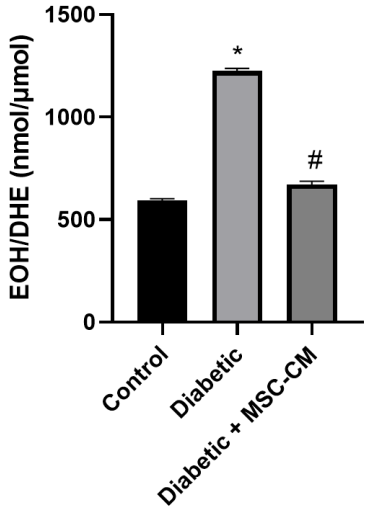


Figure 11: Measurement of superoxide production in the left ventricle using HPLC. Measurements were expressed as amount of 2-hydroxyethidium (EOH) over the amount of Dihydroethidium (DHE) in nmol/μmol. Test was performed for the following groups: Control, Diabetic, Diabetic + MSC-CM. Results are expressed for n=3 and values are mean ± SE.
*is used for $p<0.05$ vs control
is used for $p<0.05$ vs diabetic.

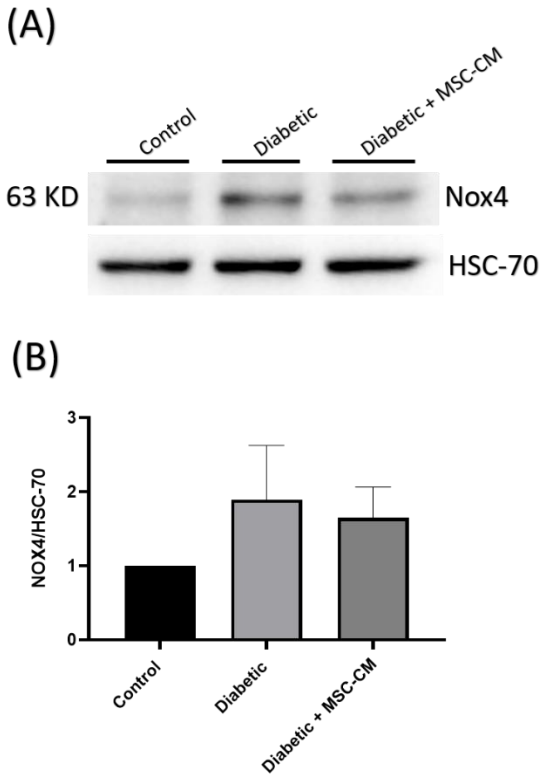


Figure 12: Expression of Nox4 protein levels in the left ventricle using western blot analysis. A) Representative Western blot of NOX4 and HSC-70. B) Histogram showing the quantification of NOX4 protein level relative to HSC-70 protein level for the following groups: Control, Diabetic, Diabetic + MSC-CM.
Results were expressed for n=3 and values are mean ± SE

CHAPTER V
DISCUSSION

Several experimental studies have reported the favorable outcomes of MSCs and MSCs-derived secretome on diabetic-induced cardiomyopathy. They demonstrate that MSCs and their secretome improved several parameters in diabetic cardiomyopathy [157, 158]. However, there were conflicting results about the extent of how much MSCs and the derived secretome may reduce blood glucose levels. The contradictory results might be due to the variability in the administration route, the time points, the frequency and the amount of injections, as well as the model used. In previous studies, multiple MSCs transplantations maintain glucose homeostasis and correct hyperglycemia in STZ-induced diabetic mice [163]. Other studies show that a single injection of MSCs into obesity-induced diabetic mice lowers blood glucose and increases glucose tolerance [164]. In contrast, other investigations show that the action of MSCs had no effect on the high levels of blood glucose. MSCs were shown to ameliorate hyperglycemia-induced damage, yet, they do not correct hyperglycemia itself [104, 165]. Consistently, our results show increased levels of HbA1c in diabetic group as well as in MSC-CM treated group. This implies that our results were not based on changes on glucose levels.

Moreover, the heart weight was significantly decreased in diabetic rats compared to the controls, indicating heart atrophy. This is compatible with previous studies in the literature that correlate STZ-induced diabetes with myocardial atrophy as opposed to hypertrophy [166]. This could be associated with the loss of contractile proteins and cardiomyocyte dropout. It is also consistent with the substrate shift that is caused by

diabetes-induced metabolic disturbance and may lead to atrophic changes myocardium [167, 168]. Other investigations have associated insulin deficiency with myocardial protein degradation, mitochondrial dysfunction and defective lysosomal enzyme activity which reduce the quantity of actin, ultimately, resulting in cellular remodeling [169, 170]. In our study, MSC-CM tend to increase heart weight in treated groups compared to untreated, yet didn't return values to normal

In addition, hemodynamic parameters were measured by echocardiography, particularly %EF and %FS, after 8 and 12 weeks of MSC-CM injections. Diabetic rats show a significant decrease in %FS and %EF in both measurements indicating a systolic dysfunction when compared to controls. %EF levels are decreased to 59% and 63% at 8^{th} and 12^{th} week of treatment respectively, indicating a heart failure with preserved ejection fraction (>50%). Although determination of cardiac dysfunction in type 1 diabetes is still controversial, it may be explained as follows: Patients having heart failure with reduced ejection fraction (HFPEF) often show dilatation ventricular dysfunction. Their heart fails to develop into a hypertrophied pump to compensate for its dysfunction [171]. Similar results have been shown in type 1 diabetic animal models where no indications of hypertrophy at the histological and molecular level of the LV are reported [168, 172]. In our study, we see a significant decrease in EF%, yet the values are still >50% indicating heart failure with preserved ejection fraction. This can be explained by the fact that LV hypertrophy is a compensatory mechanism in the diabetic heart in response to insulin deficiency-induced injuries. Accordingly, the heart increases its wall thickness to exert more force to pump blood which support the interpretation of a preserved ejection fraction. This was also convenient with our FS% and EF% measurements that were

significantly reduced in diabetic rats compared to controls indicating a systolic dysfunction. Our treatment with MSC-CM was able to significantly increase EF% and FS%. This indicates that MSC-CM ameliorates the decrease in cardiac function through reversing systolic dysfunction, which is compatible with the reported literature [19, 156].

