Glyoxalase 1 attenuates the effects of chronic hyperglycemia on explant-derived cardiac stem cells

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Abstract

Given that chronic hyperglycemia generates toxic methylglyoxal, the detoxifying effect of glyoxalase-1 (Glo1) on chronic hyperglycemia induced explant-derived cardiac stem cell (EDC) dysfunction was investigated. Wildtype (WT) and Glo1 over-expressing (Glo1TG) mice with or without streptozotocin treatment were studied. Hyperglycemia reduced overall culture yields while increasing the reactive dicarbonyl content within WT mice. These intrinsic cell changes reduced the angiogenic potential and nanoparticle production by hyperglycemic EDCs while promoting cell senescence. Compared to transplant of normoglycemic WT EDCs, hyperglycemic EDCs reduced myocardial function following infarction by inhibiting angiogenesis and endogenous repair mechanisms. In contrast, EDCs from hyperglycemic Glo1TG mice decreased reactive dicarbonyl content and restored culture yields. Intramyocardial injection of hyperglycemic Glo1TG EDCs also boosted myocardial function and reduced scarring. These findings demonstrate that, while chronic hyperglycemia decreases the regenerative performance of EDCs, over-expression of Glo1 reduces dicarbonyl stress and rescues the adverse effects of hyperglycemia on EDCs.

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List of Abbreviations

7-AAD	7-Aminoactinomycin D
AGE	Advanced glycation end product
Akt	Protein kinase B
ANOVA	Analysis of variance
ARE	Antioxidant response element
Bcl-2	B-cell lymphoma 2
BrdU	5-bromo-2’-deoxyuridine
CDC	Cardiosphere-derived cell
c-Kit	receptor tyrosine kinase
CSC	Cardiac stem cell
CSp	Cardiosphere
cTnT	Cardiac troponin T
CVD	Cardiovascular disease
DAPI	4’,6-diamidino-2-phenylindole
DHAP	Dihydroxyacetone phosphate
ECAR	Extracellular acidification rate
ECM	Extracellular matrix
EDC	Explant-derived cardiac stem cell
EPC	Endothelial progenitor cell
ERK	Extracellular signal-regulated kinase-1
FFA	Free fatty acid

Glo1	Glyoxalase 1
Glo1TG	Glyoxalase 1 over-expression
GO	Glyoxal
HbA1c	Glycated hemoglobin
HESP	Hesperetin
HUVEC	Human umbilical vein endothelial cell
IGF-1	Insulin-like growth factor 1
LC	Left coronary
MG	Methylglyoxal
MI	Myocardial infarction
MSC	Mesenchymal stem cell
Nrf2	Nuclear factor erythroid 2-related factor
OCR	Oxygen consumption rate
OCT	Optimal cutting temperature compound
PBS	Phosphate buffered solution
RAGE	Receptor for advanced glycation end products
ROS	Reactive oxygen species
SEM	Standard error mean
SDF-1α	Stromal cell-derived factor 1α
STZ	Streptozotocin
TGF-β	Transforming growth factor-β
tRES	Trans-resveratrol
VEGF	Vascular endothelial growth factor

WT	Wildtype

1.0 Introduction

1.1 Diabetes Mellitus

Given that one in ten Canadians live with diabetes, diabetes costs Canada almost $3 billion in direct healthcare costs and these numbers are projected to grow by more than 40% over the next 10 years.[1] Diabetes is a chronic metabolic disease in which the body's ability to produce or use insulin is impaired. There are 2 types of diabetes: type 1 and type 2 diabetes. Type 1 diabetes occurs when the body's ability to produce insulin is impaired because of auto-immune clearance of endogenous insulin producing pancreatic islets cells. In contrast, type 2 diabetes is mediated by increased insulin resistance within muscle and fat.[2] As a result, circulating blood glucose levels are increased with knock-on detrimental effects on pancreatic beta cells (further reducing insulin production), blood vessels and kidneys.[3] As such, chronic hyperglycemia is strongly associated with cardiovascular diseases (CVD) such as atherosclerosis and progressive heart failure.[4]

1.1.1 Pathophysiology of cardiovascular disease in diabetes

Diabetic complications can be divided into macrovascular and microvascular complications. Macrovascular complications result from inflammation which promotes arterial endothelial lining and smooth muscle cell dysfunction and leads to atherosclerosis, ischemic heart disease, cerebrovascular disease and peripheral

vascular disease. In contrast, microvascular complications affect the small vessels and results in impaired autoregulation of blood flow, altered permeability, inflammation, extracellular matrix accumulation, hypoxia, cell loss, neovascularization and fibrosis.[5–7] Insulin resistance and hyperglycemia exacerbates CVD risk factors such as hypertension and endothelial dysfunction which amplifies and accelerates disease progression.[8]

1.1.2 Effects of chronic hyperglycemia on myocardial metabolism

The heart can use a variety of substrates as fuel such as fatty acids, lactate and glucose. With fatty acids as the heart's main source of fuel, one of the consequences of chronic hyperglycemia is elevated free fatty acid (FFA) release, leading to a decrease in myocardial glucose transporter expression and reduced glucose uptake (Figure 1).[9] This substrate imbalance leads to lipotoxicity in the heart which promotes oxidative stress and apoptosis.[10] With an increase in FFA metabolism via the Kreb's cycle, there is an increase in electron donors (NADH and FADH) into the electron transport chain. This increases the voltage gradient of the mitochondrial membrane, resulting in a back up of electrons to coenzyme Q. These electrons get transferred to molecular oxygen, generating superoxides. Increased reactive oxygen species (ROS) overwhelms antioxidant reserves which promotes mitochondrial DNA damage, mitochondrial uncoupling and inefficient oxidative phosphorylation.[11–13]

1.1.3 Effects of chronic hyperglycemia on myocardial function

Abnormalities in contractile and regulatory protein expression are often associated with chronic hyperglycemia (Figure 1). There are 3 isoforms of myosin heavy-chain: V_1 (high ATPase activity), V_2 (medium ATPase activity) and V_3 (low ATPase activity). A shift in myosin isoenzyme composition (from V_1 to V_3 isoforms) typically occurs in diabetic hearts and this pattern of switch may be an attempt to maintain adequate cardiac performance in myocardial cells. In addition, observed increases in fetal β myosin heavy chain expression reduces the ATPase activity of myofibrils and resultant contractile force.[14] Furthermore, insulin secretion in response to increases in glucose is calcium dependent. In diabetic cardiomyocytes, there is a disruption in calcium homeostasis. As a result, inefficient calcium sequestering within the sarcoplasmic reticulum leads to decreases in sarcoplasmic reticulum calcium pump activity, cytosolic calcium overload and slow or incomplete relaxation of the ventricles during diastole (i.e., diastolic dysfunction).[15]

1.1.4 Effects of chronic hyperglycemia on myocardial structure

Hyperglycemic induced cellular injury commonly results from non-enzymatic glycation and oxidation of proteins or lipids to produce advanced glycation end products (AGEs).[16] Driven by excess ROS, AGEs form crosslinks within or between extracellular matrix (ECM) proteins such as myocardial collagen, laminin and elastin (Figure 1). This impairs the degradation of collagen and ultimately leads to fibrosis which increases the

stiffness of the heart (i.e., diastolic dysfunction).[17] Due to the increase in the glycolytic intermediate dihydroxyacetone phosphate (DHAP) in the diabetic setting, this increases *de novo* synthesis of diacylglycerol. Furthermore, cardiac-specific activation of the protein kinase C pathway (PKC-α, -β2, -δ, - ε isoform activation) stimulates connective tissue growth factor expression, endothelial dysfunction, and promotes cardiac fibrosis.[18,19] In a study performed with retinal pericytes, hyperglycemia was found to persistently activate PKCδ to cause vascular cell apoptosis and diabetic retinopathy.[20] As outlined in Figure 1, significant cross talk exists between these different mechanisms as activation of the diacylglycerol/protein kinase C signalling pathway can also occur through hyperglycemia-induced increases in oxidants.[21]

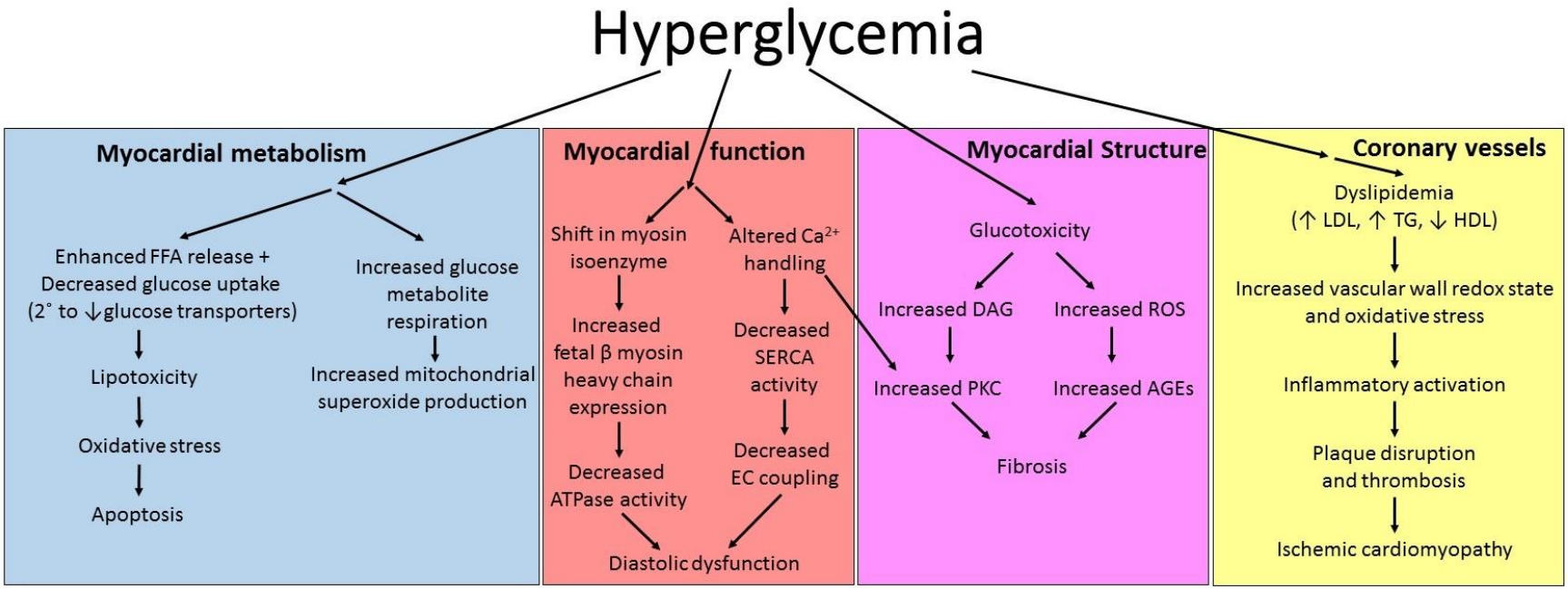


Figure 1: Summary of the adverse effects of hyperglycemia on the heart.

