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MOLECULAR MECHANISMS UNDERLYING THE
ATTENUATION OF DIABETES-INDUCED RENAL
INJURIES BY MESENCHYMAL STEM CELLS

by
CHRISTELLE ROGER AL ZAGHRINI

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submitted in partial fulfillment of the requirements
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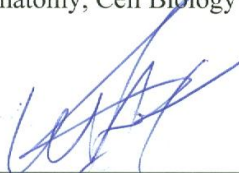
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AN ABSTRACT OF THE THESIS OF

Christelle Roger Al Zaghrini   for   Master of Science
Major: Physiology

Title: Molecular Mechanisms Underlying the Attenuation of Diabetes-Induced Renal Injuries by Mesenchymal Stem Cells.

Background: Diabetic nephropathy (DN) is one of the major complications of diabetes. It is characterized by morphological and functional renal alterations such as tubulointerstitial fibrosis, glomerulosclerosis and protenuria ultimately leading to end stage renal disease. Diabetes, among several pathological conditions, alters the redox balance causing modifications in the DNA, the proteins and the antioxidant system leading to oxidative stress.

Aims: An emerging body of studies has shown that stem cells have the potential to improve acute tubular injury recovery and glomerular repair. However, the mechanism of repair of MSCs is not yet identified. The present study aims to determine the effect of MSCs treatment on oxidative stress, specifically on ROS production and to investigate the mechanistic pathway by which MSCs attenuate renal injury.

Methods: 4 weeks streptozotocin-induced diabetic rats received weekly injections of 1×10^6 cell MSCs intravenously, while the control and diabetic groups received weekly injections of media. After eight weeks of treatment, physical, histological and biochemical parameters and kidney function were measured. Dihydroethidium (DHE) was used to assess intracellular ROS production levels. Real-time Reverse Transcription-Polymerase Chain Reaction (RT-PCR) was performed to determine mRNA levels of fibronectin and collagen IV, Nox4, CYP4A and CYP2C11 and TGF-β. The histological alterations were measured using different histochemical stains including H&E, PAS and Masson Trichrome.

Results: MSCs treatment had no effect on the blood glucose levels yet restored normal urinary albumin excretion levels markers of diabetic kidney injury, i.e. glomerulosclerosis detected by periodic acid Schiff (PAS) stain, tubulointerstitial fibrosis revealed by Masson Trichrome stain and expression of fibronectin, laminin and collagen IV in MSCs treated rats were reversed in rats treated with MSCs compared to STZ-diabetic rats. Oxidative stress was also decreased by the normalized levels of Nox4, CYP4A, CYP2C11 and TGF-β.

Conclusion: Our results suggest that MSCs present a novel therapy in the treatment of diabetes. Further in-depth mechanistic investigations are needed to understand how MSCs treatment reversed oxidative stress in diabetic nephropathy.

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ILLUSTRATIONS

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x

ABBREVIATIONS

DN: Diabetic Nephropathy

GFP: Green fluorescent protein

MSCs: Mesenchymal stem cells

NADPH: Nicotinamide adenine dinucleotide phosphate

ROS: Reactive Oxygen Species

STZ: Streptozotocin

CYP450:   Cytochrome 450

HG: High Glucose

CHAPTER I

INTRODUCTION

Diabetes mellitus (DM) is a global health issue affecting children, adolescents, and adults. The World Health Organization recent estimates indicate that 8.3% of adults -382 million people - have diabetes, and the number of people with the disease is set to rise beyond 592 million in less than 25 years. Yet, with 175 million of cases currently undiagnosed, a vast amount of people with diabetes are progressing towards unawared microvascular and macrovascular complications, including retinopathy, nephropathy, and neuropathy (microvascular) in addition to ischemic heart disease, peripheral vascular disease, and cerebrovascular disease (macrovascular). Diabetic complications result in organ and tissue damage in approximately one third to one-half of people with diabetes and accounts for approximately 8% of worldwide mortality [1]. Diabetes in all its forms imposes unacceptably high human, social, and economic costs on countries at all income levels. Despite the array of tools available to tackle the disease including effective drug therapies, advanced technology, ever-improving education and preventive strategies, the battle to protect people from diabetes and its disabling, life-threatening is being lost.

A. Diabetic nephropathy

Diabetic nephropathy (DN) is a major risk factor for cardiovascular morbidity and

mortality and is a leading cause of end-stage renal disease. DN ultimately progresses to glomerular and interstitial fibrosis associated with renal dysfunction. Renal disease will affect between 20-40% of diabetics in their lifetime [2]. DN is characterized by morphological and functional renal alterations such as glomerular and tubular hypertrophy, mesangial cell injury, thickening of the glomerular or tubular basement membrane, and podocyte dysfunction. Renal hypertrophy, matrix protein accumulation and tubulointerstitial fibrosis are major pathological features of diabetic nephropathy (DN) and contribute to the eventual decline in glomerular filtration rate in humans and experimental models of DN. The pathogenic mechanisms of DN affecting the glomerulus or the tubules are not fully understood. While strict metabolic control prevents many of the complications of diabetes, normoglycemia is often difficult to achieve and is fraught with complications. Therefore, designing adjunct therapy to prevent/treat diabetic complications including nephropathy is of great importance.

B. Reactive oxygen species and diabetic nephropathy

Diabetes and hyperglycemia are accompanied by increased generation of reactive oxygen species (ROS) in the kidney [3-6]. ROS play a central role in cellular physiology, and take part in various biological processes [7]. ROS can be either free radicals or their non-radical intermediates. Oxygen and nitrogen are the two most important elements generating free radicals in the biological system. The main sources of ROS production include the enzyme Nicotinamide Adenine Dinucleotide Phosphate (NADPH) oxidase, mitochondrial electron transport chain, Cytochrome 450 (CYP450) and other oxido -

reductases. ROS production is held in balance due to a complex system of antioxidant defense [8] .However, in diabetes, this balance is disrupted. HG induces the overproduction of superoxide and H_2O_2 by several cell types such as endothelial, vascular smooth muscle, mesangial, and epithelial cells [4, 9]. The free radical production causes a drop in the antioxidant system and damage in many biomolecules, which ultimately lead to kidney cell injury [10, 11]. In DN, ROS play a key role in apoptosis, hypertrophy, fibrosis, and other pathophysiological processes underlying renal dysfunction [3- 6, 12].

C. NADPH oxidase in diabetic nephropathy

The phagocyte respiratory burst oxidase or NADPH oxidase catalyzes the NADPH-dependent reduction of molecular oxygen to generate superoxide anion (O_2^-, which is dismutated to form hydrogen peroxide (H_2O_2) [13]. The well characterized respiratory burst oxidase consists of two plasma membrane-associated proteins, gp91^{phox} (the catalytic subunit) and p22^{phox} which comprise flavocytochrome b_{558}, and cytosolic factors, p47^{phox}, p67^{phox} and the small GTPase/Rac [14, 15]. Enzyme activation by stimuli occurs through assembly of the cytosolic factors with the membrane-associated cytochrome b_{558} [14, 15]. Studies over the last decade have documented significant NAD(P)H-dependent ROS-generation in nonphagocytic/stromal cells, including kidney cells [4, 16- 22]. A family of gp91^{phox} homologues termed Noxes (for NAD(P)H oxidases) have been discovered recently [13, 23, 24]. To date, the Nox family comprises five members (Nox1–5), of which Nox2 is gp91^{phox} or the neutrophil isoform. Although these oxidases play a role in a variety of signaling events, such as cell growth, cell death, or survival, oxygen sensing and