Masson's trichrome is used to stain collagen fibers, and *Periodic Acid-Schiff (PAS)* is used to stain polysaccharides; the glycosylated proteins, that are found in connective tissues and basal laminae. These stains are used to evaluate fibrotic changes in the tissue reflecting a histological cardiac dysfunction in diabetes. In a diabetic environment, AGE formation is increased as a result of hyperglycemia-mediated cellular injury. The increased production of AGE can alter structural proteins producing crosslinks that involve collagen and elastin. This will affect the ability of collagen to be degraded and thus being accumulated resulting in fibrosis [62, 173]. In our study, both glycated proteins and collagen deposition are significantly increased in the basement of left ventricle of diabetic rats which is also consistent with the literature [174]. MSC-CM was able to decrease the composition of collagen and glycosylated proteins in the left ventricle of diabetic treated rats. Consistent with previous studies, MSC-CM can ameliorate myocardial fibrosis in diabetic cardiomyopathy [158, 161].

Cardiomyocyte injury is characterized by activation of different intracellular signaling pathways and transcriptional mediators including extracellular matrix (ECM) remodeling. Markers of cardiac injury were studied including α-SMA, revealing an upregulation in expression in response to injury [175]. α-SMA is found in fetal life where it is responsible for cardiomyocyte differentiation. Its presence in an adult heart is a sign of hypertrophied myocytes and fibrosis. It is considered as an early marker of myopathy

[176, 177]. In this study, an increase in α-SMA levels was observed in diabetic groups compared to controls, which is consistent with previous reports, yet not significant. This could be due to the small number of animals used (n=3), especially that a significant increase in fibrosis was observed in diabetic groups at the histological level. Interestingly, our treatment with MSC-CM has significantly lower the level of α-SMA in the LV when compared to diabetic rats. This was also compatible with previous experiments that assess the effect of MSC and MSC-CM [97, 178].

Another marker of cardiac injury is fibronectin. During an embryonic heart development, fibronectin is a required ECM component for the cardiomyocyte to shape the heart [179]. Following a cardiac damage in adults, epicardium generates fibronectin organ-wide and then localized at site of injury [180]. Fibronectin deposition was reported to be associated with adverse effects like fibrosis [181, 182]. In this study, our results were consistent with literature as they show a general tendency to increase fibronectin in diabetic group and decrease its levels in MSC-CM treated groups. The lack of significance may be due to the small size of samples used (n=3). It was also found that fibronectin expression is mainly mediated by the increased level of ROS and oxidative stress [18]. We didn't elucidate concrete evidence that the increased expression of fibronectin is a result of increased levels of ROS. However, this fact could be used to add further consistency to our results as we also show an increased trend in NADPH oxidase 4 in diabetic groups, but also limited by the small number of animals (n=3).

In the same spirit, diabetes is associated with an increase in ROS levels, and oxidative stress has been implicated in the development of diabetic cardiomyopathy [183]. Although hyperglycemia has been strongly associated with generation of reactive

oxygen species (ROS), precise sources of ROS in the diabetic myocardium are still unknown. There is evidence that nicotinamide adenine dinucleotide phosphate (NADPH) oxidases of the NOX family are increased in cardiovascular disease and diabetes[184]. In this family, Nox4 is expressed in both the heart and cultured cardiac myocytes, and its effect on cardiomyocyte is still controversial. It was reported that Nox4 is an important source of ROS in the left ventricle and can contribute to cardiomyopathy at early stages of type 1 diabetes [185]. In transgenic mice, overexpression of Nox4 by hypertrophic stimuli and aging induces oxidative stress, apoptosis, and LV dysfunction [186]. In contrary, other studies investigate a cardiovascular protective role as well as a metabolism-regulating role of Nox4 in case of injury [187, 188]. In our study, a significant increase in ROS production was observed in diabetic rats. ROS production was significantly decreased in MSC-CM treated group. Thus, decreasing ROS production by MSC-CM could be a factor in attenuating diabetes-induced cardiac injuries, as hypothesized in our study. In western blot analysis, Nox4 expression was increased in the LV of the diabetic rats compared to control rats. Nox4 levels were decreased in the LV of rats treated with MSC-CM. This general tendency suggests a role of Nox4 in the pathogenesis of diabetic cardiomyopathy, but an increased sample size would draw better interpretation. In this context, other sources of ROS may also be involved including Nox1, Nox2, and cytochromes P450 enzymes.

Taken together, our data demonstrate that MSC-CM exert beneficial effects on cardiac injuries, major macrovascular complications of diabetes. MSC-CM enhance cardiac function through reversing systolic dysfunction in rats. This was observed through a significant increase in fractional shortening and ejection fraction. MSC-CM

also demonstrates a significant attenuation of myocardial fibrosis in the LV tissue through lowering collagen and glycosylated protein content. Moreover, it also shows a significant reduction in fibrosis via downregulating α-SMA; marker of hypertrophy and fibrosis. We also show that MSC-CM had a tendency to decrease markers of injury (fibronectin) and Nox4-derived ROS, yet it was not significant. However, a significant decrease of ROS production was reported upon treatment with MSC-CM. This indicates that the treatment attenuates hyperglycemia-induced oxidative stress through a decrease in ROS production, and nox4 as well as other sources of ROS might be involved. Importantly, further markers of cardiac injury, inflammation, apoptosis, and angiogenesis are worth to be evaluated in future directions. Definitely, increasing number of sample is a necessary step to do later. It is worth noting that a novel therapeutic strategy using MSC-CM could be a safe and efficient treatment for diabetic cardiomyopathy and other diabetic complications. Indeed, further *in-vivo* and *in-vitro* applications are required for a better mechanistic understanding of MSC-CM to open the door for more clinical approaches.

CHAPTER VI
LIMITATIONS AND FUTURE PERSPECTIVES

In our study, the small number of animals was a limiting factor for the molecular experiments. Each group consisted of three rats only. In the future, we will increase the number of animals which might add more accuracy and reliability to our results. Moreover, we need to study other NOX isoforms such as NOX1 and NOX2 as well as other sources of ROS including cytochromes P450 enzymes to better understand sources of ROS in the pathogenies of diabetic cardiomyopathy. In the future perspectives, it is important to study the array of factors present in MSC-CM and assess signaling pathway crosstalk. Also further markers of inflammation and apoptosis are worth to be investigated including IL-1β, IL-6, BAX, TNF, and caspase-3, which are known to be altered in diabetic cardiomyopathy. Last but not least, it would be interesting to perform the study on T2DM and examine the effect of MSC-CM on other tissues such as the kidney, liver and pancreas.