1.1.5 Effects of chronic hyperglycemia on coronary vessels

In the hyperglycemic environment, vascular homeostasis (i.e., the balance maintained between vasoactive factors that control permeability and adhesiveness) is disrupted; permitting the infiltration of oxidized low-density lipoprotein, lipids and immune cells into the vascular wall (Figure 1).[22] This infiltration contributes to the formation of plaques which progress to chronically limit blood flow. Acute rupture of thinned unstable plaques can occur which leads to acute thrombus formation. If left untreated, this leads to apoptotic/necroptic myocyte loss and eventual scar formation.[23] Normally, endothelial cells release prostacyclin and nitric oxide which induce relaxation of the smooth muscle cells and reduce aggregation of platelets. However, in diabetic patients, dysfunctional endothelial cells lead to accelerated atherosclerosis. In this case, there is an increase in platelet aggregation leading to increased vasospasms and eventual tissue ischemia. Ischemia in the heart due to prolonged vasospasms can lead to a myocardial infarction.[24] A single large myocardial infarction or multiple recurrent infarctions can lead to adverse cardiac remodeling and ischemic cardiomyopathy. Given that hyperglycemia impairs parasympathetic and sympathetic nervous system activity, a substantial number of myocardial infarcts in patients with chronic hyperglycemia are asymptomatic or associated with only minor atypical symptoms which delays and even precludes treatment.[25,26]

1.2 Impaired adult stem cell function in patients with chronic hyperglycemia

Towards the end of the last century, prevailing dogma posited that the adult heart was a terminally post-mitotic organ incapable of regeneration.[27] This notion has since been refuted with the discovery that adult cardiomyocytes exhibit a capacity for self-renewal. Bergmann et al. demonstrated that fewer than 50% of adult cardiomyocytes are renewed over an individual's lifespan. Taking advantage of atmospheric carbon-14 release from nuclear bomb testing during the Cold War, they used carbon dating to identify carbon-14 integration into genomic DNA. Using this technique, they estimated a turnover rate of approximately 1% per year.[28] Other groups have since confirmed the existence of self-renewing cardiomyocytes using various techniques such as the incorporation of cell cycle markers Ki67 and the nucleoside analog 5-bromo-2'-deoxyuridine (BrdU) in healthy and diseased adult hearts.[29,30] These discoveries raised the tantalizing prospect that, if endogenous repair could be harnessed or enhanced, patients may be saved from the inevitable progression of chronic heart failure. To address this challenge, several groups investigated the ability of *ex vivo* cultured stem cells from the heart and other organs to promote myocardial repair after cardiac injury. Over the past 20 years, protocols have been developed to culture stem cells from the blood, bone marrow, skeletal muscle, fat and heart for cellular cardiomyoplasty. Unfortunately, increasing medical co-morbidities inhibit the performance of these adult stem cell products.[31] Understanding these mechanisms may help develop our understanding and treatment of diabetic complications.

1.2.1 Mesenchymal stem sells

Mesenchymal stem cells (MSCs) are adult stem cells found in the bone marrow, peripheral blood, umbilical cord blood and fat tissue. MSCs are defined as cells that: 1) adhere under basic culture conditions; 2) express CD90, CD73 and CD105 without lineage (CD79α, CD14 or CD11b, CD45, CD19, CD34 and HLA-DR) and 3) differentiate *in vitro* into chondrocytes, osteoblasts and adipocytes.[32] Human derived MSCs sourced from diabetic patients exhibit impaired proliferative potential compared to non-diabetic patients.[33] Myocardial apoptosis has also been reported to be greater.[34] Treatment of injured hearts with diabetic MSCs demonstrated lesser improvements in myocardial function 4 weeks after cell injection as compared to MSCs sourced from normoglycemic patients. Mechanistically, this reduction has been attributed to less paracrine-mediated myocardial salvage (i.e., reduced apoptosis of endogenous myocardium) as indicated by lower levels of the anti-apoptotic transcript B-cell lymphoma 2 (Bcl-2) within infarcted myocardium, as well as the anti-apoptotic cytokines vascular endothelial growth factor (VEGF)[35] and insulin-like growth factor 1 (IGF-1).[36]

1.2.2 Endothelial progenitor cells

Endothelial progenitor cells (EPCs) are a separate type of adult stem cell isolated from bone marrow defined by surface marker expression (i.e., CD34+) or isolated using inductive cell culture conditions (containing signals that change cell behavior, shape, differentiation, mitotic activity, signal cascades and/or gene expression). These cells

are self-renewing and capable of differentiating into endothelial cells which contribute to the thin layer of cells that line the inner surface of vessels.[37] The number of circulating EPCs is inversely correlated with fasting blood sugar and HbA1c levels.[38] Hyperglycemia negatively impacts the proliferative capacity of EPCs in culture, and high glucose concentrations culture conditions markedly promotes apoptosis.[38] Hyperglycemia also impairs transplanted EPC adherence to endothelium and incorporation into blood vessels.[39] Hyperglycemic EPCs also demonstrate an impaired ability to respond to cytokine signals responsible for recruitment to areas of injury (i.e., stromal cell-derived factor 1α (SDF-1α)) which delays cell homing and vascular repair.[28,40] Finally, retained hyperglycemic cells demonstrate reduced indirect repair of the damaged endothelium as the pro-angiogenic paracrine signature is markedly attenuated.[39,40]

1.2.3 c-Kit+ cardiac stem cells

In 2003, Beltrami et al. identified a cell population within the adult heart that expressed the stem cell marker receptor tyrosine kinase (c-Kit), transcription factors associated with cardiomyocyte development (such as Nkx2.5 and MEF2C) and most notably, did not express hematopoietic lineage markers (such as CD34 and CD45). In culture, isolated c-Kit+ cells were shown to be multipotent, capable of clonogenic self-renewal and differentiation into endothelial cells, smooth muscle cells and myocytes. Once injected into an heart after ischemic injury, cardiac-derived c-Kit+ cell treatment increased vessel and myocyte formation.[41,42] Recently, the ability of endogenous *in situ*

c-Kit+ cells to provide cardiac repair have come into question with several groups suggesting that endogenous c-Kit+ cells contribute minimally to the generation of new cardiomyocytes in the adult heart.[43,44] These observations raise the possibility that prolonged *ex vivo* expansion of a relatively rare cell population (estimated to be 1/10,000 cardiac cells) to relevant cell doses may profoundly alter the regenerative performance of c-Kit+ cells, but this remains to be clearly shown.

In a manner akin to other adult stem cells, hyperglycemia has been shown to promote c-Kit+ cardiac stem cell (CSC) aging through the generation of ROS, ultimately shortening telomeres, upregulating senescent associated proteins (such as p53) and increasing apoptosis.[45] Hyperglycemia also decreases c-Kit+ cell culture yields, slows proliferation and decreases resistance to hydrogen peroxide stress.[36,46] c-Kit+ CSCs from hyperglycemic hosts are also less able to adopt cardiac (Tnnt2), endothelial (Flk1) or smooth muscle (Acta2) lineages after exposure to different differentiation conditions. Chronic hyperglycemia impairs the ability of CSCs to respond to insulin and results in persistent activation of the insulin signaling cascade, as evidenced by the inability of hyperglycemic CSCs to phosphorylate protein kinase B (Akt) upon exposure to insulin- despite having higher basal Akt phosphorylation and phosphorylation of tyrosine residues of insulin receptors as compared to non-diabetic CSCs.[36,47]

Hyperglycemia also impairs the mitochondrial activity of c-Kit+ CSCs. Mitochondrial consumption of oxygen (OCR) is lower in diabetic CSCs while basal extracellular acidification rates (ECAR) are modestly increased.[47] This increased reliance on glycolytic activity results in disproportionate partitioning of glucose into the pentose phosphate pathway and the glycerolipid pathway- thereby uncoupling ancillary

biosynthetic pathways of glucose metabolism. This higher glycolytic phenotype diminishes mitochondrial activity and reduces proliferation, which ultimately decreases cell survival and the capacity to differentiate.[47]

Over the past 15 years, the impressive c-Kit+ literature has literature has largely arisen from one group in the United States. Recent concerns regarding ethical use of government funds[48], retractions from the literature[49] and expressions of concern regarding the conduct of the only cardiac c-Kit+ cell trial to date[50] may lead to more retractions in the coming months/years. As such, the c-Kit literature needs to be reviewed with caution and not blindly extended to other *ex vivo* proliferated cell products.