inflammatory processes, their *bona fide* functions and regulation as well as molecular composition are largely unknown. The Nox catalytic subunit, Nox4, is expressed in mesangial cells [22], in podocytes [4] and in tubular cells [6]. The isoform Nox4/Renox was cloned from the kidney and found to be highly expressed. Nox4 is a 578 amino acid protein that exhibits 39% identity to $gp91^{phox}$/Nox2 with special conservation in membrane-spanning regions and binding sites for NADPH, FAD and heme, the electron transfer centers that are required to pass electrons from NAD(P)H to oxygen to form superoxide [13]. There is evidence that NAD(P)H oxidases mediate HG-induced oxidative stress and downstream signaling [3, 5, 6]. Indeed, enzyme activity or subunits expression are up-regulated in response to HG in renal cells as well as in experimental models of diabetes [3-6, 25]. For instance, a $p22^{phox}$- and $p47^{phox}$-containing oxidase contributes to ROS generation after exposure of MCs to HG [25]. Similarly, in streptozotocin-induced diabetic rats, expression of certain components of the oxidase $p47^{phox}$, $p67^{phox}$, $p22^{phox}$ or Nox4 is augmented in the kidney [25]. Collectively, these observations indicate that NAD(P)H oxidases of the Nox family are major contributors to the increased oxidative stress in diabetes.

D. Cytochrome P450 in diabetic nephropathy

Arachidonic acid (AA) is present in the cell membrane esterified to glycerophospholipids. Phospholipase activation leads to the liberation of AA from the phospholipids pools [26]. Subsequently, Arachidonic acid (AA) is metabolized by several cytochrome 450 (CYP) isoforms to produce 20-hydroxyeicosatetraenoic acid (20-HETE) and epoxyeicosatrienoic acids (EETs). CYPs of the 4A and 4F subfamilies are highly

expressed in the kidneys and form 20-HETE [27, 28]. CYPs of the 2B, 2C and 2J subfamilies form EETs. Cytochromes P450 of the 4A family and the cytochromes P450 of the CYP2C family are the most abundant in the rat kidneys. CYPs have been shown to be significant sources of oxidative stress in kidneys and other organs, i.e. coronary arteries and liver [3, 29, 30]. In DN, the expression and the activity of CYP450 and their metabolites are impaired [3, 5, 6].

E. TGF-β and diabetic nephropathy

Transforming growth factor (TGF-β) is a signaling molecule involved in cell proliferation, migration, differentiation, and apoptosis. It has three isoforms, TGF-β1, TGF-β2 and TGF-β3. Of the three, TGF-β1 isoform is thought to be a major key player in fibrosis [31, 32]. It has been shown that in addition to hyperglycemia, increased expression of growth factors such as TGF-β are major contributors to diabetic kidney injury [33- 36]. Prolonged activation of TGF-β leads to ECM accumulation and tubulointerstitial fibrosis [37], followed by organ dysfunction [38]. We have previously shown that increased TGF-β1 expression in the kidney in diabetes mellitus mediates the detrimental effects of hyperglycemia to promote cellular hypertrophy and stimulate extracellular matrix biosynthesis [5]. Furthermore, in the kidney, oxidative stress up regulates the transforming growth factor TGF-β, leading to injury [39].

F. Mesenchymal stem cells in diabetic nephropathy

Kidneys are considered to have a low cell turnover and minimal capacity for regeneration [40]. An emerging body of studies has shown that stem cells have the potential to improve acute tubular injury recovery and glomerular repair. However their mechanism of action is not well defined [41, 43-45]

Mesenchymal stem cells (MSCs) are multipotent cells present in the bone marrow and other tissues. They are defined by their surface markers expression: CD73, CD90, CD105. They are easy to isolate and have the ability to differentiate both *in vitro* and *in vivo* into several cell types including chondrocytes, adipocytes, osteocytes, myocytes, hemopoietic supporting stromal cells, in addition to nonmesodermal cells from brain and kidney tissues [41- 43]. Although, MSCs have been shown to possess renotropic properties and are able to improve organ functions, however little is known about their regeneration mechanisms [44]. Several studies using diverse models of renal damage proved that MSCs administration led up to improvements in the kidney function and repair [45]. In one of first performed studies, intravenously injected MSCs improved the renal structure and enhanced the proliferation of the renal cells in a drug-induced acute injury model [46]. Semedo et al. demonstrated that a MSCs treatment attenuated renal fibrosis in a remnant model kidney [47], and Morogi et al. showed that hCB-MSCs improved renal regeneration apoptosis, and inflammation through a paracrine action on tubular cells in a murine model [48]. However, the mechanism by which MSCs attenuate diabetic kidney injury, hasn't been tackled yet and needs to be elucidated.

G. Hypothesis and aims of the study

While strict metabolic control prevents many of the complications of diabetes, euglycemia is often difficult to achieve and is fraught with complications [49, 50]. Understanding the mechanism by which glucose exert their deleterious effects will help design adjunct therapy in addition to metabolic control to prevent or reverse diabetic nephropathy.

It has been suggested that chronic kidney disease may be due to an imbalance between mechanisms of injury and the regenerative potential within specific renal tissue compartments. The possibility of using stem cells to attenuate damage and/or enhance regeneration of injured organs is emerging as a new therapeutic approach in regenerative medicine. Numerous studies affirm that mesenchymal stem cells derived from bone marrow (MSCs) can restore kidney function after acute injury through paracrine-modulation [46]. However, the mechanism of repair is not yet identified.

It has been shown that HG dysregulates the expression levels of NADPH oxidases and CYPs which leads to ROS generation, and results in the alteration of growth factors expression including TGF-β ultimately leading to renal injury[3- 6].In this project, we aim to evaluate whether MSCs could exert a protective role against diabetic renal injury.

CHAPTER II

MATERIALS AND METHODS

A. Isolation, cell labeling and characterization of MSCs

The green fluorescent protein GFP-labeled MSCs were a gift from King's College London. The MSCs were isolated according to the following protocol:

1. Euthanize rats by CO2 asphyxiation.

2. Harvest hind limbs from one mouse. Remove skin and as much muscle and connective tissue as possible. Cut the limb above the hip and below the ankle joint (including some of the foot); it is important to maintain the bone ends to ensure sterility of the bone marrow. After severing the limb, carefully break apart the knee joint and strip remaining connective tissue from both ends of femur and tibia if ends remain intact.

3. Briefly wipe bones with gloved fingers dipped in 7% ethanol and place bones in dish of sterile PBS on ice. Collect all bones in pool in same dish.

4. Move pooled dish of bones into sterile tissue culture hood. Wash bones by transferring through sterile PBS 6 times.

5. Snip off ends of each bone with scissors (keeping as close to end as possible to extract more bone marrow) and gently place in sterile PBS.

6. Fill 10cc syringe with complete conditioned media and attach 25 gauge needle. With forceps, grab one bone. Use syringe to force media through bone shaft to extract all red marrow into 150mm plate. Can flip bone over and do from opposite side. Repeat a few

times to ensure all marrow is removed. Bone should look very white when finished. Continue until all bones are demarrowed.

7. Pipette cell mixture up and down a few times so dissociate cells- can also use syringe to pull large marrow pieces through needle to dissociate further.

8. Pass cell suspension through cell strainer (70μm size) to remove any large cell clumps or bone particles. Count cell suspension. Add 10μm cells to 10μl trypan blue. Use hemocytometer to count.