REFERENCES

1. Bhutani, J. and S. Bhutani, *Worldwide burden of diabetes.* Indian journal of endocrinology and metabolism, 2014. **18**(6): p. 868-870.
2. Barnard-Kelly, K.D., et al., *Suicide and Self-inflicted Injury in Diabetes: A Balancing Act.* J Diabetes Sci Technol, 2019: p. 1932296819891136.
3. Saeedi, P., et al., *Global and regional diabetes prevalence estimates for 2019 and projections for 2030 and 2045: Results from the International Diabetes Federation Diabetes Atlas, 9(th) edition.* Diabetes Res Clin Pract, 2019. **157**: p. 107843.
4. Lin, J., et al., *Projection of the future diabetes burden in the United States through 2060.* Population Health Metrics, 2018. **16**(1): p. 9.
5. *Economic Costs of Diabetes in the U.S. in 2017*. 2018; dci180007]. Available from: https://care.diabetesjournals.org/content/diacare/early/2018/03/20/dci18-0007.full.pdf.
6. *Diagnosis and Classification of Diabetes Mellitus.* Diabetes Care, 2014. **37**(Supplement 1): p. S81.
7. Fowler, M.J., *Microvascular and Macrovascular Complications of Diabetes.* Clinical Diabetes, 2008. **26**(2): p. 77.
8. Bianchi, C. and S. Del Prato, *Metabolic memory and individual treatment aims in type 2 diabetes--outcome-lessons learned from large clinical trials.* The review of diabetic studies : RDS, 2011. **8**(3): p. 432-440.
9. Gaster, B. and I.B. Hirsch, *The Effects of Improved Glycemic Control on Complications in Type 2 Diabetes.* Archives of Internal Medicine, 1998. **158**(2): p. 134-140.
10. Westermann, D., et al., *Gene deletion of the kinin receptor B1 attenuates cardiac inflammation and fibrosis during the development of experimental diabetic cardiomyopathy.* Diabetes, 2009. **58**(6): p. 1373-81.
11. Balakumar, P., U.K. Maung, and G. Jagadeesh, *Prevalence and prevention of cardiovascular disease and diabetes mellitus.* Pharmacol Res, 2016. **113**(Pt A): p. 600-609.
12. Berezin, A.E., *Biomarkers for cardiovascular risk in patients with diabetes.* Heart, 2016. **102**(24): p. 1939.
13. Murarka, S. and M.R. Movahed, *Diabetic cardiomyopathy.* Journal of cardiac failure, 2010. **16**(12): p. 971-979.
14. Cagalinec, M., et al., *Morphology and Contractility of Cardiac Myocytes in Early Stages of Streptozotocin-Induced Diabetes Mellitus in Rats.* Physiological research / Academia Scientiarum Bohemoslovaca, 2013. **62**.
15. Jia, G., V.G. DeMarco, and J.R. Sowers, *Insulin resistance and hyperinsulinaemia in diabetic cardiomyopathy.* Nature reviews. Endocrinology, 2016. **12**(3): p. 144-153.
16. Harmancey, R. and H. Taegtmeyer, *The complexities of diabetic cardiomyopathy: Lessons from patients and animal models.* Current Diabetes Reports, 2008. **8**(3): p. 243-248.
17. Cai, L., et al., *Hyperglycemia-induced apoptosis in mouse myocardium: mitochondrial cytochrome C-mediated caspase-3 activation pathway.* Diabetes, 2002. **51**(6): p. 1938-48.
18. Murdoch, C.E., et al., *Endothelial NADPH oxidase-2 promotes interstitial cardiac fibrosis and diastolic dysfunction through proinflammatory effects and endothelial-mesenchymal transition.* J Am Coll Cardiol, 2014. **63**(24): p. 2734-41.

19. Li, J.H., N. Zhang, and J.A. Wang, *Improved anti-apoptotic and anti-remodeling potency of bone marrow mesenchymal stem cells by anoxic pre-conditioning in diabetic cardiomyopathy.* J Endocrinol Invest, 2008. **31**(2): p. 103-10.
20. Arslan, F., et al., *Mesenchymal stem cell-derived exosomes increase ATP levels, decrease oxidative stress and activate PI3K/Akt pathway to enhance myocardial viability and prevent adverse remodeling after myocardial ischemia/reperfusion injury.* Stem Cell Res, 2013. **10**(3): p. 301-12.
21. Shabbir, A., et al., *Heart failure therapy mediated by the trophic activities of bone marrow mesenchymal stem cells: a noninvasive therapeutic regimen.* Am J Physiol Heart Circ Physiol, 2009. **296**(6): p. H1888-97.
22. Kinnaird, T., et al., *Local delivery of marrow-derived stromal cells augments collateral perfusion through paracrine mechanisms.* Circulation, 2004. **109**(12): p. 1543-9.
23. Vizoso, F.J., et al., *Mesenchymal Stem Cell Secretome: Toward Cell-Free Therapeutic Strategies in Regenerative Medicine.* Int J Mol Sci, 2017. **18**(9).
24. Maguire, G., *Stem cell therapy without the cells.* Commun Integr Biol, 2013. **6**(6): p. e26631.
25. Vishnubhatla, I., et al., *The development of stem cell-derived exosomes as a cell-free regenerative medicine.* Journal of Circulating Biomarkers, 2014. **3**(Godište 2014): p. 3-2.
26. Echtay, A., et al., *Comprehensive analysis of the financial burden of diabetes mellitus at Rafik Hariri University Hospital: the economic implications from the public sector perspective in Lebanon.* 2015. **2**: p. 6.
27. Meo, S., A. Usmani, and E.J.E.R.M.P.S. Qalbani, *Prevalence of type 2 diabetes in the Arab world: impact of GDP and energy consumption.* 2017. **21**(6): p. 1303-12.
28. *2. Classification and Diagnosis of Diabetes: <em>Standards of Medical Care in Diabetes—2020</em&gt.* Diabetes Care, 2020. **43**(Supplement 1): p. S14.
29. Alberti, K.G. and P.Z. Zimmet, *Definition, diagnosis and classification of diabetes mellitus and its complications. Part 1: diagnosis and classification of diabetes mellitus provisional report of a WHO consultation.* Diabet Med, 1998. **15**(7): p. 539-53.
30. American Diabetes, A., *Standards of medical care in diabetes--2010.* Diabetes Care, 2010. **33 Suppl 1**: p. S11-61.
31. Kahanovitz, L., P.M. Sluss, and S.J. Russell, *Type 1 Diabetes - A Clinical Perspective.* Point of care, 2017. **16**(1): p. 37-40.
32. Altobelli, E., et al., *Genetic and environmental factors affect the onset of type 1 diabetes mellitus.* Pediatr Diabetes, 2016. **17**(8): p. 559-566.
33. Guillausseau, P.J., et al., *Abnormalities in insulin secretion in type 2 diabetes mellitus.* Diabetes Metab, 2008. **34 Suppl 2**: p. S43-8.
34. Zaccardi, F., et al., *Pathophysiology of type 1 and type 2 diabetes mellitus: a 90-year perspective.* Postgraduate Medical Journal, 2016. **92**(1084): p. 63.
35. Leong, A., et al., *Type 2 Diabetes Genetic Predisposition, Obesity, and All-Cause Mortality Risk in the U.S.: A Multiethnic Analysis.* Diabetes Care, 2016. **39**(4): p. 539.
36. Halpern, A., et al., *Metabolic syndrome, dyslipidemia, hypertension and type 2 diabetes in youth: from diagnosis to treatment.* Diabetology & metabolic syndrome, 2010. **2**: p. 55-55.
37. Noctor, E. and F.P. Dunne, *Type 2 diabetes after gestational diabetes: The influence of changing diagnostic criteria.* World journal of diabetes, 2015. **6**(2): p. 234-244.
38. de la Monte, S.M. and J.R. Wands, *Alzheimer's disease is type 3 diabetes-evidence reviewed.* J Diabetes Sci Technol, 2008. **2**(6): p. 1101-13.
39. Leszek, J., et al., *Type 3 Diabetes Mellitus: A Novel Implication of Alzheimers Disease.* Curr Top Med Chem, 2017. **17**(12): p. 1331-1335.