1.2.4 Explant-derived cardiac stem cells and cardiosphere-derived cells

In 2004, Messina and colleagues demonstrated the isolation of undifferentiated cells from subcultures of human and murine heart biopsies. In this methodology, spontaneous outgrowth obtained from explant tissue plated on fibronectin coated dishes are collected using mild enzymatic digestion and re-plated onto poly-D-lysine coated flasks to form self-aggregating cardiospheres (CSps). The initial explant derived cells contain a complimentary admixture of cells that express cardiac (c-Kit+), endothelial (CD34) and mesenchymal (CD90) progenitor cell markers.[51] The 3D formation of CSps was believed to enhance and enrich the proliferation of the cardiac (c-Kit+) progenitor cell populations by increasing the stimulation of the extracellular signal-regulated kinase-1 (ERK) and VEGF pathway within the niche-like environment- although this

remains to be clearly shown.[52] Given the large diameter of CSps (70-100 μm), intra-coronary delivery felt to be problematic as coronary obstruction would result in myocardial infarction.[52–55] To address this issue, CSps were re-plated to form 2D adherent cultures. This provided a single cell expanded cell population called cardiosphere-derived cells (CDCs). In 2010, Chimenti and colleagues demonstrated the salutary effects of CDC therapy is mediated by paracrine stimulation of endogenous repair[56] which has since been attributed to a wide array of CDC-sourced cytokines, nanoparticles and matrix metalloproteinases.[57–60] Early clinical trials (CADUCEUS, CArdiosphere-Derived aUtologous stem CElls to reverse ventricUlar dySfunction,[61] ALLSTAR, ALLogeneic Heart STem Cells to Achieve Myocardial Regeneration and DYNAMIC, Dilated cardiomYopathy iNtervention with Allogeneic MyocardIally-regenerative Cells)[62] have established that CDCs are safe with promising hints of efficacy that include decreased scar size, increases in viable myocardium and improvements in regional function 12 months after treatment.

Given the limited evidence supporting “cardiosphering” and the markedly greater ability of explant derived cardiac stem cells (EDCs) to adopt a cardiac lineage 1000 fold greater than CDCs[63] (determined by using bioluminescent imaging of NCX1-driven luciferase expression following culture conditions known to favour cardiac differentiation), the Davis lab focuses on using EDCs for cell therapy. As outlined above, EDCs are a heterogenous mixture of cardiac (c-Kit, SSEA-1), endothelial (CD31, CD34) and mesenchymal (CD90) progenitors.[54] EDCs provide therapeutic benefits after myocardial infarction through direct differentiation into working myocardium and the secretion of cardioprotective, pro-angiogenic and pro-healing cytokines.[64–67] Akin to

other adult stem cell sources, increasing patient co-morbidities attenuate the regenerative performance of EDCs through diminished production of cardiogenic cytokines, pro-healing nanoparticles and the increased expression of the pro-inflammatory cytokine IL-6.[31] Pertinent to this study, hyperglycemia has been shown to reduce overall EDC cell numbers, stimulation of angiogenesis and post infarct EDC-mediated cardiac repair.[68] Given that hyperglycemia has negligible effects on the cytokine profile of EDCs,[68] the mechanism underlying hyperglycemic effects on EDCs is unknown and represents the focus of this thesis.

1.3 Role of nanoparticles in EDC-induced repair

Extracellular vesicles are endogenous nanoparticles that can deliver biological information between cells. These small vesicles range in size between 30-1000 nm-which includes both exosomes and microvesicles.[69] Exosomes are a homogenous population of extracellular vesicles released from cells when multivesicular endosomes fuse with the plasma membrane and release its contents. They range in size between 30-100 nm. Microvesicles range in size between 100-1000 nm and are formed from the budding and shedding of the cellular membrane. However, the size ranges of these extracellular vesicles may overlap and are variable across different cell types.[70] These vesicles often carry similar cellular receptors and transmembrane proteins on their surface from the cells that they were released from. While there is no established marker for microvesicles, exosomes frequently express CD9, CD63, CD81, HSP70 and TSG101.[71] These extracellular vesicles are known to carry and transport protein and

genomic material such as mRNA, siRNA, lnRNA and miRNA. The transportation of miRNA within these vesicles and the role that they play in cell-mediated cardiac regeneration have received the most attention recently.[72,73] Previous work suggests that that the delivery of extracellular vesicles sourced from CDCs into infarcted mouse hearts reproduced CDC-induced therapeutic regeneration.[60] One of the miRNAs identified to play an important role in mediating the therapeutic effect was miR-146a.[60] Similarly, a different group showed that the release of nanoparticles from CPCs accounted for the cardioprotective and proangiogenic paracrine activities of human CPCs, and that these vesicles were enriched with miR-210 and miR-132.[74] Another class of non-coding RNAs known as “Y RNAs” have been found to be highly abundant in CDC sourced extracellular vesicles and that these Y RNAs also confer cardioprotection.[75] Clearly, these endogenous nanoparticles play an important role in the paracrine mechanism of cell-mediated repair.

1.4 Role of dicarbonyl stress in chronic hyperglycemia

Dicarbonyl stress occurs when toxic α-oxoaldehyde metabolites accumulate through an imbalance between the formation and detoxification of dicarbonyl metabolites. Dicarbonyl stress becomes excessive in diseases such as obesity, hyperglycemia, CVD and renal failure.[76] Dicarbonyl metabolites include glyoxal (GO), methylglyoxal (MG) and 3-deoxyglucosone.[77] Typical concentrations of GO and MG in the human plasma range between 50-150 nM. Impaired glucose tolerance in patients

can cause a 3-fold increase in plasma and tissue concentrations of MG - leading to the nucleic acid and protein modifications which contribute to cell and tissue dysfunction.[78]

1.4.1 Advanced glycation end products

Dicarbonyl stress has detrimental biochemical and physiological consequences. Firstly, it increases the rate of dicarbonyl reactions with proteins and nucleotides resulting in advanced glycation end products (AGEs). Dicarbonyl glycation of cellular and extracellular matrix proteins cause mitochondrial protein dysfunction, mitochondrial pathway activated apoptosis, cell detachment, increased levels of ROS, and the upregulation of the receptor for advanced glycation end products (RAGE).[79–81] Interactions between RAGE primed cells and increasing concentrations of AGE ligands amplifies pro-inflammatory gene activation to further exacerbate dicarbonyl stress.[80]

Dicarbonyl glycation of proteins is usually directed against arginine residues, forming dihydroxyimidazolidine and hydroimidazolone adducts. Given that arginine residues are often present in the functional domains of proteins, MG modifications of arginine are detrimental, with MG-H1 (a hydroimidazolone adduct) being the most abundant AGE in physiological systems .[82] Other important but less frequent AGE-modification include lysine glycations (such as Nε-(carboxymethyl)lysine and Nε-(carboxyethyl)lysine).[76]

Dicarbonyl glycation of DNA is another source of AGEs as dicarbonyls avidly glycate deoxyguanosine residues of DNA to form 8-dihydro-8-oxo-2′-deoxyguanosine and imidazopurinone adducts. These glycation reactions eventually result in DNA

breaks, nucleotide transversions, cross-link formation and glycation of nucleosomal histone proteins which combine to limit cell viability and function.[82]

1.4.2 Formation, metabolism and dysregulation of methylglyoxal

MG is the most important precursor to AGEs. MG is a toxic by-product produced by several metabolic pathways in which triosephosphates are metabolic intermediates such as glycolysis, gluconeogenesis, glyceroneogenesis and photosynthesis. MG is formed at a relatively high flux mainly through the degradation of glyceraldehyde-3-phosphate and DHAP. These triosephosphate intermediates increase with an increased flux of glucose into the glycolytic pathway.[83] Approximately 99% of MG is metabolized by the rate limiting enzyme glyoxalase-1 (Glo1). This glutathione-dependent system recycles toxic MG into non-toxic D-lactate, which is then useful again in cellular metabolism. In the presence of reduced glutathione, MG is non-enzymatically converted into a hemithioacetal. Glo1 catalyzes the isomerization of the hemithioacetal into S-D-lactoylglutathione, after which glyoxalase-2 hydrolyzes S-D-lactoylglutathione to produce D-lactate and restore glutathione (Figure 2).[84] Given that Glo1 is the rate limiting enzyme in this system, I chose to focus my attention on this particular enzyme for my thesis.

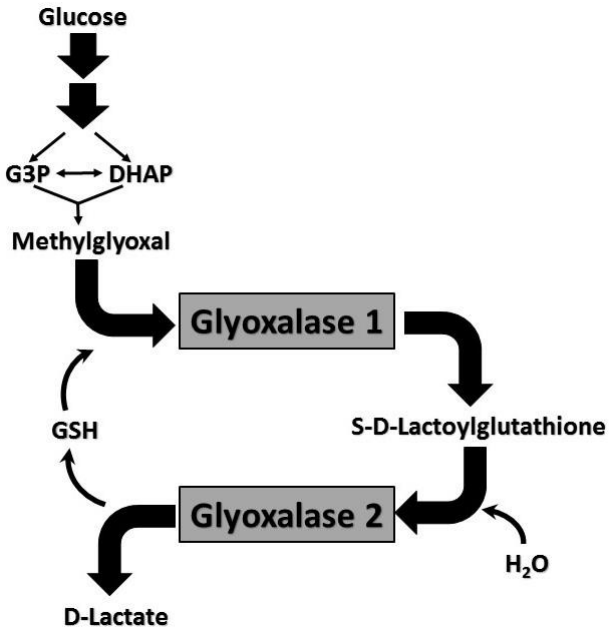


Figure 2: Formation and detoxification of methylglyoxal via the glyoxalase system.