9. Incubate cells until cells adhere and look nearly confluent. Remove media and wash once with PBS to remove nonadherent cells. Trypsinize and split.

10. Expand cells until 70%-90% confluent, changing media every 3-4 days. At this point, can either split further for experiments of freeze in liquid nitrogen for further use.

11. At this time, cells can be seeded for different assays including differentiation antibody staining on gelatinized cover slips.

B. Cell culture conditions

The GFP-labeled MSCs were plated in a Dulbecco's Modified Eagle Medium (DMEM) (Sigma-Aldrich, Steinheim, Germany) plus 10% Fetal Bovine Serum (FBS) (Sigma-Aldrich) and 1% Penicillin/Streptomycin (P/S) (Sigma-Aldrich) and were allowed to adhere to the plate bottom. The cell line was incubated under standard conditions at 37ºC in a 5% CO_2 incubator. The medium was changed every 3 days. At 80% confluence, cells were harvested by trypsin and reseeded. Cells of passage 9 were used for the *in vivo* experiment. GFP-labeled MSCs were administered via tail vein at a concentration of 1.10^6cell/ml saline once a week for eight continuous weeks.

C. Experimental animal

Male Sprague-Dawley rats weighing between 200 and 225 g were divided into 3 groups of five animals each. Group 2 rats were injected intravenously via the tail vein with one single dose of 55 mg/kg body wt Streptozotocin (STZ) (Sigma-Aldrich, Steinheim, Germany) in sodium citrate buffer (0.01 M, pH 4.5) to induce type 1 diabetes. Group 3 rats were injected with STZ followed by GFP-labeled MSCs once a week intravenously (1.10^6 cell/ml saline). Group 1 rats were injected with an equivalent amount of sodium citrate buffer alone (control group).After 2 days, rats with a blood glucose level above 250mg/dL were considered diabetic. All rats were kept in a temperature controlled room and on a 12/12 dark/light cycle and had free food and water access. All protocols were approved by the Institutional Animal Care and Use Committee of the American University of Beirut. After 84 days of treatment, all rats were euthanized, and both kidneys were removed and weighed. A slice of kidney cortex at the pole was fixed with 4% formalin for immunohistochemical analysis or flash-frozen in liquid nitrogen and stored at −80°C for biochemical and image analysis.
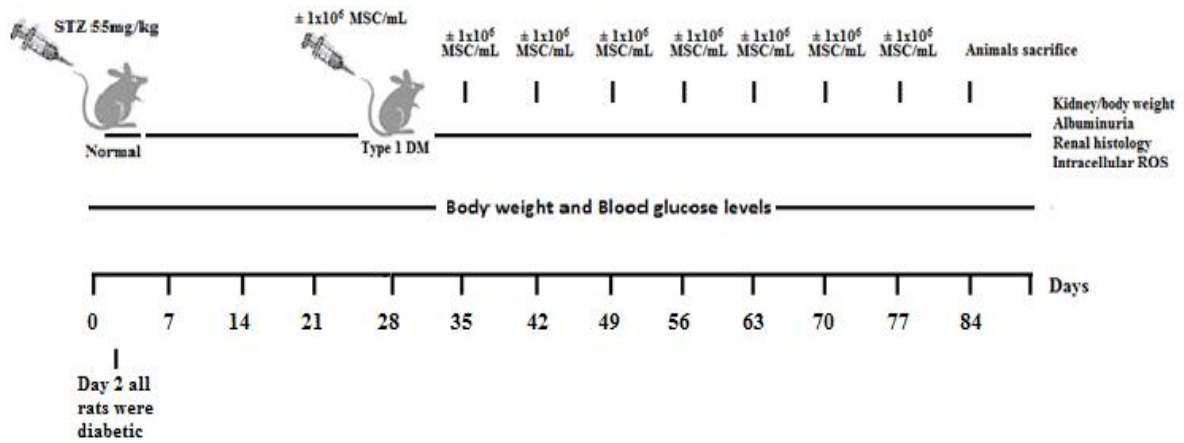
Figure 1. Mesenchymal stem cells administration and experimental design. On day 0, the rats were injected with 55mg/kg STZ to induce diabetes. 28 days after the STZ injection, the rats received 1.10^6MSC/ml saline intravenously. On day 84, post diabetes induction, the rats were sacrificed and the kidneys were harvested and processed for histological and molecular evaluations.

D. Physical and biochemical analysis

The body weight and the blood glucose levels were monitored every 48h periodically. Blood samples were taken from the tail vein and glucose levels were determined with the glucometer system Accu-Chek. Rats were considered diabetic if glycemia was above 250mg/dL. At day 63, rats were kept in metabolic cages for 24 hours. Urine samples were collected and albumin concentrations were determined by the medical laboratory at the American University of Beirut Medical Center (AUBMC).

E. Real-time RT-PCR

mRNA expression in renal cortex was analyzed by real-time RT-PCR using the $\Delta\Delta C_t$ method and the SYBR green system. Total RNA was extracted from the renal cortex lysate using TRIZOL reagent (Sigma Aldrich, Steinheim, Germany) and converted into

11

cDNA using the Revert First Strand cDNA Synthesis Kit according to the protocol. cDNA

was quantified using Real-time PCR Biorad CFX96 with SYBR green dye and rat

RT^{2}qPCR Primers (Integrated DNA Technologies, Inc., Coralville, IA, USA) for collagen

IV, fibronectin, Nox4,CYP4A, CYP2C11 and *TGF*-β. GAPDH was used as internal

reference gene.

Primers	Sequence	Annealing TºC
ColloagenIV	F: 5'- CGT GGA TGG TGT ACC TGG TC -3' R: 5'- ACC TCG TGA GCC ATT GTA GC -3'	57ºC
Fibronectin	F: 5'- GGA GAA CCA GGA GAG CAC AC -3' R: 5'- CGG GAA CAT CAT CGG ATC GT -3'	57ºC
Nox 4	F: 5'- TTC GGG TGG CTT GTT GAA GT -3' R: 5'-TGG GGT CCG GTT AAG ACT GA -3'	57ºC
TGF-β	F: 5'- TGG CCA GAT CCT GTC CAA AC -3' R: 5'- GTT GTA CAA AGC GAG CAC CG -3'	57ºC
CYP4A	F: 5'- ATC CAG AGG TGT TTG ACC CTT AT -3' R: 5'- AAT GAG ATG TGA GCA GAT GGA GT -3'	57ºC
CYP2C11	F: 5'- ACA AGG ACA ATC CTC GGG AC -3' R: 5'- AAG GGT AAA CTC AGA CTG CGG -3'	57ºC
GAPDH	F: 5'- GGG GCT CTC TGC TCC TCC CTG -3' R: 5'- CGG CCA AAT CCG TTC ACA CCG -3'	57ºC

Table 1: Oligonucleotide primer sequences and conditions employed for real-time PCR.

F. Immunohistochemical analysis

Kidney cortex tissues of three rats from each group were fixed in a 4% formalin

solution and embedded in paraffin block. Samples were cut into 5 μm thick sections and

placed on glass slides. The kidney sections were stained with periodic acid Schiff (PAS)

reagent to assess mesangial accumulation, masson trichrome staining to evaluate the collagenIV fibers deposition and hematoxylin and eosin stain (H&E) to review the morphologic changes in the kidney. A quantitative measurement for 5 randomly sampled glomeruli and 3 randomly samples proximal tubular areas was performed for 3 rats from each group by a blinded observer using Image J software.