40. Yang, G.-R., T.D. Dye, and D. Li, *Association between diabetes, metabolic syndrome and heart attack in US adults: a cross-sectional analysis using the Behavioral Risk Factor Surveillance System 2015.* BMJ Open, 2019. **9**(9): p. e022990.
41. McAllister, D.A., et al., *Incidence of Hospitalization for Heart Failure and Case-Fatality Among 3.25 Million People With and Without Diabetes Mellitus.* Circulation, 2018. **138**(24): p. 2774-2786.
42. Kannel, W.B., M. Hjortland, and W.P. Castelli, *Role of diabetes in congestive heart failure: The Framingham study.* The American Journal of Cardiology, 1974. **34**(1): p. 29-34.
43. Stratton, I.M., et al., *Association of glycaemia with macrovascular and microvascular complications of type 2 diabetes (UKPDS 35): prospective observational study.* BMJ, 2000. **321**(7258): p. 405-12.
44. Gottdiener, J.S., et al., *Outcome of Congestive Heart Failure in Elderly Persons: Influence of Left Ventricular Systolic Function: The Cardiovascular Health Study.* Annals of Internal Medicine, 2002. **137**(8): p. 631-639.
45. Nieminen, M.S., et al., *EuroHeart Failure Survey II (EHFS II): a survey on hospitalized acute heart failure patients: description of population.* European Heart Journal, 2006. **27**(22): p. 2725-2736.
46. Kloch-Badelek, M., et al., *Prevalence of left ventricular diastolic dysfunction in European populations based on cross-validated diagnostic thresholds.* Cardiovascular ultrasound, 2012. **10**: p. 10-10.
47. Boyer, J.K., et al., *Prevalence of ventricular diastolic dysfunction in asymptomatic, normotensive patients with diabetes mellitus.* Am J Cardiol, 2004. **93**(7): p. 870-5.
48. Höke, U., et al., *Influence of Diabetes on Left Ventricular Systolic and Diastolic Function and on Long-Term Outcome After Cardiac Resynchronization Therapy.* Diabetes Care, 2013. **36**(4): p. 985.
49. Brunvand, L., et al., *Early reduced myocardial diastolic function in children and adolescents with type 1 diabetes mellitus a population-based study.* BMC cardiovascular disorders, 2016. **16**: p. 103-103.
50. Abudiab, M.M., et al., *Cardiac output response to exercise in relation to metabolic demand in heart failure with preserved ejection fraction.* European journal of heart failure, 2013. **15**(7): p. 776-785.
51. Berceanu, M., et al., *The Significance of Right Ventricle in Young Subjects with Diabetes Mellitus Type 1. An echocardiographyic study.* Current health sciences journal, 2019. **45**(2): p. 174-178.
52. *Diabetic cardiomyopathy: where we are and where we are going FAU - Lee, Wang-Soo FAU - Kim, Jaetaek.* Korean J Intern Med, 2017. **32**(3): p. 404-421.
53. Yu, W., et al., *Curcumin alleviates diabetic cardiomyopathy in experimental diabetic rats.* PloS one, 2012. **7**(12): p. e52013-e52013.
54. Tsai, T.-H., et al., *Deletion of RasGRF1 Attenuated Interstitial Fibrosis in Streptozotocin-Induced Diabetic Cardiomyopathy in Mice through Affecting Inflammation and Oxidative Stress.* International journal of molecular sciences, 2018. **19**(10): p. 3094.
55. Rubler, S., et al., *New type of cardiomyopathy associated with diabetic glomerulosclerosis.* Am J Cardiol, 1972. **30**(6): p. 595-602.
56. Westermeier, F., et al., *New Molecular Insights of Insulin in Diabetic Cardiomyopathy.* Front Physiol, 2016. **7**: p. 125.
57. Du, X., et al., *Inhibition of GAPDH activity by poly(ADP-ribose) polymerase activates three major pathways of hyperglycemic damage in endothelial cells.* The Journal of clinical investigation, 2003. **112**(7): p. 1049-1057.