Glo1 is ubiquitously expressed in all prokaryotic and eukaryotic cells.[85] The promoter region contains several regulatory elements, including: metal response element, insulin response element, as well as an antioxidant response element (ARE). The Glo1 gene is also a hotspot for copy number variations in many species, with copy numbers often being linked to an increased risk of obesity, diabetes and aging.[86]

Glo1 activity is dependent on the regulation of gene expression and post-translational modifications. Glo1 expression is under the stress-responsive control of nuclear factor erythroid 2-related factor 2 (Nrf2). In normal physiologic conditions, Nrf2

binds to the regulatory ARE in the promoter regions to protect against dicarbonyl stress. In high dicarbonyl stress environments such as chronic hyperglycemia, constitutive activation of the NF-kB complex antagonizes the activity of Nrf2- thereby impairing Glo1 activity.[87] Hypoxia is also an important physiological driver of dicarbonyl stress as MG formation is increased while hypoxia inducible factor 1α[88] and RAGE combine to limit Glo1 expression.[86] Clearly, an adequate balance between MG and Glo1 activity is necessary to ensure cell survival and the detoxification of MG.

1.4.3 Preventing methylglyoxal accumulation as a form of treatment

In diabetes, dicarbonyl stress is thought to play a role as MG promotes insulin resistance and beta-cell toxicity. One strategy to limit dicarbonyl stress is to decrease MG formation using scavengers. However, given the ubiquitous nature of MG, MG scavenging requires a high reactivity to be effective which can be associated with toxicity and instability.[89] Metformin is a commonly used medication to treat type 2 diabetes which is capable of scavenging MG, but recent kinetic studies suggest the noted improvements in dicarbonyl stress are largely attributable to improved glycemic control alone.[90]

Glo1 inducers such as trans-resveratrol (tRES) and hesperetin (HESP) have also been proposed as a means of limiting dicarbonyl stress. With a functional ARE in the promoter region of Glo1, Nrf2 activators recruit accessary proteins and increase the concentration of functionally active Nrf2 to levels required to increase the expression of Glo1.[91] The first clinical evaluation of a tRES/HESP binary combination produced

improvements in metabolic and vascular health in overweight and obese patients by increasing Glo1 activity in peripheral blood mononuclear cells and decreasing plasma MG and MG-protein activation.[87,92,93]

Several studies have used Glo1 over-expression to better understand the pathophysiology of chronic hyperglycemic induced dicarbonyl stress. Transgenic mouse strains have shown that increased constitutive expression of human Glo1 attenuates oxidative damage in the diabetic heart,[94] endothelial dysfunction and renal damage.[95] Pertinent to this study, over-expression of Glo1 has been shown to restore the ability of bone marrow-derived circulating angiogenic cells sourced from hyperglycemic donors to promote ischemic hindlimb perfusion.[96]

In the Davis lab, we have shown that the forced over-expression of Glo1 reverses the effects of chronic hyperglycemia on EDCs by restoring the ability of cells to promote post-infarct neovascularization *in vitro*.[68] These findings hint that MG content plays a pivotal role in the hyperglycemia and the regenerative performance of adult stem cells. Clearly, more mechanistic insight is needed to understand the hyperglycemic-induced dysfunction and, hopefully, reverse these adverse effects to improve cellular therapy for the individuals most in need of future cellular cardiomyoplasty.

2.0 Study Aims, Hypothesis and Specific Objectives

2.1 Study aim

This project is designed to determine the effect of altered reactive dicarbonyl content on EDC-mediated repair of ischemic cardiomyopathy.

2.2 Study hypothesis

Preventing the accumulation of reactive dicarbonyls within diabetic EDCs through direct genetic engineering to over-express Glo1 will enhance EDC-mediated cardiac repair.

2.3 Specific objectives

1. To explore the effect of Glo1 over-expression on the proliferative capacity of EDCs cultured from transgenic murine models of chronic hyperglycemia.
2. To explore the effects of Glo1 over-expression on the ROS content of EDCs cultured from transgenic murine models of chronic hyperglycemia.
3. To explore the effect of Glo1 over-expression on the survival of EDCs cultured from transgenic murine models of chronic hyperglycemia.
4. To explore the effect of Glo1 over-expression on the ability of EDCs cultured from transgenic murine models of chronic hyperglycemia to promote angiogenesis and secrete nanoparticles.
5. To assess the functional effects of Glo1 over-expression on EDC-mediated cardiac repair after injection into a mouse model of myocardial infarction: ventricular function, scar size, re-vascularization and cellular proliferation.

3.0 Methods

3.1 Cell culture

All animal protocols were reviewed and approved by the University of Ottawa Animal Care Committee. Murine cardiac tissue was obtained from 3.4±1.3 month old wild-type C57Bl/6 (WT) or Glo1 over-expressing (Glo1TG) transgenic mice under isoflurane sedation. As described previously,[96] mice that over-express human Glo1 gene were used. The cDNA encoding human Glo1 with an amino terminal c-myc epitope tag was cloned into the Not1-digested PEP8 plasmid resulting in the human Glo1 insert to be under the control of the murine pre-proendothelin promoter. Cardiac tissue was minced, enzymatically digested with collagenase (1mg/mL; Thermo Fisher) and plated as cardiac explants on fibronectin coated dishes. Cardiac explants were cultured in physiological 5% oxygen conditions[97] using custom formulated glucose-free Iscove's Modified Dulbecco's Medium supplemented with 5 mmol/l D-Glucose (physiological glucose), 20 mmol/l D-mannitol, 20% fetal bovine serum, 10% penicillin streptomycin, 2 mmol/l L-glutamine, and 0.1 mmol/l 2-mercaptoethanol (all from Thermo Fisher). EDCs emigrating from the plated tissue was harvested using mild trypsinization (0.05% trypsin; Thermo Fisher) once a week for 4 weeks for direct experimentation.[31,64–68] Human umbilical vein endothelial cells (HUVECs) were cultured in standard media (CC-2517, Lonza Group) at 21% oxygen conditions.

3.2 STZ induced hyperglycemia

Hyperglycemia was induced in C57 or Glo1 mice by intraperitoneal injection of STZ (50 mg/kg for 5 days) in 0.05 M sodium citrate. Normoglycemic control mice received equal volumes of 0.05 M sodium citrate. Fasting blood glucose and glycated hemoglobin (HbA1c) measurements were measured prior to sacrifice using Ascensia Contour Blood Glucose Meter (Bayer) and enzyme-linked immunosorbent assay (CSB-E08141m, Cusabio).

3.3 Measurement of senescence

EDC senescence within 5% oxygen, 1% exosome-free serum, 5mM (physiological) glucose conditions was quantified using overnight staining for senescence-associated β-galactosidase activity (KAA002, EMD Millipore) as per the manufacturer's directions. Briefly, EDCs were seeded on fibronectin-coated 12-well culture plates (Corning) and cultured for 5 days under 1% oxygen. The percentage of β-galactosidase cells was quantified using phase-contrast microscopy to count stained cells in five random fields per cell line assayed.

3.4 Measurement of bioenergetic determinations

This experiment was performed in collaboration with Dr. Mary Ellen Harper at the University of Ottawa. EDCS were seeded onto a Seahorse XF24 Cell Culture Microplate

overnight. Cells were washed and the culture media was replaced with Seahorse medium (bicarbonate-free DMEM, 5mM D-glucose, 4mM L-glutamine, 1mM sodium pyruvate; pH 7.4) and incubated in a non-CO2 incubator at 37ºC for 30 minutes. The assay cartridge was hydrated with XF calibrant solution one day prior to experiment and left at 37ºC overnight. Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) measurements were determined using a Seahorse XF24 Extracellular Flux Analyser (Seahorse Bioscience, Agilent Technologies). Calibration was conducted prior to collection of data. Leak and maximal respiration were measured after injection of 2µM oligomycin and 1µM FCCP, respectively (Sigma-Aldrich). Non-mitochondrial respiration was measured after injection of 1µM antimycin A (Sigma-Aldrich). Following the experiment, cells were lysed with 50µL of 0.5M NaOH to conduct protein quantification determination (Bradford assay). Rates were normalized to protein content in each well.

3.5 Measurement of EDC proliferation

EDCs proliferation within 1% oxygen,1% exosome-free serum, 5 mM (physiological) glucose conditions was quantified using a colorimetric assay (Cell Counting Kit-8, Dojindo) as per manufacturer's directions with confirmatory manual haemocytometer cell counts. Briefly, EDCs were seeded on fibronectin-coated 96-well culture plates (Corning) and cultured overnight. CCK-8 solution was added to each well and the plate was incubated for 3 hours at 5% oxygen and 5% carbon dioxide. Absorbance was measured at 450nm (BioTek Instruments).

3.6 Measurement of apoptosis resistance

The capacity of EDCs to resist apoptosis within 21% oxygen, 1% exosome-free serum, 5 mM (physiological) glucose conditions was quantified using flow cytometry (Guava easyCyte, EMD Millipore) for phycoerythrin-Annexin A5 (PE-Annexin V) and 7-Aminoactinomycin D (7-AAD) (559763, BD Biosciences). Briefly, EDCs were seeded on fibronectin coated 6-well plates (Corning) and incubated with 0.1 µM of staurosporine (A8192, ApexBio) for 24 hours. EDCs and media were collected and stained for PE-Annexin V and 7-AAD for 15 minutes at room temperature in the dark. EDCs were analyzed by flow cytometry.

3.7 Measurement of reactive oxygen species content

EDC reactive oxidant species content was in 5% oxygen, 20% exosome-free serum, 5 mM (physiological) glucose conditions using the 2',7-dichlorofluorescein diacetate fluorometric assay (ab113851, Abcam) as per the manufacturer's directions. Briefly, EDCs were seeded on fibronectin-coated 96-well culture plates (Corning) and cultured overnight. 25mM 2',7-dichlorofluorescein diacetate was added to each well and incubated for 45 minutes at 5% oxygen and 5% carbon dioxide. Cells were washed and fluorescence excitation and emission was quantified (BioTek Instruments) at 495 nm and 529nm respectively.