G. Western Blot analysis

Homogenates from the renal cortex were prepared in 500 µL of radioimmunoprecipitation assay (RIPA) buffer containing 0.1% sodium dodecyl sulfate (SDS) ,0.5% sodium deoxycholate, 150 mM sodium chloride, 50 mMTris-hydrochloride, 100 mM EDTA, 1% Tergitol (NP40), and 1% of the protease and phosphatase inhibitors using a dounce homogenizer. Homogenates were incubated for 2h at 4 °C and centrifuged at 13,600 rpm for 30 minutes at 4 °C. Proteins in the supernatants were measured using the Lowry Protein Assay. For immunoblotting, proteins (30µg) were separated on 10% Polyacrylamide gel Electrophoresis (Bio-Rad Laboratory, CA, USA) and transferred to nitrocellulose membranes (Bio-Rad Laboratory, CA, USA). The membranes were blocked with 5% low fat milk in Tris-buffered saline and then incubated with rabbit polyclonal anti-laminin (1:1000, abcam) and anti-fibronectin (1:5000, Sigma). The primary antibodies were detected using horseradish peroxidase–conjugated IgG (1:7000, Bio-Rad). Bands were visualized by enhanced chemiluminescence. Densitometric analysis was performed using Image J software.

H. NADPH Oxidase Assay

NADPH oxidase activity was calculated by the lucigenin-enhanced chemiluminescence method as described [3, 4]. Superoxide production was expressed as relative chemiluminescence (light) units (RLU)/mg protein. Protein content was measured using the Bio-Rad protein assay reagent.

I. Intracellular ROS detection

Dihydroethidium (DHE), which is relatively specific for superoxide anion measurement, is an oxidative fluorescent dye that undergoes a two-electron oxidation to form the DNA-binding fluorophoreethidium bromide. The DHE staining for superoxide was carried out as previously described [51]. Briefly, cortical frozen samples were cut into 4 µm thick sections and placed on glass slides. DHE (20 µmol/l) was applied to each tissue section, and the slides were incubated in a light-protected humidified chamber at 37°C for 30 min. Fluorescent images of ethidium-stained tissue were obtained with a laser-scanning confocal microscope (Zeiss, LSM 710) at t=30 mins. Fluorescence was detected at 561 nm long-pass filter. Superoxide generation was demonstrated by red fluorescent labeling. The average of three sections stained with DHE was taken as the value for each animal. Quantification was done using Zen light Software.

J. Statistical analysis

Results are expressed as means ± SEM (n=3) statistical significance was assessed by student's unpaired *t* test. Significance was determined as probability (p) less than 0.05.

CHAPTER III

RESULTS

A. Blood glucose levels throughout the study.

Treatment with MSCs did not change blood glucose levels throughout the study (Table 2).

Group	n	Body weight day 1 (g)	Body weight day 84 (g)	Glucose levels day 1 (mg/dL)	Glucose levels day 84 (mg/dL)
Control	5	249.6 ± 6.4	564.8 ± 34.4	133 ± 5.2	104.4 ± 3.5
Diabetic	5	$203.4 \pm 6*$	$380.2 \pm 21.1*$	348.6 ± 24.7	515.4 ± 47.3
Diabetic + MSCs	5	192.2 ± 11.7	$289.8 \pm 31*$	463.6 ± 66.6	533 ± 38.2

Table 2: Body weights and blood glucose levels of control rats, type 1 diabetic rats, and type 1 diabetic rats treated with MSCs.