58. Szabo, C., *PARP as a Drug Target for the Therapy of Diabetic Cardiovascular Dysfunction.* Drug News Perspect, 2002. **15**(4): p. 197-205.
59. Aragno, M., et al., *Oxidative stress-dependent impairment of cardiac-specific transcription factors in experimental diabetes.* Endocrinology, 2006. **147**(12): p. 5967-74.
60. Chiu, J., et al., *PARP mediates structural alterations in diabetic cardiomyopathy.* J Mol Cell Cardiol, 2008. **45**(3): p. 385-93.
61. Braz, J.C., et al., *PKC-alpha regulates cardiac contractility and propensity toward heart failure.* Nat Med, 2004. **10**(3): p. 248-54.
62. Susic, D., et al., *Collagen cross-link breakers: a beginning of a new era in the treatment of cardiovascular changes associated with aging, diabetes, and hypertension.* Curr Drug Targets Cardiovasc Haematol Disord, 2004. **4**(1): p. 97-101.
63. Norton, G.R., G. Candy, and A.J. Woodiwiss, *Aminoguanidine prevents the decreased myocardial compliance produced by streptozotocin-induced diabetes mellitus in rats.* Circulation, 1996. **93**(10): p. 1905-12.
64. Petrova, R., et al., *Advanced glycation endproduct-induced calcium handling impairment in mouse cardiac myocytes.* J Mol Cell Cardiol, 2002. **34**(10): p. 1425-31.
65. Belke, D.D., E.A. Swanson, and W.H. Dillmann, *Decreased Sarcoplasmic Reticulum Activity and Contractility in Diabetic db/db Mouse Heart.* Diabetes, 2004. **53**(12): p. 3201.
66. Penpargkul, S., et al., *Depressed cardiac sarcoplasmic reticular function from diabetic rats.* J Mol Cell Cardiol, 1981. **13**(3): p. 303-9.
67. Trost, S.U., et al., *Overexpression of the sarcoplasmic reticulum Ca(2+)-ATPase improves myocardial contractility in diabetic cardiomyopathy.* Diabetes, 2002. **51**(4): p. 1166-71.
68. Kumar, R., et al., *Intracardiac intracellular angiotensin system in diabetes.* American journal of physiology. Regulatory, integrative and comparative physiology, 2012. **302**(5): p. R510-R517.
69. Privratsky, J.R., et al., *AT1 blockade prevents glucose-induced cardiac dysfunction in ventricular myocytes: role of the AT1 receptor and NADPH oxidase.* Hypertension, 2003. **42**(2): p. 206-12.
70. Bugger, H. and E.D. Abel, *Molecular mechanisms of diabetic cardiomyopathy.* Diabetologia, 2014. **57**(4): p. 660-71.
71. Giacco, F. and M. Brownlee, *Oxidative stress and diabetic complications.* Circulation research, 2010. **107**(9): p. 1058-1070.
72. Lee, W.S. and J. Kim, *Diabetic cardiomyopathy: where we are and where we are going.* Korean J Intern Med, 2017. **32**(3): p. 404-421.
73. Miki, T., et al., *Diabetic cardiomyopathy: pathophysiology and clinical features.* Heart failure reviews, 2013. **18**(2): p. 149-166.
74. Larijani, B., et al., *Stem cell therapy in treatment of different diseases.* Acta Med Iran, 2012. **50**(2): p. 79-96.
75. Kalra, K., P.J.A.J.o.P. Tomar, and C. Therapeutics, *Stem cell: basics, classification and applications.* 2014. **2**(7): p. 919-930.
76. Zakrzewski, W., et al., *Stem cells: past, present, and future.* Stem Cell Res Ther, 2019. **10**(1): p. 68.
77. Menasché, P., et al., *Human embryonic stem cell-derived cardiac progenitors for severe heart failure treatment: first clinical case report.* Eur Heart J, 2015. **36**(30): p. 2011-7.
78. Schwartz, S.D., et al., *Human embryonic stem cell-derived retinal pigment epithelium in patients with age-related macular degeneration and Stargardt's macular dystrophy: follow-up of two open-label phase 1/2 studies.* Lancet, 2015. **385**(9967): p. 509-16.

79. Li, M. and S. Ikehara, *Stem cell treatment for type 1 diabetes.* Frontiers in cell and developmental biology, 2014. **2**: p. 9-9.
80. Cosmi, B. and G. Palareti, *Is there a role for oral anticoagulant therapy in patients with peripheral arterial disease?* Curr Drug Targets Cardiovasc Haematol Disord, 2004. **4**(3): p. 269-73.
81. *The Challenges of Stem Cell Therapy.* 2018.
82. Zhou, J.Y., Z. Zhang, and G.S. Qian, *Mesenchymal stem cells to treat diabetic neuropathy: a long and strenuous way from bench to the clinic.* Cell Death Discov, 2016. **2**: p. 16055.
83. Lukomska, B., et al., *Challenges and controversies in human mesenchymal stem cell therapy.* 2019. **2019**.
84. Giannini, D. and A. Balbarini, *Thrombolytic therapy in peripheral arterial disease.* Curr Drug Targets Cardiovasc Haematol Disord, 2004. **4**(3): p. 249-58.
85. Abushouk, A.I., et al., *Mesenchymal Stem Cell Therapy for Doxorubicin-Induced Cardiomyopathy: Potential Mechanisms, Governing Factors, and Implications of the Heart Stem Cell Debate.* 2019. **10**(635).
86. Jin, L., et al., *Mesenchymal stem cells ameliorate myocardial fibrosis in diabetic cardiomyopathy via the secretion of prostaglandin E2.* Stem Cell Res Ther, 2020. **11**(1): p. 122.
87. Sun, D.Z., et al., *Harnessing the mesenchymal stem cell secretome for regenerative urology.* Nature Reviews Urology, 2019. **16**(6): p. 363-375.
88. Friedenstein, A.J., et al., *Stromal cells responsible for transferring the microenvironment of the hemopoietic tissues. Cloning in vitro and retransplantation in vivo.* Transplantation, 1974. **17**(4): p. 331-40.
89. Ding, D.C., W.C. Shyu, and S.Z. Lin, *Mesenchymal stem cells.* Cell Transplant, 2011. **20**(1): p. 5-14.
90. Jackson, W.M., L.J. Nesti, and R.S. Tuan, *Potential therapeutic applications of muscle-derived mesenchymal stem and progenitor cells.* Expert opinion on biological therapy, 2010. **10**(4): p. 505-517.
91. Karnieli, O., et al., *Generation of insulin-producing cells from human bone marrow mesenchymal stem cells by genetic manipulation.* Stem Cells, 2007. **25**(11): p. 2837-44.
92. Weyand, B., et al., *Mesenchymal Stem Cells-Basics and Clinical Application II*. 2013: Springer.
93. Dissaranan, C., et al., *Rat mesenchymal stem cell secretome promotes elastogenesis and facilitates recovery from simulated childbirth injury.* Cell transplantation, 2014. **23**(11): p. 1395-1406.
94. Shake, J.G., et al., *Mesenchymal stem cell implantation in a swine myocardial infarct model: engraftment and functional effects.* Ann Thorac Surg, 2002. **73**(6): p. 1919-25; discussion 1926.
95. Chen, J., et al., *Intravenous bone marrow stromal cell therapy reduces apoptosis and promotes endogenous cell proliferation after stroke in female rat.* J Neurosci Res, 2003. **73**(6): p. 778-86.
96. Tang, Y.L., et al., *Autologous mesenchymal stem cell transplantation induce VEGF and neovascularization in ischemic myocardium.* Regul Pept, 2004. **117**(1): p. 3-10.
97. Mias, C., et al., *Mesenchymal stem cells promote matrix metalloproteinase secretion by cardiac fibroblasts and reduce cardiac ventricular fibrosis after myocardial infarction.* Stem Cells, 2009. **27**(11): p. 2734-43.
98. Chen, J., et al., *Therapeutic benefit of intravenous administration of bone marrow stromal cells after cerebral ischemia in rats.* Stroke, 2001. **32**(4): p. 1005-11.
99. Ding, D.C., et al., *The role of endothelial progenitor cells in ischemic cerebral and heart diseases.* Cell Transplant, 2007. **16**(3): p. 273-84.