3.8 Effects of conditioned media on angiogenesis

Conditioned medium was prepared from confluent cultures of EDCs following 48 hours of hypoxic culture (1% O_2) in physiological glucose medium containing 1% exosome-free FBS (System Bioscience). The angiogenic potential of EDC conditioned media was evaluated using a cytokine depleted matrigel assay (ECM625, Millipore).[31,64,66–68] Briefly, human umbilical vein endothelial cells (HUVECs) were seeded on matrigel and incubated in EDC-conditioned media for 16 hours within 20% oxygen conditions. All phase contrast fields were compiled and cumulative tubular growth was determined using Image J software plug-in, NeuronJ (National Institutes of Health).

3.9 Nanoparticle purification

Conditioned media was collected from EDCs plated in 1% oxygen, 1% exosome-free serum, 5 mM (physiological) glucose conditions for 2-5 days. Nanoaparticles were isolated using ExoQuick-TC Exosome Precipitation Solution (System Biosciences) for nanoparticle tracking analysis (NanoSight LM10; Malvern Instruments). Briefly, conditioned media was centrifuged at 3000 x g for 15 minutes to remove cellular debris. Media was transferred to a sterile tube and 20% of ExoQuick-TC Exosome Precipitation Solution was added and refrigerated overnight. Media mixture was centrifuged at 1500 x g for 30 minutes. Supernatant was aspirated and residual mixture was centrifuged at

1500 x g for 5 minutes. Resulting pellet was resuspended in 250 µl of 1x Phosphate buffered solution (PBS).

3.10 *In vivo* cardiac repair

The ability of EDCs to promote cardiac repair following permanent left coronary (LC) artery ligation was evaluated using male murine EDCs injected into female wild-type C57 mice (Figure 3).[31,64–68] Animals were injected with buprenorphine (0.05 mg/kg; subcutaneous) 1 hour prior to surgery and twice daily thereafter for 3 days. During the ligation, mice were intubated, anesthetized using isoflurane (maintained at 2–3 %) and maintained under physiological temperatures. One week after LC ligation, animals were randomized into one of 7 groups to receive a myocardial injection consisting ($1x10^5$ cells divided into 2 injections at the apex and ischemic border zone) of: (1) sham treated WT male EDCs (not exposed to STZ), (2) STZ-treated WT male EDCs, (3) sham treated GLO1TG male EDCs (not exposed to STZ), (4) STZ-treated GLO1TG male EDCs (not exposed to STZ), or (7) vehicle (PBS). EDCs or vehicle were injected into using echocardiographic guidance. Myocardial function was evaluated using echocardiography (VisualSonics V1.3.8) and invasive hemodynamics (Transonic ADV500). During intramyocardial injection of cells/vehicle or physiological measures, animals were intubated, anesthetized using isoflurane (maintained at 2–3 %) and maintained under physiological temperatures. After the final echocardiogram, the hearts were excised and randomly allocated to histological analysis or quantitative polymerase chain reaction for retained male (transplanted) cells.[98] Hearts allocated to histology

were fixed with 4% paraformaldehyde, embedded in optimal cutting temperature compound (OCT) and sectioned. Tissue viability within the infarct zone was calculated from Masson's trichrome stained sections by tracing the infarct borders manually and then using ImageJ software to calculate the percent of viable myocardium within the overall infarcted area. To evaluate stem cell proliferation and differentiation, co-staining with BrdU (11778-1-AP; Proteintech Group, USA) and cardiac troponin T (cTnT) (ab66133; Abcam) was used. Capillary density within the infarct border zone was assessed by staining for isolectin B4 expression (B-1205; Vector Laboratories) in conjunction with DAPI (Sigma, Canada). The total number of nuclei within one image field of the border zone were counted and assessed for isolectin B4 expression.

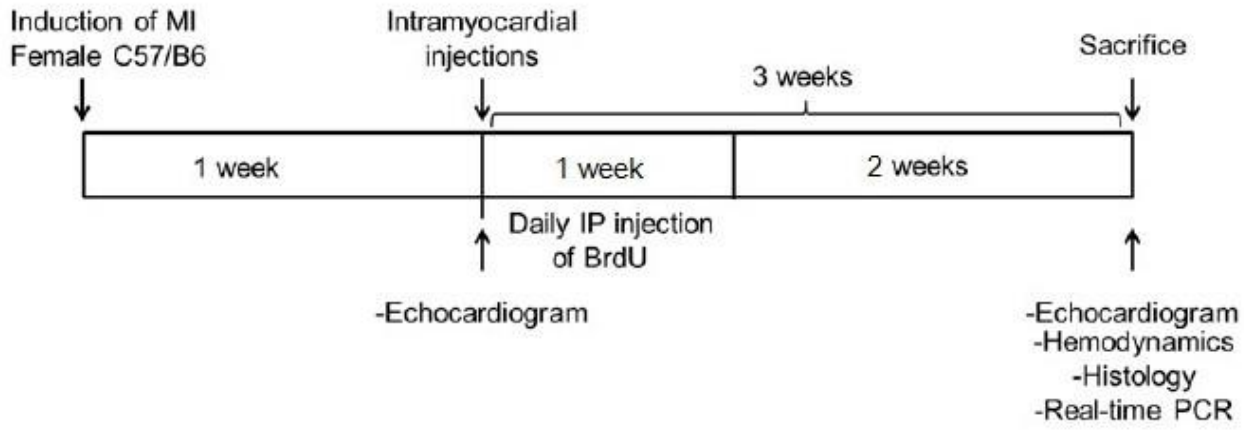


Figure 3: Experimental outline of *in vivo* study.

3.11 Data and statistical analysis

All procedures and analyses were performed blinded to animal or cell identity. Sample size calculations were performed to determine the number of animals needed

for each experiment (α= 0.05, β= 0.2, effect size= 6% change – extrapolated from clinical relevant improvements in disease based on clinical trials used for β-blocker drugs[99]). All data is presented as mean ± standard error mean (SEM). To determine if differences existed within groups, data was analyzed by a one-way analysis of variance (ANOVA). If such differences existed, Bonferroni's corrected t-test was used to determine the group(s) with the difference(s) (Prism 5.00; GraphPad Software, Inc.). Differences in categorical measures were analyzed using a Chi Square test. A final value of $P \leq 0.05$ was considered significant for all analyses.[66]

4.0 Results

4.1 Over-expression of Glo1 reduces the adverse effects of hyperglycemia on EDCs

EDCs were cultured from the cardiac biopsies of 16-week old wild type (WT) or Glo1 over-expressing (Glo1TG) mice 2 months after treatment with STZ or vehicle. Fasting blood glucose (FBG) obtained at time of sacrifice was markedly higher in STZ treated animals as compared to vehicle treated controls (22.2±1.0 versus 6.6±0.2 mmol/L, p≤0.001). Poor glycemic control was also confirmed by measuring glycated hemoglobin (HbA1c) levels in hyperglycemic mice (Figure 4). Chronically impaired glycemic control resulted in a 2.0±0.1-fold increase in glycated hemoglobin (p=0.001 vs vehicle treated mice) within both WT and Glo1TG STZ treated mice (p=0.27 for strain differences).

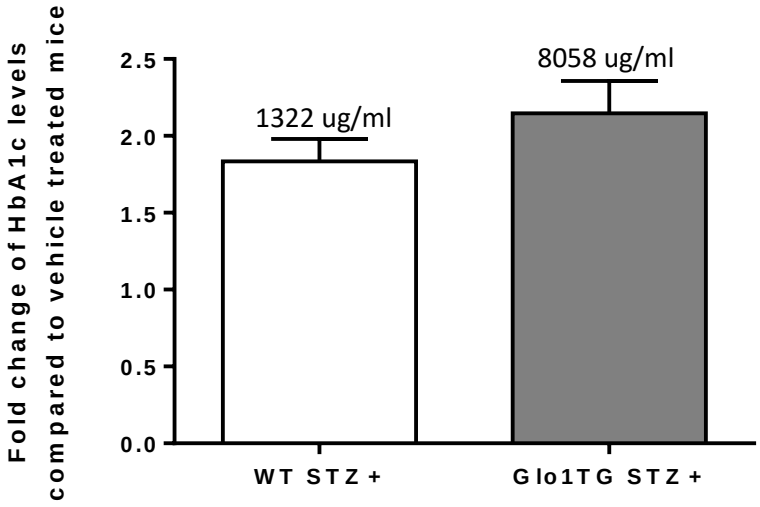


Figure 4: HbA1c levels in WT STZ+ (n=5) and Glo1TG STZ+ (n=6) treated animals.

Despite having no influence on the amount of tissue available for plating, chronic hyperglycemia reduced the cumulative number of EDCs cultured from plated cardiac biopsies by 86±3% ($p=0.0009$ versus WT vehicle treated controls; Figure 5A). This observation may be in part be attributable to the ~2 fold greater number of senescent EDCs found in cell lines sourced from STZ-treated WT mice ($p=0.03$; Figure 5B and Supplemental Figure 1).