B. Mesenchymal stem cells treatment attenuates glomerular and cortical tubular injury.

Immunohistochemical studies were performed to determine the degree of renal cellular damage. Fibrosis and matrix protein accumulation were assessed in the renal cortices of the three groups of rats: the control group, the diabetic group and the diabetic rats treated with MSCs group. Histological examination using PAS staining showed that there was an increase in the ECM protein accumulation in the glomerulus and fibrosis in the tubules compared with the control cortex (Figure 4). MSCs treatment reversed the observed injury in the diabetic rats. PAS staining showed an increase in fibrotic deposition in the cortex of the diabetic rats while rats treated with MSCs displayed a decrease of tubular carbohydrate accumulation with respect to the diabetic rats. Masson Trichrome stain showed a significant increase in the collagen IV fibers expression in both the glomerulus and the tubules in the diabetic kidney compared with the control. Treatment with MSCs significantly decreased the collagen IV fibers expression in the cortex compared to those observed in the kidney of the diabetic rats (Figure 3). H&E stain showed an increase in the glomerular size when compared with the control. MSC treatment restored glomerular size (Figure 2). These results provide evidence that MSCs injection reverse tubular and glomerular injury observed during the development of diabetes.
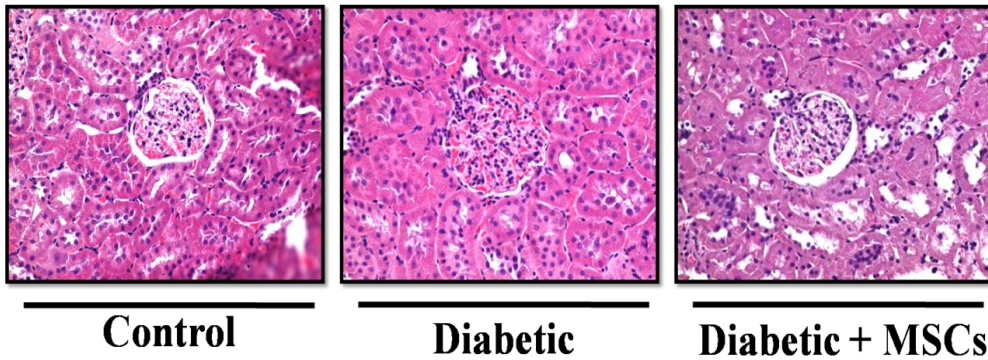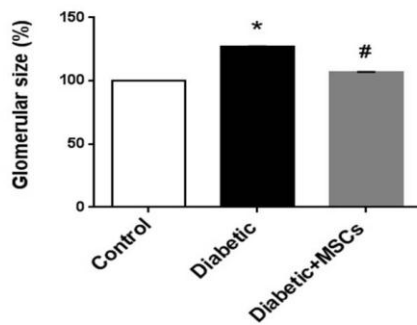
Figure 2: MSC injections reduce kidney injury by restoring normal glomerular space and size. (A) Representative images stained by H&E, showing the glomeruli and tubules morphology in the kidney cortex of the three groups. (B) Histogram showing the quantitation of the glomerular size in the glomeruli using ImageJ software. The results are means ± SEM of 5 glomeruli from 3 different rats in each group. *$P<0.05$ vs. control, #$P<0.05$ vs. diabetic.
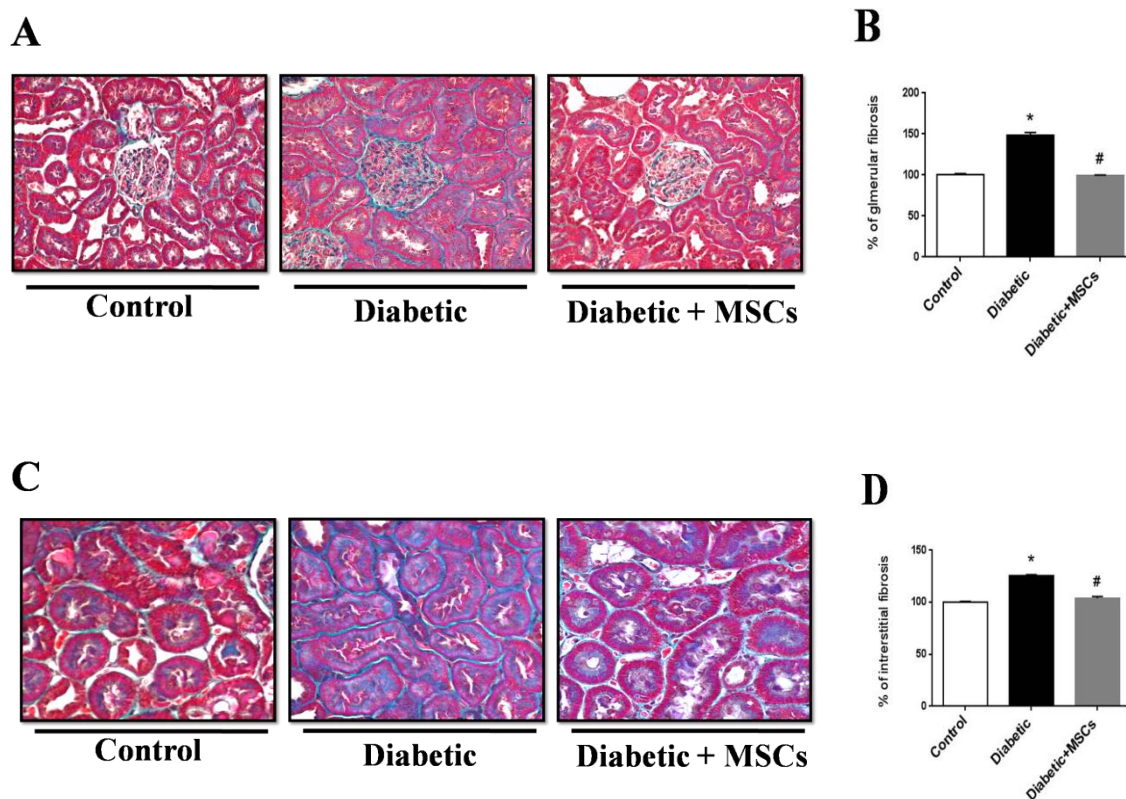
Figure 3: MSCs injections reverse diabetes-induced glomerular and tubulointerstitial fibrosis. Kidney cortices stained with Masson trichrome and the diversity of the collagen *IV* fibers expression was measured in the different groups of rats. (A) Representative histolagical analysis of glomeruli with Masson trichrome staining (fibrosis: blue color) from the different groups of rats. (B) Histogram showing the quantitation of trichrome staining in the glomeruli using ImageJ software. (C) Representative histolagical analysis of tubules with Masson trichrome staining (fibrosis: blue color) from the different groups of rats. (B) Histogram showing the quantitation of trichrome staining in the tubules using ImageJ software. The results are means ± SEM of 5 glomeruli and 3 tubular areas from 3 different rats in each group. $^*P<0.05$ vs. control, $^\#P<0.05$ vs. diabetic.
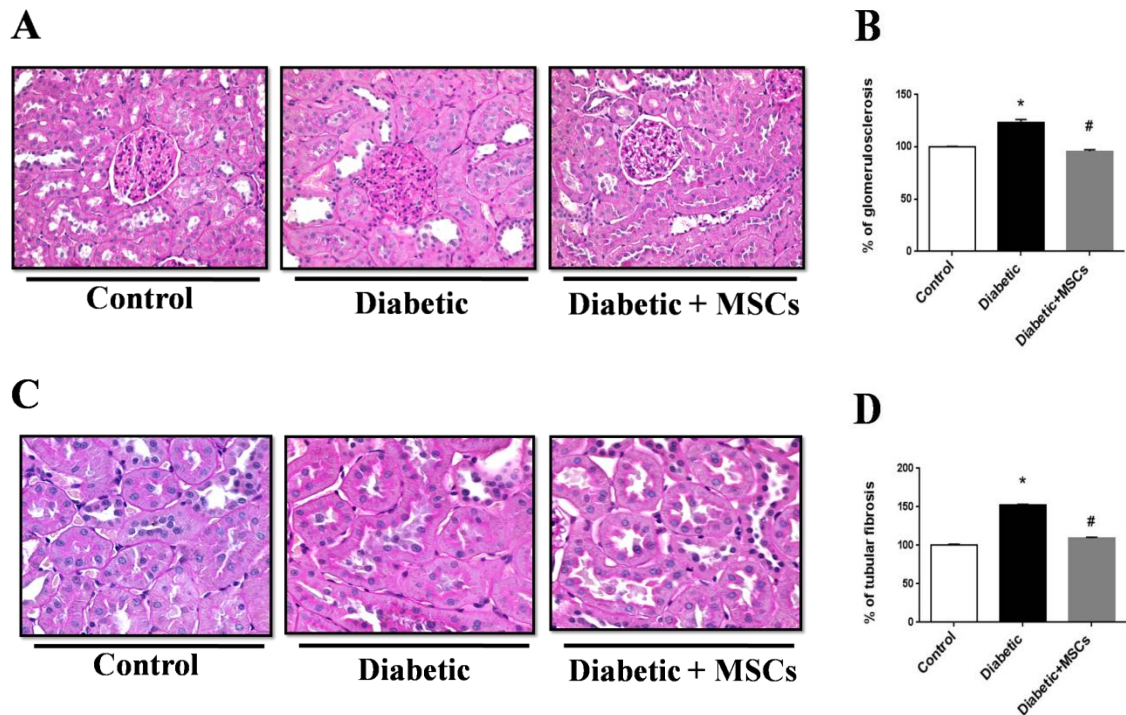
Figure 4: MSCs injections reverse diabetes-induced glomerulosclerosis and tubular fibrosis. Kidney cortices stained with PAS and the diversity of the mesangial deposition was measured in the different groups of rats. (A) Representative histolagical analysis of glomeruli with PAS staining from the different groups of rats. (B) Histogram showing the quantitation of PAS staining in the glomeruli using ImageJ software. (C) Representative histolagical analysis of tubules with PAS staining from the different groups of rats. (D) histogram showing the quantitation of PAS staining in the tubules. The results are means ± SEM of 5 glomeruli and 3 tubular areas from 3 different rats in each group. $^{*}P<0.05$ vs. control, $^{#}P<0.05$ vs. diabetic.

C. Mesenchymal stem cells treatment attenuates protenuria observed in diabetic rats.

Albuminuria is characterized clinically as an early predictor for progression of diabetic nephropathy. Protenuria (macroalbuminuria) is the universal finding in progressive renal disease, and is viewed as a measure of the severity and determinant for diabetic renal disease progression. After 5 weeks of MSCs injections, albumin urine levels increased significantly in the diabetic with respect to the control rats (Figure 5). MSCs reversed diabetes- induced albuminuria. This indicates that MSCs exert antialbuminuric effects.
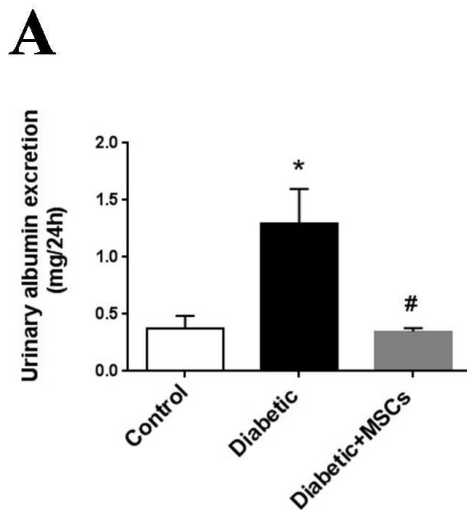

Figure 5: MSCs attenuate protenuria observed in diabetic rats.
(A) Urinary albumin excretion was determined in urine samples after 5 weeks of MSCs injections. Histogram showing the Urinary albumin excretion levelsin the three groups. The results are means ± SEM from 4 different rats in each group. $^{*}P<0.05$ vs. control, $^{#}P<0.05$ vs. diabetic.