100. Kim, S.Y., et al., *Mesenchymal stem cell-conditioned media recovers lung fibroblasts from cigarette smoke-induced damage.* Am J Physiol Lung Cell Mol Physiol, 2012. **302**(9): p. L891-908.
101. Monsel, A., et al., *Therapeutic Effects of Human Mesenchymal Stem Cell-derived Microvesicles in Severe Pneumonia in Mice.* Am J Respir Crit Care Med, 2015. **192**(3): p. 324-36.
102. Xagorari, A., et al., *Protective effect of mesenchymal stem cell-conditioned medium on hepatic cell apoptosis after acute liver injury.* Int J Clin Exp Pathol, 2013. **6**(5): p. 831-40.
103. Yang, J., et al., *Extracellular Vesicles Derived from Bone Marrow Mesenchymal Stem Cells Protect against Experimental Colitis via Attenuating Colon Inflammation, Oxidative Stress and Apoptosis.* PLoS One, 2015. **10**(10): p. e0140551.
104. Yu, B., et al., *Exosomes secreted from GATA-4 overexpressing mesenchymal stem cells serve as a reservoir of anti-apoptotic microRNAs for cardioprotection.* Int J Cardiol, 2015. **182**: p. 349-60.
105. Devine, S.M., et al., *Mesenchymal stem cells are capable of homing to the bone marrow of non-human primates following systemic infusion.* Exp Hematol, 2001. **29**(2): p. 244-55.
106. Karp, J.M. and G.S. Leng Teo, *Mesenchymal stem cell homing: the devil is in the details.* Cell Stem Cell, 2009. **4**(3): p. 206-16.
107. Baek, S.J., S.K. Kang, and J.C. Ra, *In vitro migration capacity of human adipose tissue-derived mesenchymal stem cells reflects their expression of receptors for chemokines and growth factors.* Exp Mol Med, 2011. **43**(10): p. 596-603.
108. Honczarenko, M., et al., *Human bone marrow stromal cells express a distinct set of biologically functional chemokine receptors.* Stem Cells, 2006. **24**(4): p. 1030-41.
109. Zhuang, Y., et al., *Chemokine stromal cell-derived factor 1/CXCL12 increases homing of mesenchymal stem cells to injured myocardium and neovascularization following myocardial infarction.* Chin Med J (Engl), 2009. **122**(2): p. 183-7.
110. Krabbe, C., J. Zimmer, and M. Meyer, *Neural transdifferentiation of mesenchymal stem cells--a critical review.* APMIS, 2005. **113**(11-12): p. 831-44.
111. Chermansky, C.J., et al., *Intraurethral muscle-derived cell injections increase leak point pressure in a rat model of intrinsic sphincter deficiency.* Urology, 2004. **63**(4): p. 780-5.
112. Li, Y., et al., *Human marrow stromal cell therapy for stroke in rat: neurotrophins and functional recovery.* Neurology, 2002. **59**(4): p. 514-23.
113. Beggs, K.J., et al., *Immunologic consequences of multiple, high-dose administration of allogeneic mesenchymal stem cells to baboons.* Cell transplantation, 2006. **15**(8-9): p. 711-721.
114. Keyser, K.A., K.E. Beagles, and H.-P. Kiem, *Comparison of mesenchymal stem cells from different tissues to suppress T-cell activation.* Cell transplantation, 2007. **16**(5): p. 555-562.
115. Gatti, S., et al., *Microvesicles derived from human adult mesenchymal stem cells protect against ischaemia-reperfusion-induced acute and chronic kidney injury.* Nephrol Dial Transplant, 2011. **26**(5): p. 1474-83.
116. Zhou, Y., et al., *Exosomes released by human umbilical cord mesenchymal stem cells protect against cisplatin-induced renal oxidative stress and apoptosis in vivo and in vitro.* Stem Cell Res Ther, 2013. **4**(2): p. 34.
117. Mao, F., et al., *Exosomes Derived from Human Umbilical Cord Mesenchymal Stem Cells Relieve Inflammatory Bowel Disease in Mice.* Biomed Res Int, 2017. **2017**: p. 5356760.
118. Harrell, C.R., et al., *Molecular Mechanisms Responsible for Therapeutic Potential of Mesenchymal Stem Cell-Derived Secretome.* Cells, 2019. **8**(5).

119. English, K., et al., *Cell contact, prostaglandin E(2) and transforming growth factor beta 1 play non-redundant roles in human mesenchymal stem cell induction of CD4+CD25(High) forkhead box P3+ regulatory T cells.* Clin Exp Immunol, 2009. **156**(1): p. 149-60.
120. Maggini, J., et al., *Mouse bone marrow-derived mesenchymal stromal cells turn activated macrophages into a regulatory-like profile.* PLoS One, 2010. **5**(2): p. e9252.
121. Chiesa, S., et al., *Mesenchymal stem cells impair in vivo T-cell priming by dendritic cells.* Proc Natl Acad Sci U S A, 2011. **108**(42): p. 17384-9.
122. Spaggiari, G.M., et al., *Mesenchymal stem cells inhibit natural killer-cell proliferation, cytotoxicity, and cytokine production: role of indoleamine 2,3-dioxygenase and prostaglandin E2.* Blood, 2008. **111**(3): p. 1327-33.
123. Luz-Crawford, P., et al., *Mesenchymal stem cells generate a CD4+CD25+Foxp3+ regulatory T cell population during the differentiation process of Th1 and Th17 cells.* Stem Cell Res Ther, 2013. **4**(3): p. 65.
124. Li, Y., et al., *Exosomal sphingosine 1-phosphate secreted by mesenchymal stem cells regulated Treg/Th17 balance in aplastic anemia.* IUBMB Life, 2019. **71**(9): p. 1284-1292.
125. Gazdic, M., et al., *Mesenchymal stem cells: a friend or foe in immune-mediated diseases.* Stem Cell Rev Rep, 2015. **11**(2): p. 280-7.
126. Thorgeirsson, S.S., *Hepatic stem cells in liver regeneration.* Faseb j, 1996. **10**(11): p. 1249-56.
127. Levy, V., et al., *Distinct stem cell populations regenerate the follicle and interfollicular epidermis.* Dev Cell, 2005. **9**(6): p. 855-61.
128. Anthony, D.F. and P.G. Shiels, *Exploiting paracrine mechanisms of tissue regeneration to repair damaged organs.* Transplant Res, 2013. **2**(1): p. 10.
129. Chen, L., et al., *Paracrine factors of mesenchymal stem cells recruit macrophages and endothelial lineage cells and enhance wound healing.* PLoS One, 2008. **3**(4): p. e1886.
130. Semedo, P., et al., *Mesenchymal stem cells attenuate renal fibrosis through immune modulation and remodeling properties in a rat remnant kidney model.* Stem Cells, 2009. **27**(12): p. 3063-73.
131. Meng, X.M., D.J. Nikolic-Paterson, and H.Y. Lan, *Inflammatory processes in renal fibrosis.* Nat Rev Nephrol, 2014. **10**(9): p. 493-503.
132. Takahashi, M., et al., *Cytokines produced by bone marrow cells can contribute to functional improvement of the infarcted heart by protecting cardiomyocytes from ischemic injury.* Am J Physiol Heart Circ Physiol, 2006. **291**(2): p. H886-93.
133. Eiró, N., et al., *Potential therapeutic effect of the secretome from human uterine cervical stem cells against both cancer and stromal cells compared with adipose tissue stem cells.* Oncotarget, 2014. **5**(21): p. 10692-708.
134. Kinnaird, T., et al., *Marrow-derived stromal cells express genes encoding a broad spectrum of arteriogenic cytokines and promote in vitro and in vivo arteriogenesis through paracrine mechanisms.* Circ Res, 2004. **94**(5): p. 678-85.
135. Tögel, F., et al., *Vasculotropic, paracrine actions of infused mesenchymal stem cells are important to the recovery from acute kidney injury.* Am J Physiol Renal Physiol, 2007. **292**(5): p. F1626-35.
136. Li, L., et al., *Hypoxia promotes bone marrow-derived mesenchymal stem cell proliferation through apelin/APJ/autophagy pathway.* Acta Biochim Biophys Sin (Shanghai), 2015. **47**(5): p. 362-7.
137. Zanotti, L., et al., *Mouse mesenchymal stem cells inhibit high endothelial cell activation and lymphocyte homing to lymph nodes by releasing TIMP-1.* Leukemia, 2016. **30**(5): p. 1143-54.