Unexpectedly, overall cell culture yields were also significantly decreased in the non-STZ treated Glo1TG mice as compared to non-STZ treated WT mice (36±8% fewer cells cultured from equivalent amounts of plated myocardial tissue, $p\leq0.001$; Figure 5A)- suggesting that apart from differences in Glo1 expression important differences in cell metabolism may exist within the inbred transgenic mouse line. This possibility was critically interrogated using an Agilent Seahorse Analyzer. In collaboration with Dr. Mary Ellen Harper's lab at RGN, we analyzed EDC's mitochondrial respiration by measuring oxygen consumption rate (OCR) and glycolytic activity by measuring the extracellular acidification rate (ECAR) in response to various metabolic stressors. Preliminary data suggests that Glo1TG mice exhibit potentially reduced mitochondrial activity, demonstrated by possessing a poorer baseline metabolism than normoglycemic WT EDCs, and appear to be ineffective in projecting towards a more energetic phenotype under stressful conditions. (Figure 5C). Exposure to hypoxic low serum cell conditions highlighted these potential differences as non-STZ Glo1TG EDCs took 1.5±0.2 fold longer to proliferate (Figure 5E).

Consistent with the effects of Glo1 somatic gene transfer,[68] Glo1 over-expression attenuated the impact of hyperglycemia on EDC function. EDCs cell culture

demonstrated that 29±9% fewer cells were obtained from culture of Glo1TG STZ-treated mice as compared to Glo1TG vehicle treated mice ($p \leq 0.001$; Figure 5A). Despite the changes noted within the mitochondrial metabolism of Glo1TG mice, over-expression of Glo1 reduced the proportion of senescent EDCs to a level similar to normoglycemic WT controls (p=n.s, Figure 5B). Chronic hyperglycemia also had profound lasting effects on the proliferative capacity of plated EDCs as demonstrated by the marked increase in population doubling time in WT EDCs (30±4% slower growth, p=0.04; Figure 5D). However, consistent with previous publications,[68] when EDCs were incubated with the known apoptotic inducer staurosporine (protein kinase inhibitor and activator of caspases), no effect of chronic hyperglycemia was observed on EDC apoptotic susceptibility (p=n.s; Figure 5E).

EDCs sourced from WT STZ-treated mice demonstrated a marked increase in ROS content as compared to normoglycemic controls (30072±1223 a.u. versus 23598±846 a.u., p=0.0006; Figure 5F). Over-expression of Glo1 markedly reduced the ROS content within STZ treated EDCs (30072±1223 a.u. versus 21864±1225 a.u., p=0.0007; Figure 5F).

Taken together, this data suggests that Glo1 over-expression protects cardiac tissue during chronic hyperglycemia to restore EDC yield when compared to hyperglycemic WT EDCs.

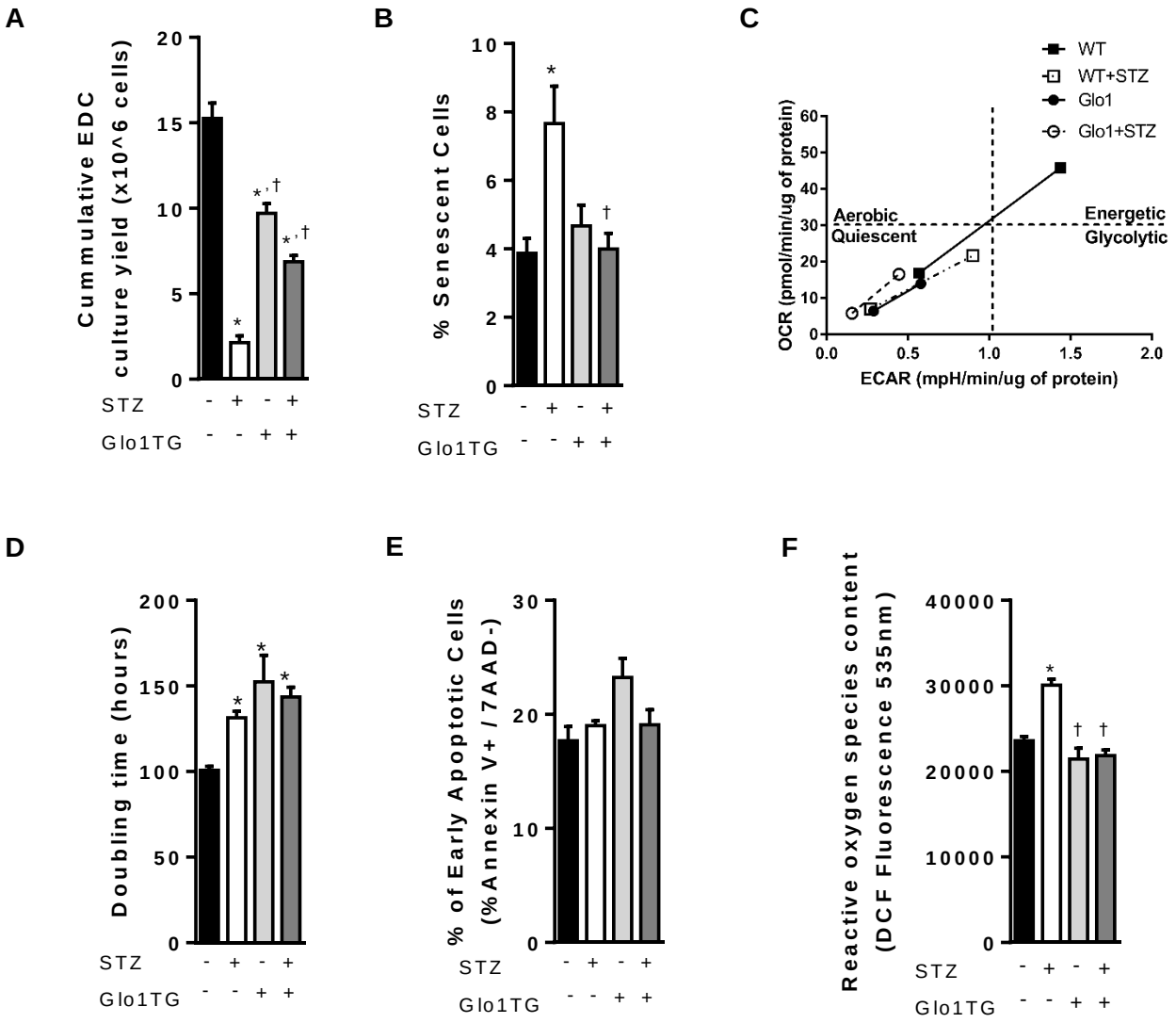


Figure 5: *In vitro* profile of Glo1 over-expressing EDCs. (A) EDC yield from normoglycemic control (n=10) and hyperglycemic (n=10) murine hearts under physiological culture conditions. (B) Proportion of senescent cells was measured by random field imaging of cells displaying senescence associated β-galactosidase activity (n=12). (C) Phenotype test of the metabolic potential of normoglycemic (n=10) and hyperglycemic (n=10) EDCs. OCR = Oxygen Consumption Rate. ECAR = Extracellular Acidification Rate (D) Population doubling times of EDCs of normoglycemic (n=8) and hyperglycemic (n=8) EDCs after 48 hours in low serum/hypoxic conditions. (E) Apoptotic susceptibility of EDCs was measured by flow cytometry after 24 hours incubation with 0.1µM staurosporine (n=12). (F) Basal ROS levels were measured in EDCs derived from hyperglycemic (n=6) and normoglycemic (n=6) myocardium. Data are expressed as the 2', 7' – dichlorofluorescein (DCF) fluorescence intensity. Values are mean ± SEM. *p<0.05 vs. non-STZ WT; †p<0.05 vs. WT+STZ

4.2 Over-expression of Glo1 reverses the adverse effects of hyperglycemia on EDC-mediated repair of injured myocardium

Given that the transgenic over-expression of Glo1 reduces the adverse effects of hyperglycemia, we explored the influence of Glo1 over-expression on EDC-mediated repair of injured myocardium 1 week after LC artery ligation. As shown in Table I in the Data Supplement, cardiac dimensions and function was similar at the time of randomization to cell or vehicle treatment. Three weeks after receiving vehicle alone, cardiac function progressively declined as adverse remodeling ensued. Hyperglycemia impaired the ability of WT EDCs to promote myocardial repair as evidenced by markedly reduced echocardiographic measures (Figure 6A) and hemodynamic measures (Figure 6B) of myocardial function as compared to treatment with EDCs cultured rom normoglycemic WT biopsies. In contrast, Glo1 over-expression prevented chronic hyperglycemia from adversely influencing the ability of EDCs to promote myocardial function (p=ns compared to normoglycemic WT and Glo1TG EDCs).

Hyperglycemia had similar effects on the ability of EDCs to prevent ventricular scarring, as the transplant of EDCs sourced from hyperglycemic WT mice demonstrated greater ventricular scars as compared to WT controls (33.9±1.8% versus 22.2±1.5%, p=0.002; Figure 6C and Figure 2 in the Data Supplement). In contrast, transplant of EDCs sourced from both normoglycemic and hyperglycemic Glo1TG mice demonstrated ventricular scarring comparable to mice treated with normoglycemic WT EDCs. Transplant of EDCs from WT STZ-treated mice resulted in fewer vessels within the peri-infarct region (1.6±0.3 versus 3.3±0.3 isolectin B4+ vessels/field, p=0.008

versus normoglycemic WT EDCs; Figure 6D and Figure 3 in the Data Supplement). Transplant of EDCs from STZ-treated or normoglycemic Glo1TG provided treated hearts with vessel density similar to those that received normoglycemic WT EDCs. Finally, transplant of EDCs cultured from hyperglycemic WT mice decreased cell proliferation (BrdU+, p=0.02) and the generation of new cardiomyocytes (BrdU+/cTnT+, p=0.001) in the infarct and peri-infarct region as compared to normoglycemic WT EDCs (Figure 6E and Figure 4 in the Data Supplement). In contrast, intra-myocardial injection of STZ treated Glo1TG EDCs stimulated proliferation of endogenous cells akin to that of the non-STZ treated WT EDCs. Quantitative polymerase chain restriction analysis of ventricular lysates for the Y-chromosome marker Rbmy failed to detect any retained transplanted cells 3 weeks after intra-myocardial injection (data not shown)- suggesting that although transplanted cells may have persisted this was below the detection threshold of the assay.[100] Thus, over-expression of Glo1 prevented the adverse effects of chronic hyperglycemia on EDC-mediated repair of ischemic myocardium.