D. MSCs treatment attenuates tubulointerstitial changes observed in the diabetic milieu.

Tubulointerstitial changes can be assessed by measuring matrix proteins. Consistent with the immunohistochemistry results, there was a significant increase in the mRNA levels of fibronectin and collagen IV fibers and in laminin fibers protein expression in the diabetic cortices compared with the controls (Figure 6). MSCs injections treatment significantly decreased fibronectin, collagen IV and laminin expression.
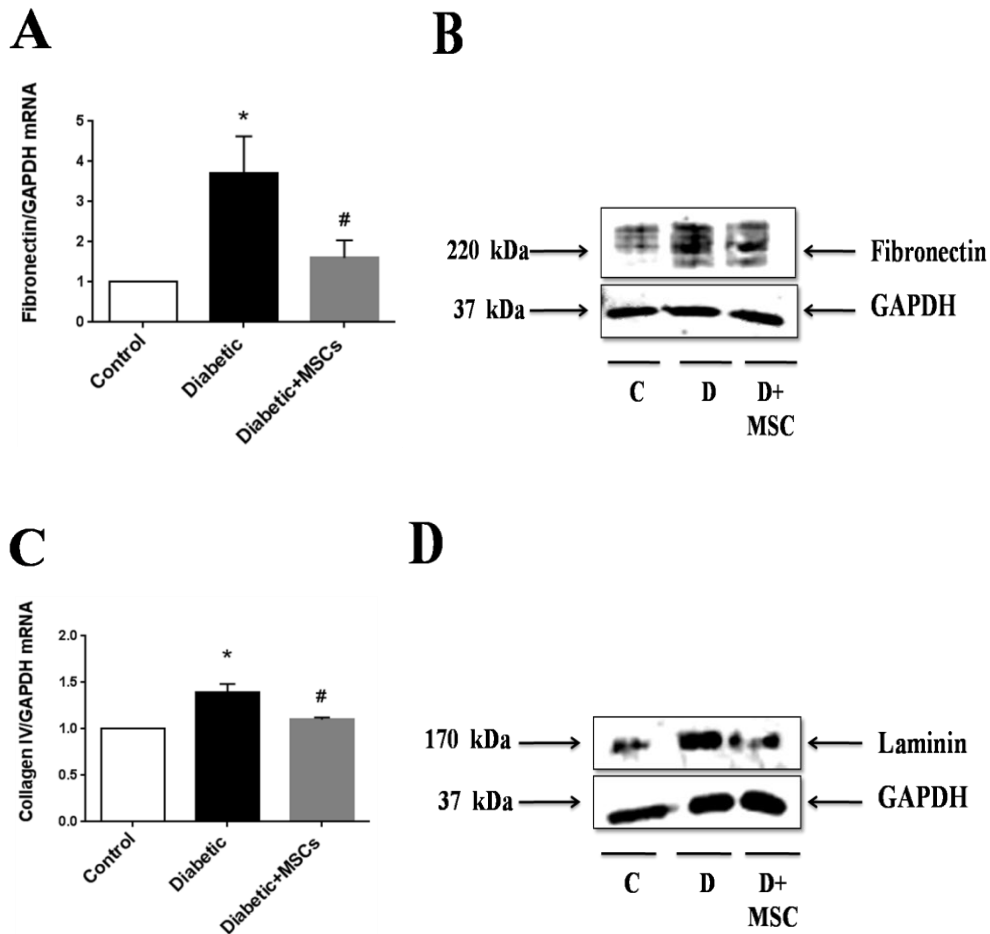
Figure 6: MSCs injections attenuate kidney injury. Fibronectin and collagen IV expressions were assessed using RT-PCR. Also, collagen IV and laminin expressions were assessed using western blot . (A)Histogram showing fibronectin mRNA levels relative to GAPDH mRNA levels. (B) Representative western blot of fibronectin from the different groups of rats. (C)Histogram showing collagen IV mRNA levels relative to GAPDH mRNA levels. (D) Representative western blot of laminin from the different groups of rats The results are means ± SEM from 3 different rats in each group. $^*P<0.05$ vs. control, $^#P<0.05$ vs. diabetic.

E. ROS generation mediates kidney injury.

Since diabetes and hyperglycemia are accompanied by increased generation of reactive oxygen species, ROS production assessed by superoxide measurements was performed in the different groups of rats. As expected, hyperglycemia induced ROS production in diabetic rats (Figure 7). Intracellular ROS production was measured using dihydroethidium DHE staining by laser-scanning confocal microscopy technique for the different groups of rats. Hyperglycemia induced ROS production was inhibited in diabetic rats upon MSCs treatment.

A

| Control | Diabetic | Diabetic + MSCs |
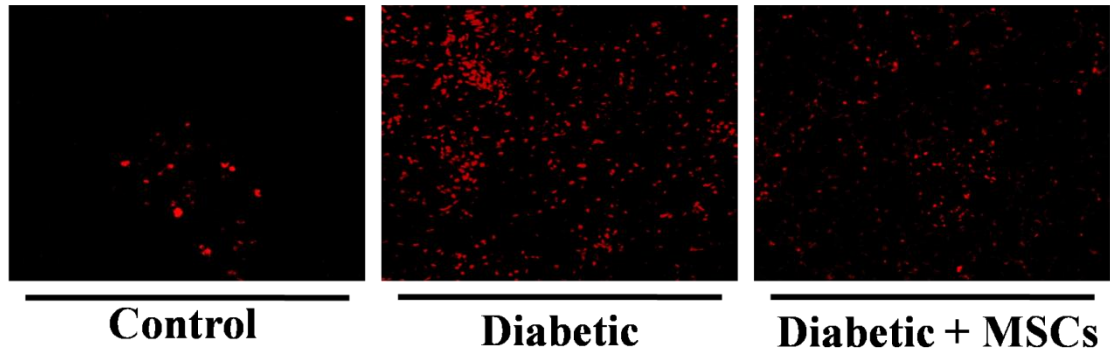
B

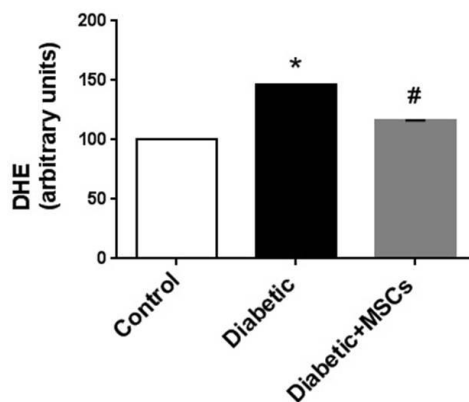**Figure 7: MSCs injections attenuate kidney injury by reducing reactive oxygen species (ROS) production.** (A)Superoxide production assessed by DHE staining. (B) Histogram showing the quantification of ROS production in the kidney cortex using Zen light Software. The results are means ± SEM of 3 different cortical areas from 3 different rats in each group. $^{*}P<0.05$ vs. control, $^{#}P<0.05$ vs. diabetic.