138. Zhang, T., et al., *Bone marrow-derived mesenchymal stem cells promote growth and angiogenesis of breast and prostate tumors.* Stem Cell Res Ther, 2013. **4**(3): p. 70.
139. Larijani, B., et al., *Stem Cell Therapy in Treatment of Different Diseases.* Acta medica Iranica, 2012. **50**: p. 79-96.
140. Volarevic, V., et al., *Interleukin-1 receptor antagonist (IL-1Ra) and IL-1Ra producing mesenchymal stem cells as modulators of diabetogenesis.* Autoimmunity, 2009. **43**: p. 255-63.
141. Yang, Z., et al., *Amelioration of diabetic retinopathy by engrafted human adipose-derived mesenchymal stem cells in streptozotocin diabetic rats.* Graefes Arch Clin Exp Ophthalmol, 2010. **248**(10): p. 1415-22.
142. Scalinci, S.Z., et al., *Potential role of intravitreal human placental stem cell implants in inhibiting progression of diabetic retinopathy in type 2 diabetes: neuroprotective growth factors in the vitreous.* Clin Ophthalmol, 2011. **5**: p. 691-6.
143. Ezquer, M.E., et al., *MSC transplantation: a promising therapeutic strategy to manage the onset and progression of diabetic nephropathy.* Biol Res, 2012. **45**(3): p. 289-96.
144. Lee, R.H., et al., *Multipotent stromal cells from human marrow home to and promote repair of pancreatic islets and renal glomeruli in diabetic NOD/scid mice.* Proc Natl Acad Sci U S A, 2006. **103**(46): p. 17438-43.
145. Chen, Y., et al., *Bone marrow-derived mesenchymal stem cells ameliorate nephrosis through repair of impaired podocytes.* Clin Invest Med, 2017. **40**(1): p. E13-E24.
146. Zhou, H., et al., *Mesenchymal stem cells transplantation mildly ameliorates experimental diabetic nephropathy in rats.* Chin Med J (Engl), 2009. **122**(21): p. 2573-9.
147. Wang, S., et al., *Mesenchymal stem cells ameliorate podocyte injury and proteinuria in a type 1 diabetic nephropathy rat model.* Biol Blood Marrow Transplant, 2013. **19**(4): p. 538-46.
148. Shibata, T., et al., *Transplantation of bone marrow-derived mesenchymal stem cells improves diabetic polyneuropathy in rats.* Diabetes, 2008. **57**(11): p. 3099-107.
149. Siniscalco, D., et al., *Long-lasting effects of human mesenchymal stem cell systemic administration on pain-like behaviors, cellular, and biomolecular modifications in neuropathic mice.* Front Integr Neurosci, 2011. **5**: p. 79.
150. Liu, L., et al., *Exosomes Derived from Mesenchymal Stem Cells Rescue Myocardial Ischaemia/Reperfusion Injury by Inducing Cardiomyocyte Autophagy Via AMPK and Akt Pathways.* Cell Physiol Biochem, 2017. **43**(1): p. 52-68.
151. Sun, X., et al., *Intravenous mesenchymal stem cell-derived exosomes ameliorate myocardial inflammation in the dilated cardiomyopathy.* Biochem Biophys Res Commun, 2018. **503**(4): p. 2611-2618.
152. Huang, P., et al., *New strategies for improving stem cell therapy in ischemic heart disease.* Heart Fail Rev, 2016. **21**(6): p. 737-752.
153. Zhang, Z., et al., *Pretreatment of Cardiac Stem Cells With Exosomes Derived From Mesenchymal Stem Cells Enhances Myocardial Repair.* J Am Heart Assoc, 2016. **5**(1).
154. Gong, X.H., et al., *Exosomes derived from SDF1-overexpressing mesenchymal stem cells inhibit ischemic myocardial cell apoptosis and promote cardiac endothelial microvascular regeneration in mice with myocardial infarction.* J Cell Physiol, 2019. **234**(8): p. 13878-13893.
155. Ma, T., et al., *MicroRNA-132, Delivered by Mesenchymal Stem Cell-Derived Exosomes, Promote Angiogenesis in Myocardial Infarction.* Stem Cells Int, 2018. **2018**: p. 3290372.