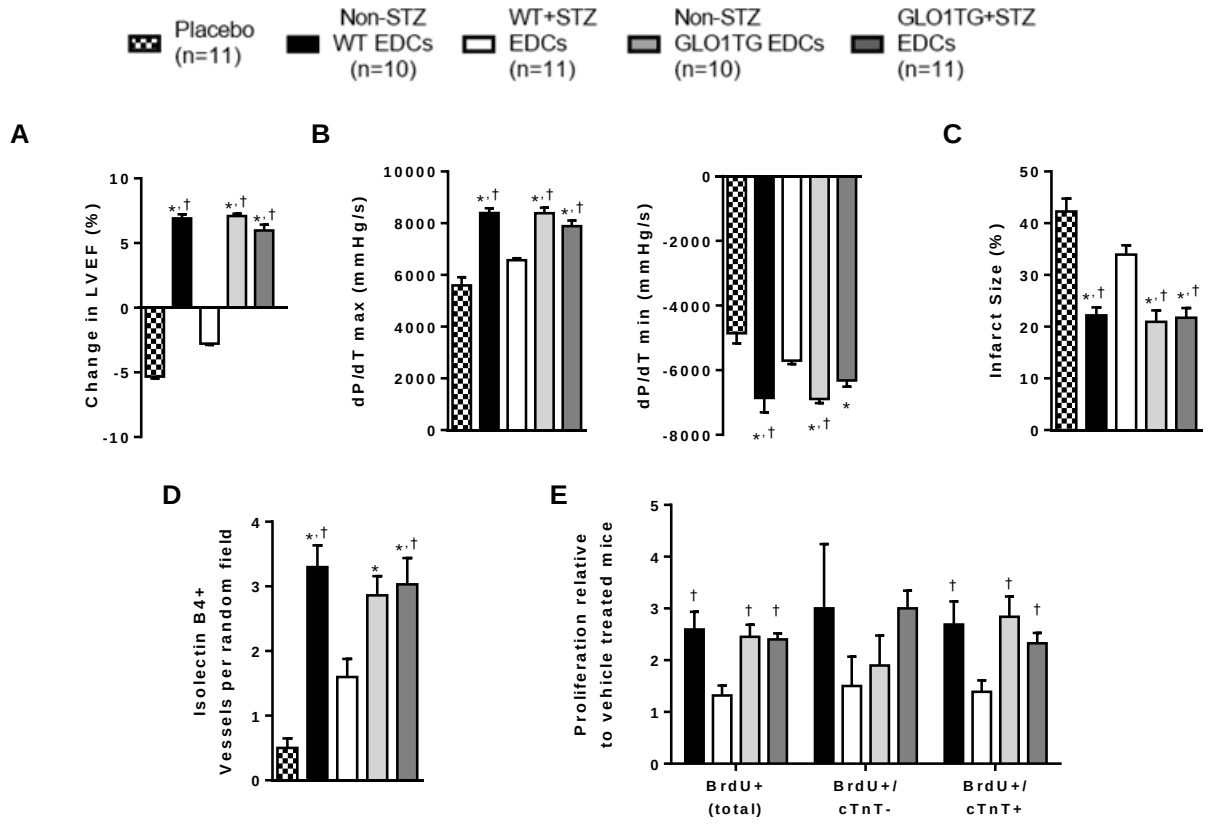


Figure 6: Measurement of EDC- mediated functional and structural improvements. (A) Change of left ventricular ejection fraction 4 weeks post-MI. (B) dP/dT measurements 4 weeks post-MI. (C) Size of infarct 4 weeks post-MI. (D) Vessel density of treated hearts 4 weeks post-MI. (E) Mice injected with BrdU daily for 7 days after PBS or EDC injection to evaluate endogenous cellular regeneration. Values are mean ± SEM. *p<0.05 vs. Placebo; †p<0.05 vs. WT+STZ

4.3 Glo1 over-expression limits the effects of hyperglycemia on EDC-mediated angiogenesis by enhancing nanoparticle production

Given the transient presence of transplanted cardiac-derived cells and the important role of indirect (paracrine-mediated) cardiac repair in cell treatment outcomes, the effects of hyperglycemia on the paracrine profile of EDCs was evaluated using media conditioned under low serum hypoxic conditions designed to mimic the harsh infarct environment. Consistent with previous publications,[1] chronic hyperglycemia reduced the ability of EDC conditioned media to promote formation of tubules by 74±2% as compared to WT non-STZ EDC conditioned media (Figure 7A and Figure 5 in the Data Supplement). Although media conditioned by Glo1TG mouse EDCs demonstrated a reduced ability to stimulate tubule formation, over-expression of Glo1 eliminated the anti-angiogenic effects of hyperglycemia.

Chronic hyperglycemia also markedly decreased the production of nanoparticles within EDC conditioned media by 43±6% (Figure 7B). EDCs sourced from STZ treated Glo1TG mice demonstrated a similar decrease in production of nanoparticles within EDC conditioned media when compared to non-diabetic Glo1 mice. The marked increase in nanoparticle production by Glo1TG EDCs compared to WT EDCs could in part be an effect of differences in mouse lines. Consistent with previous publications,[60,101] nanoparticles secreted by EDCs are nanosized vesicles ranging between ~30-150 nm in size (Figure 7C).

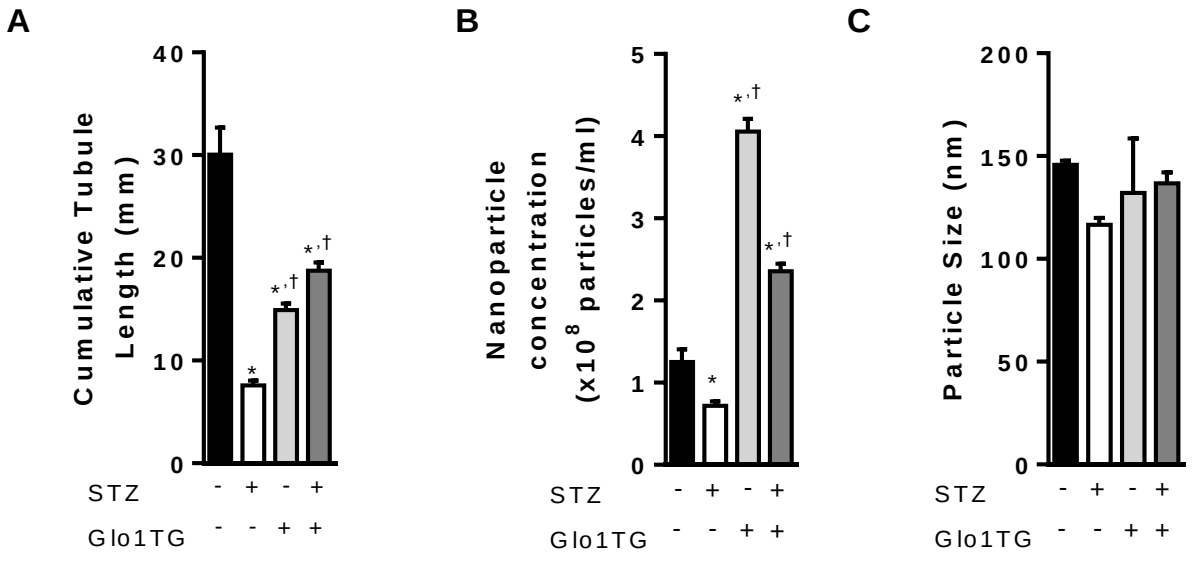


Figure 7: Paracrine potency of Glo1 over-expressing EDCs. (A) Total tubule length of human umbilical vein endothelial cells (HUVECs) after 16 hours exposure to conditioned media prepared from hyperglycemic EDCs (n=6) and normoglycemic EDCs (n=6). (B) Nanoparticle content of hyperglycemic (n=7) and normoglycemic (n=6) EDC-conditioned media using NanoSight Tracking Analysis. Media was conditioned under exosome-free low serum, hypoxic environment for 48 hours. (C) Nanoparticle particle size of hyperglycemic (n=6) and normoglycemic (n=6) EDC-conditioned media using NanoSight Tracking Analysis. Media was conditioned under exosome-free low serum, hypoxic environment for 48 hours. Values are mean ± SEM. *p<0.05 vs. non-STZ WT; †p<0.05 vs. WT+STZ

5.0 Discussion

As the significant role that stem cells play in tissue regeneration continue to be uncovered, regenerative medicine is proving to be a promising alternative to traditional pharmaceutical medicine. Cardiac-derived cell therapy has developed rapidly and shown that it is possible to harness endogenous regenerative mechanisms to reverse heart damage. By extracting and growing cells from a cardiac patient's own biopsy, it is possible to transplant these cells back into myocardium to stimulate repair. [53,63,102] Given that these EDCs were thought to differentiate into working myocardium, it was surprising that very few of these cells persisted despite clear evidence for its benefits. It has become accepted that the functional gains seen after EDC therapy are leveraged upon the ability of transplanted cells to recruit other host stem cells and rescue reversibly damaged myocardium through cytokine and nanoparticle secretion.[51,56,60,61,101,103]

Recently, we have been shown that the function of *ex vivo* proliferated EDCs may be impaired by accumulating patient co-morbidities[31] and, akin to other adult stem cell products, hyperglycemia impairs the regenerative performance of EDCs.[68] This impairment threatens to limit the impact of autologous EDCs for ill diabetic patients who may need cellular cardiomyoplasty in the future. Since hyperglycemia promotes the generation of MG, the influence of the key detoxification enzyme Glo1 on chronic hyperglycemia induced EDC dysfunction was investigated in this thesis.