F. NADPH oxidase mediates ROS production; thus inducing renal injury.

Our results also showed that NADPH dependent superoxide generation was increased in diabetic rats when compared to control rats and this increase was inhibited by MSCs treatment. These results suggest that MSC treatment acts on kidney cells by inhibiting ROS production and NADPH oxidase activation known to play a major role in diabetic nephropathy. NADPH oxidase assay revealed an increase in the NADPH dependent superoxide production in the diabetic rats (Figure 8). This increase was inhibited by the MSCs injections.
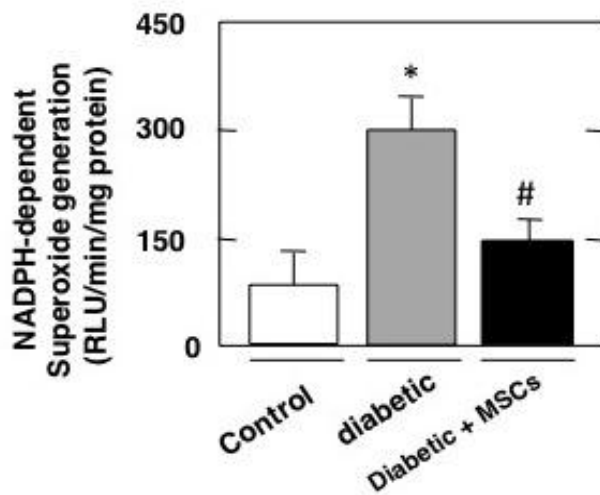

Figure 8: MSCs injections attenuate kidney injury by reducing NADPH-derived reactive oxygen species production. NADPH oxidase activity in cortical homogenates. NADPH dependent superoxide production was expressed as RLU/min/mg protein. The results are means ± SEM of different cortical areas from 5 different rats in each group. $^*P<0.05$ vs. control, $^\#P<0.05$ vs. diabetic.

G. MSCs injections decrease Nox4-derived reactive oxygen species production.

The kidney is well known to express NAD(P)H oxidase and produce ROS. The isoform Nox4, also known as RENOX, was cloned from the kidney and found to be highly expressed in this organ. It is expressed in the tubules, fibroblasts, podocytes and mesangial cells. In STZ-induced diabetic rats, the increase in NADPH dependent ROS generation in kidney cortex is associated with an increase of Nox 4 expression [52]. This is the first study to reveal the effect of MSCs treatment on the Nox4 expression. The Nox4 expression levels were assessed in the different groups of rats. Consistent with DHE and the NADPH oxidase assay results, Nox 4 mRNA levels increased significantly in the diabetic rats compared with the control. As expected, the activation of Nox 4 was notably reduced in the diabetic rats treated with MSCs (Figure 9).
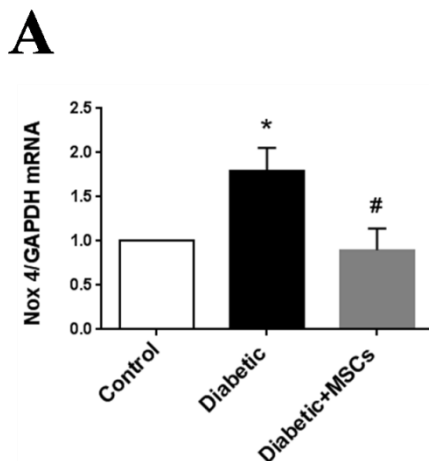

Figure 9: MSCs injections attenuate kidney injury by decreasing Nox4-derived reactive oxygen species production. Nox 4 expression was assessed using RT-PCR. (A)Histogram showing Nox4 mRNA levels relative to GAPDH mRNA levels. The results are means ± SEM from 3 different rats in each group. $^*P<0.05$ vs. control, $^#P<0.05$ vs. diabetic.

H. MSCs injections attenuate kidney injury through CYP2C11 and CYP4A.

The CYP4A and CYP2C11 mRNA levels were assessed in the different groups of rats. In a previous study, Eid S. et al. demonstrated that HG increased ROS production was associated with an upregulation of CYP4A and CYP2C11 [5]. The results are consistent with the recent findings, CYP4A mRNA levels increased in the diabetic rats compared with the controls. Predictably, MSCs injections greatly decreased CYP4A mRNA levels with respect to the diabetic rats (Figure 10- A). Furthermore, CYP2C11 mRNA levels decreased in the diabetic rats compared with the controls. As expected, these levels were notably increased in the MSCs treated rats (Figure 10-B).

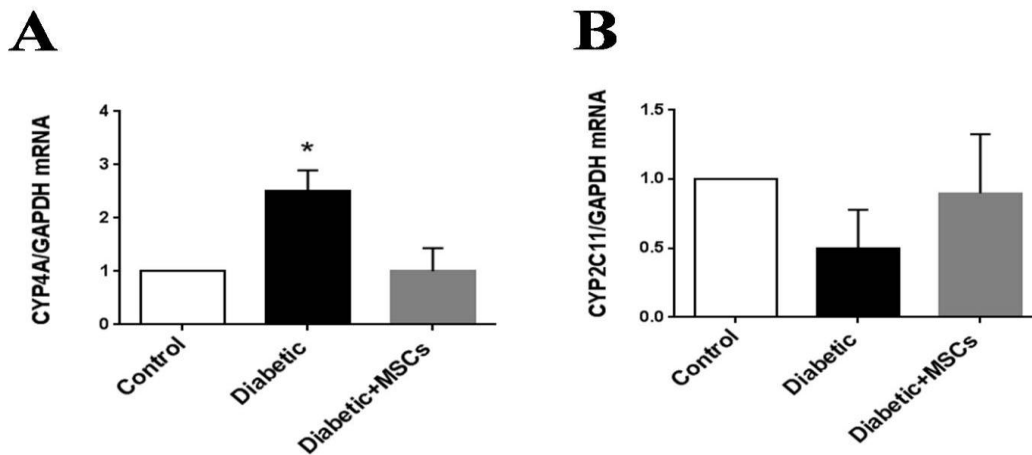**Figure 10: MSCs injections attenuate kidney injury through CYP4A and CYP2C11 levels.** CYP4A and CYP2C11 levels were assessed by RT-PCR. (A) Histogram showing CYP4A mRNA levels relative to GAPDH mRNA levels.(B) Histogram showing CYP2C11 mRNA levels relative to GAPDH mRNA levels. The results are means ± SEM from 3 different rats in each group. $^{*}P<0.05$ vs. control, $^{\#}P<0.05$ vs. diabetic.

I. MSCs injections attenuate kidney injury through TGF-β.

The growth factor TGF-beta has emerged as a key participant in the cascade of events, which leads to kidney sclerosis. Increased TGF-beta expression in the kidney in diabetes mellitus mediates the renal actions of hyperglycemia to promote cellular hypertrophy and stimulate extracellular matrix biosynthesis. However the crosstalk between MSC treatment and TGF-beta alteration in DN is unknown. The TGF-β mRNA levels were assessed in the different groups of rats. Interestingly, our results demonstrate that hyperglycemia resulted in an increase in TGF-B mRNA levels, an effect that was prevented by treatment with MSCs (Figure 11). These results suggest that MSCs exert their role by inhibiting TGF-beta and ROS production thus reducing kidney injury.
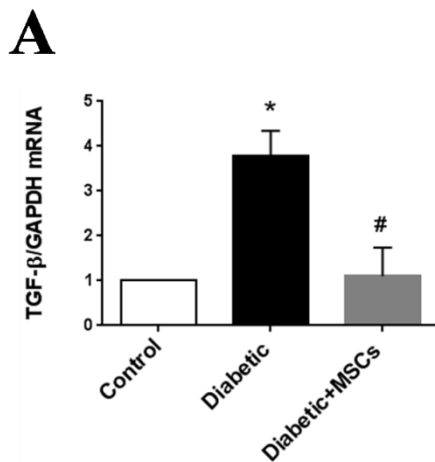

Figure 11: MSCs injections attenuate kidney injury by normalizing TGF-β levels.
TGF-β levels were assessed by RT-PCR. (A) Histogram showing TGF-β levels relative to GAPDH mRNA levels. The results are means ± SEM from 3 different rats in each group. $^{*}P<0.05$ vs. control, $^{\#}P<0.05$ vs. diabetic.