156. Hare, J.M., et al., *A randomized, double-blind, placebo-controlled, dose-escalation study of intravenous adult human mesenchymal stem cells (prochymal) after acute myocardial infarction.* J Am Coll Cardiol, 2009. **54**(24): p. 2277-86.
157. Zhang, N., et al., *Bone marrow mesenchymal stem cells induce angiogenesis and attenuate the remodeling of diabetic cardiomyopathy.* Exp Clin Endocrinol Diabetes, 2008. **116**(2): p. 104-11.
158. Nagaya, N., et al., *Transplantation of mesenchymal stem cells improves cardiac function in a rat model of dilated cardiomyopathy.* Circulation, 2005. **112**(8): p. 1128-35.
159. Calligaris, S.D. and P. Conget, *Intravenous administration of bone marrow-derived multipotent mesenchymal stromal cells has a neutral effect on obesity-induced diabetic cardiomyopathy.* Biol Res, 2013. **46**(3): p. 251-5.
160. Khan, M., et al., *Preconditioning diabetic mesenchymal stem cells with myogenic medium increases their ability to repair diabetic heart.* Stem Cell Res Ther, 2013. **4**(3): p. 58.
161. Jin, L., et al., *Mesenchymal stem cells ameliorate myocardial fibrosis in diabetic cardiomyopathy via the secretion of prostaglandin E2.* Stem Cell Research & Therapy, 2020. **11**(1): p. 122.
162. Abushouk, A.I., et al., *Mesenchymal Stem Cell Therapy for Doxorubicin-Induced Cardiomyopathy: Potential Mechanisms, Governing Factors, and Implications of the Heart Stem Cell Debate.* Frontiers in Pharmacology, 2019. **10**(635).
163. Ho, J.H., et al., *Multiple intravenous transplantations of mesenchymal stem cells effectively restore long-term blood glucose homeostasis by hepatic engraftment and β-cell differentiation in streptozocin-induced diabetic mice.* Cell Transplant, 2012. **21**(5): p. 997-1009.
164. Cao, M., et al., *Adipose-derived mesenchymal stem cells improve glucose homeostasis in high-fat diet-induced obese mice.* Stem cell research & therapy, 2015. **6**: p. 208-208.
165. Hare, J.M., et al., *A randomized, double-blind, placebo-controlled, dose-escalation study of intravenous adult human mesenchymal stem cells (prochymal) after acute myocardial infarction.* Journal of the American College of Cardiology, 2009. **54**(24): p. 2277-2286.
166. Bilim, O., et al., *Diacylglycerol kinase ζ inhibits myocardial atrophy and restores cardiac dysfunction in streptozotocin-induced diabetes mellitus.* Cardiovascular Diabetology, 2008. **7**(1): p. 2.
167. Depre, C., et al., *Streptozotocin-induced changes in cardiac gene expression in the absence of severe contractile dysfunction.* J Mol Cell Cardiol, 2000. **32**(6): p. 985-96.
168. Nemoto, O., et al., *Left ventricular dysfunction and remodeling in streptozotocin-induced diabetic rats.* Circ J, 2006. **70**(3): p. 327-34.
169. Young, L.H., et al., *Physiological hyperinsulinemia inhibits myocardial protein degradation in vivo in the canine heart.* Circ Res, 1992. **71**(2): p. 393-400.
170. Howarth, F.C., et al., *Chronic effects of streptozotocin-induced diabetes on the ultrastructure of rat ventricular and papillary muscle.* Acta Diabetol, 2000. **37**(3): p. 119-24.
171. Seferovic, P.M. and W.J. Paulus, *Clinical diabetic cardiomyopathy: a two-faced disease with restrictive and dilated phenotypes.* Eur Heart J, 2015. **36**(27): p. 1718-27, 1727a-1727c.
172. Salem, K.A., et al., *The direct effects of streptozotocin and alloxan on contractile function in rat heart.* Pharmacol Res, 2009. **59**(4): p. 235-41.
173. Yan, S.F., et al., *RAGE and its ligands: a lasting memory in diabetic complications?* Diab Vasc Dis Res, 2004. **1**(1): p. 10-20.

174. Goldin, A., et al., *Advanced glycation end products: sparking the development of diabetic vascular injury.* Circulation, 2006. **114**(6): p. 597-605.
175. Aoyagi, T. and T. Matsui, *The Cardiomyocyte as a Source of Cytokines in Cardiac Injury.* Journal of cell science & therapy, 2011. **2012**(S5).
176. Maalouf, S.W., S. Theivakumar, and D.M. Owens, *Epidermal alpha6beta4 integrin stimulates the influx of immunosuppressive cells during skin tumor promotion.* J Dermatol Sci, 2012. **66**(2): p. 108-18.
177. Kern, S., et al., *Up-regulation of alpha-smooth muscle actin in cardiomyocytes from non-hypertrophic and non-failing transgenic mouse hearts expressing N-terminal truncated cardiac troponin I.* FEBS Open Bio, 2013. **4**: p. 11-7.
178. Meier, R., et al., *Microencapsulated human mesenchymal stem cells decrease liver fibrosis in mice.* Journal of Hepatology, 2014. **62**.
179. George, E.L., H.S. Baldwin, and R.O. Hynes, *Fibronectins are essential for heart and blood vessel morphogenesis but are dispensable for initial specification of precursor cells.* Blood, 1997. **90**(8): p. 3073-81.
180. Wang, J., et al., *Fibronectin is deposited by injury-activated epicardial cells and is necessary for zebrafish heart regeneration.* Dev Biol, 2013. **382**(2): p. 427-35.
181. Knowlton, A.A., et al., *Rapid expression of fibronectin in the rabbit heart after myocardial infarction with and without reperfusion.* J Clin Invest, 1992. **89**(4): p. 1060-8.
182. Willems, I.E., J.W. Arends, and M.J. Daemen, *Tenascin and fibronectin expression in healing human myocardial scars.* J Pathol, 1996. **179**(3): p. 321-5.
183. Giacco, F. and M. Brownlee, *Oxidative stress and diabetic complications.* Circ Res, 2010. **107**(9): p. 1058-70.
184. Hansen, S.S., E. Aasum, and A.D. Hafstad, *The role of NADPH oxidases in diabetic cardiomyopathy.* Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease, 2018. **1864**(5, Part B): p. 1908-1913.
185. Maalouf, R.M., et al., *Nox4-derived reactive oxygen species mediate cardiomyocyte injury in early type 1 diabetes.* American journal of physiology. Cell physiology, 2012. **302**(3): p. C597-C604.
186. Ago, T., et al., *Upregulation of Nox4 by hypertrophic stimuli promotes apoptosis and mitochondrial dysfunction in cardiac myocytes.* Circulation research, 2010. **106**(7): p. 1253-1264.
187. Rajaram, R.D., et al., *Potential benefits and harms of NADPH oxidase type 4 in the kidneys and cardiovascular system.* Nephrology Dialysis Transplantation, 2018. **34**(4): p. 567-576.
188. Morawietz, H., *Cardiovascular protection by Nox4.* Cardiovascular Research, 2018. **114**(3): p. 353-355.