CHAPTER IV

DISCUSSION

MSCs play an important role in reversing kidney injury. In a diabetic milieu, there is a macrophage accumulation secreting proinflammatory, antiangiogenic and profibrotic elements that leads ultimately to renal injury. It has been demonstrated that MSCs treatment markedly reduced the number of macrophages in glomeruli of many animal models [53]. Furthermore, it has been shown that MSCs exert a paracrine effect. MSCs downregulated the proinflammatory cytokines such as IL-1b, TNF- α, IFN-r and upregulated the secretion of IL-10, bFGF, TGF-β anti-inflammatory cytokines [44]. Of equal importance, MSCs are thought to secrete the angiogenic vascular endothelial growth factor VEGF responsible for the regulation of capillary repair of damaged glomeruli [54]. Also, given that MSCs secrete large amounts of VEGF, they may be involved in the inhibition of TNF-αthat leads to fibrosis [45]. However, Schrijvers et al. revealed that VEGF mediates glomerular hypertrophy and proteinuria [55].

Moreover, Morigi et al. were the first to report the importance of human BM-MSCs treatment on survival. BM-MSCs injected in an acute kidney injury AKI model protected the mice from renal dysfunction and ultimately death by targeting the tubular cell damage. In this context, BM-MSCs improved tubular proliferation and reduced renal apoptosis by secreting prosurvival growth factor such as IGF-1 [56].

On the other hand, *in vitro* studies revealed that MSCs could undergo transformation or enhance the development of existing tumors. Equally important to tumor formation, MSCs showed to promote fibrosis development. In fact, MSCs are fibroblast-like cells and can be stimulated by TGF-β, an important factor in the progression of fibrosis [43]. Several studies demonstrated that MSCs treatment improved the biochemical, physical parameters in DN [57]. However, there were conflicting results about how much intravenously injected MSCs reduced blood glucose levels. Ho et al. revealed that several intravenous MSCs injections corrected hyperglycemia in STZ-induced diabetic mice [58]. Of equal importance, Ezquer FE et al. proved that MSCs corrected hyperglycemia by regenerating new yet smaller and less abundant beta-pancreatic islets [42]. By contrast, other studies found that MSCs had no effect on hyperglycemia [59, 60]. In this study, multiple injections of MSCs had no effect on 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 MSCs injected. Under normal conditions, podocytes foot processes maintain a selectively permeable diaphragm to proteins thus, forming the glomerular filtration barrier. In DN, the podocytes processes and the slit diaphragm are damaged causing albumin to leak i.e. microalbuminuria and subsequently protenuria. Treatment with MSCs returned the normal expression levels of nephrin and podocin [60]. Our results are consistent with the previous study [60] demonstrating that MSCs exert antialbuminuric effects by reducing urine albumin levels in the diabetic rats.

In addition to the fact that MSCs recover renal function, MSCs reduce renal fibrosis. The reason may be related to a transdifferentiation, a fusion, an enhancement of the tissue-endogenous progenitor cells proliferation, or a paracrine action [61, 62]. Also recently, it

has been declared that also intrarenal cells constitute a major source for kidney regeneration and homeostasis [59]. In their study, Shasha et al. verified that MSCs decreased the expression of ECM proteins such as fibronectin and collagen I in addition to the glomerulosclerosisin kidney tissues of DN [57].The present study provides clear evidence that MSCs intravenous injections remarkably reduce the histopathologycal features of DN including glomerulosclerosis and tubulointerstitial fibrosis.

It is well known that ROS is involved in the development of STZ-induced diabetes. In fact, it is established that oxidative stress initiates and develops diabetic nephropathy through an activation of several enzymatic and non-enzymatic sources of ROS in the kidney [57, 63]. How do MSCs decrease ROS production? Zhuoet al. proved that MSCs administration reduced the oxidative damage consequently renal injury in Ischemia/reperfusion injury implying that MSCs have antioxidant properties [64]. A single recent study revealed that MSCs treatment markedly inhibited oxidative stress; this was due to the reduction in both blood glucose levels and intracellular glucose levels [57].

However, the mechanistic pathway is unknown. To our knowledge, this is the first study to demonstrate that MSCs reverse ROS production in type 1 diabetes. We and others showed that NADPH oxidases are expressed in the kidneys and that Nox enzymes contribute to ROS generation in HG [3, 7]. Of the seven Nox isoforms, Nox4 was found to be the most expressed in the kidney and the most involved in renal ROS production [7, 52]. In this study, we found that ROS production, was decreased significantly in DHE staining and in the NADPH oxidase assay in the MSCs treated cortices, suggesting that MSCs down regulated the activity of the NADPH oxidase enzyme.

Also, in their study, Gorin et al. showed that the increase in the Nox4-derived ROS leads to oxidative stress and mediates renal hypertrophy and fibronectin overexpression. Currently, we demonstrated that the HG upregulated Nox4 enzyme expression and MSCs injections restored normal Nox4 expression levels.

Another major source for ROS and for the progression of tubular injury is the cytochromes P450 and their corresponding metabolites. In our lab, we showed that HG induced ROS production, tubulointerstitial changes, and tubular hypertrophy through the activation of both CYP4Aand NADPH oxidases. CYP4A-derived 20-HETE activated ROS production, ECM proteins accumulation, and induced renal hypertrophy. Conversely, we showed that HG decreases CYP2C11 protein expression and EETs production leading to an increase in ECM proteins amassing and cellular hypertrophy [5]. In this study, we revealed that CYP4A was downregulated and CYP2C11 was upregulated with the MSCs treatment.

In DN, it is well known that transforming growth factor TGF-β plays a key role in the progression of renal hypertrophy and ECM deposition. TGF-β mRNA and protein levels increase extensively in a diabetic milieu [32].A study established by Sharma K. et al. provides strong evidence that neutralizing the activity of TGF-β using anti-TGF-β antibodies decreased glomerular enlargement and inhibited the genes highly activated in ECM accumulation [65]. One of the findings in our lab was that in diabetes, the increased expression TGF-β goes along with the upregulation of CYP4A and its 20-HETE metabolite resulting in an excessive ROS production [5].

Correspondingly, it was found that in diabetes, MSCs treatment reduced or repressed TGF-β in the mesangial cells [57]. In this study, we demonstrated that MSCs reduced the

expression of TGF-β thus preventing the damaging effects of TGF-β and reversing the progression of DN.

Taken together, our data demonstrated that MSCs are beneficial for the treatment of renal injury, a major complication of diabetes. It is important to realize that there are indefinite advantages of MSCs treatment including the ease of isolation and the lack of ethical problems linked to their use. Further in-depth mechanistic studies are required to comprehend how MSCs attenuate diabetic nephropathy.

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