In this study, we demonstrated that chronic hyperglycemia decreases EDC yield, resistance to oxidative stress, pro-angiogenic capacity, nanoparticle secretion, resistance to cellular senescence and EDC-mediated cardiac repair. However, the constitutive over-expression of Glo1 in EDCs reduces dicarbonyl content and rescued impaired chronic hyperglycemic EDC-mediated cardiac repair through new cardiomyocyte/vessel formation and the recovery of reversibly damaged myocardium.

The novelty of this study includes: 1) being the first to show that the over-expression of Glo1 can rescue EDC-mediated cardiac repair, 2) identifying the mechanism underlying impaired cardiac repair by diabetic EDCs, and 3) being the first to show that adverse effects of chronic hyperglycemia persists despite culture within physiological glucose (5 mmol/L) and oxygen (5% oxygen) conditions.

Current EDC culture conditions in clinical testing subject cells to 25 mmol/L glucose culture conditions due to the use of the commercially sourced basal media formulation (Iscove's Modified Dulbecco's Medium). Previous data highlights that exposure of EDCs to high-glucose culture reduced angiogenic capacity and cardiac repair potential of EDCs.[68] Recent reports hint that physiological oxygen tensions in culture are important in promoting the expansion of healthy, biologically potent stem cells that maintain genomic stability during prolonged cell culture.[97,104] As such, *ex vivo* proliferated EDCs in this study were cultured under physiological glucose (5 mmol/L) and oxygen (5%) conditions to examine the effect of STZ-induced hyperglycemia. Despite these efforts, the adverse effects of chronic hyperglycemia persisted in culture

as EDCs sourced from STZ-treated WT mice demonstrated a reduced ability to proliferate from plated tissue, promote angiogenesis, avoid cellular senescence and maintain the secretion of nanoparticles.

Unexpectedly, data suggesting poorer baseline metabolism in the inbred Glo1TG mice hints that these mice may harbor mitochondrial defects.[105] It is important to note that due to limitations in equipment, these experiments were performed outside of physiological oxygen conditions (21% oxygen). Literature has shown that physiological oxygen levels are very important for EDCs, and that the exposure of EDCs to a hyperoxic state may lead to oxidative stress and genomic instability.[104] It is possible that fluctuations in oxygen tension may have aggravated the potentially compromised Glo1TG EDCs. It is also possible that the constitutive over-expression of Glo1 itself could have compromised the EDCs. Despite these possibilities, Glo1 over-expression attenuated the impact of hyperglycemia. This is supported by several studies demonstrating that reducing MG levels enhances cellular and tissue function.[106,107] Recently, researchers demonstrated that the over-expression of Glo1 or the use of MG scavenging using a synthetic peptide reduced thermal hyperalgesia in MG-treated and diabetic mice.[106] MG scavenging has also been effective in cell culture. As seen in a similar study exploring the effect of the MG scavenger deferoxamine on the performance of human aortic endothelial cells cultured under high glucose condition. Here, co-incubation with deferoxamine for 5 days reduced the toxic effects of MG to baseline, decreased cellular ROS levels and restored hypoxia-induced VEGF production.[107]

Although elucidating the exact mechanism in which Glo1 over-expressing EDCs promote myocardial repair is challenging, the present study suggests that the stimulation of angiogenesis, reduction of fibrotic scar tissue and the generation of new cardiomyocytes underlies the observed boost in therapeutic regeneration. In mice treated with non Glo1 EDCs, excess MG impairs neovascularization of ischemic tissues and increased RAGE expression induces fibrosis via the upregulation of transforming growth factor-β (TGF-β).[17,108] The enhanced regenerative performance of Glo1 over-expressing EDCs *in vivo* in the face of modest engraftment[65] emphasizes that EDC repair is largely driven by the paracrine release of growth factors and their trophic actions on damaged myocardial tissue.

In terms of study limitations, EDCs were sourced from STZ treated wildtype and Glo1 over-expressing transgenic mice. We also acknowledge that the STZ model of diabetes reflects insulin deficient hyperglycemia while most patients who would require cellular cardiomyoplasty would be type 2 diabetics. With type 2 diabetes involving both hyperglycemia and insulin resistance, this multifactorial disease is complicated to recapitulate in an animal model. Although the mechanism in which elevated blood sugar occurs is different between the STZ model and type 2 diabetes, both models result in hyperglycemia and elevated levels of MG and reactive dicarbonyls. Using an STZ model provides an unbiased platform to observe the effects of chronic hyperglycemia. A second limitation of this study is that Glo1 over-expression and Glo1 activity in EDCs sourced from the transgenic mice was not assessed at the time this thesis was

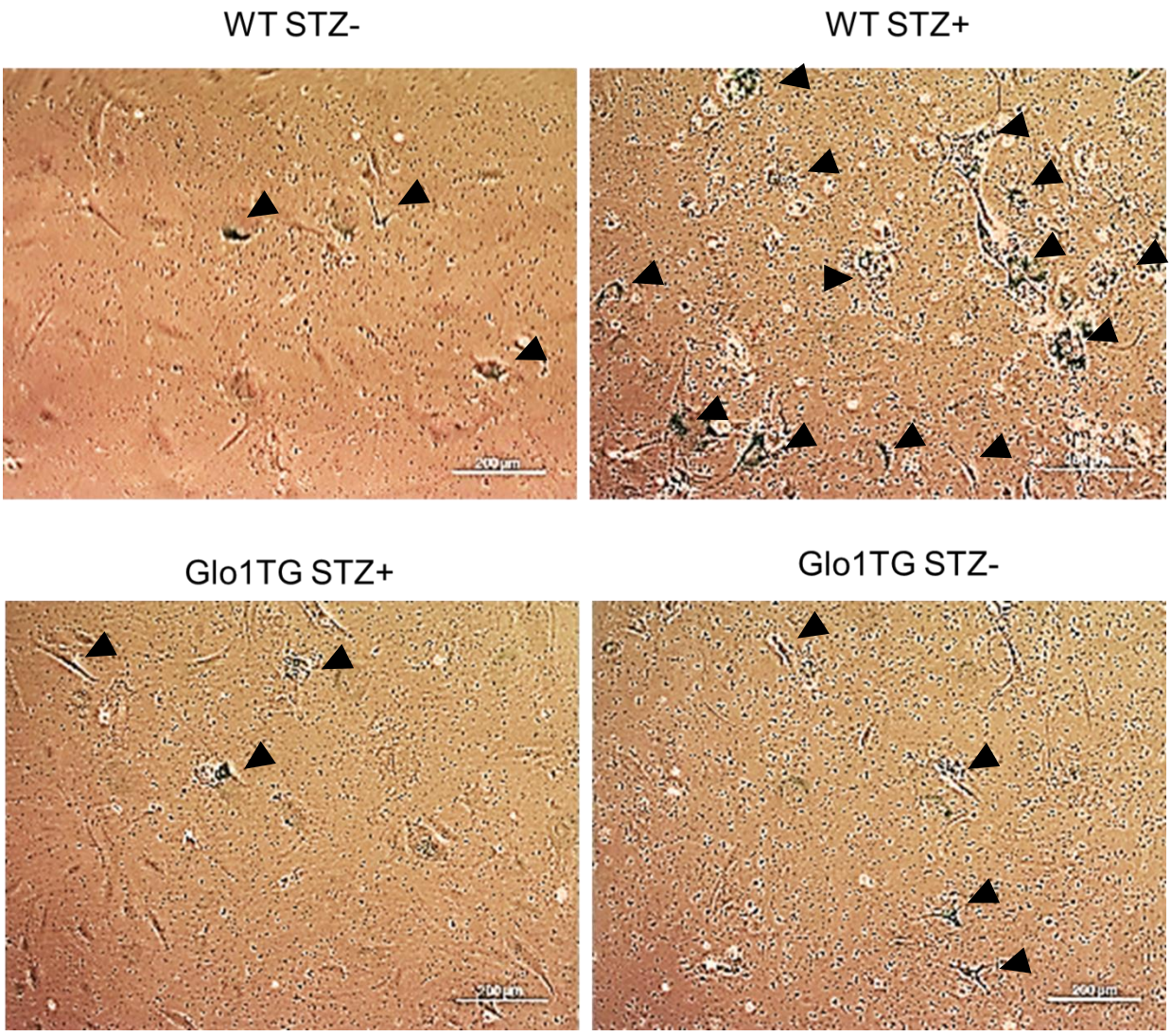
submitted. Without confirming this, other possibilities for their improved function may exist. For example, the enhanced function of EDCs from Glo1TG mice may be due to secondary effects conferred by other cells in the myocardium that over-express Glo1. Finally, a third limitation in this work lies in the study design that highlights the persistent effects of Glo1 over-expression on *ex vivo* proliferated hyperglycemic EDCs without extension to cell performance after transplantation back into a hyperglycemic host. Based on our findings, we could assume that EDC function would be preserved, but this important point deserves further study. Questions also remain as to what the effect of chronic hyperglycemia and Glo1 over-expression is on the ability of EDCs to secrete cardioprotective cytokines and on the miRNA content and identity within EDC-sourced nanoparticles. Emerging literature suggests that nanoparticles play key roles in EDC-induced repair of ischemic damage and is mediated through key miRNAs.[60,101] Given that hyperglycemia appears to have marked effects on the nanoparticle production by EDCs, it would be of interest to profile miRNA expression to identify key mediators that underlie the observed beneficial effects of EDCs sourced from Glo1 over-expressing mice.

6.0 Conclusions

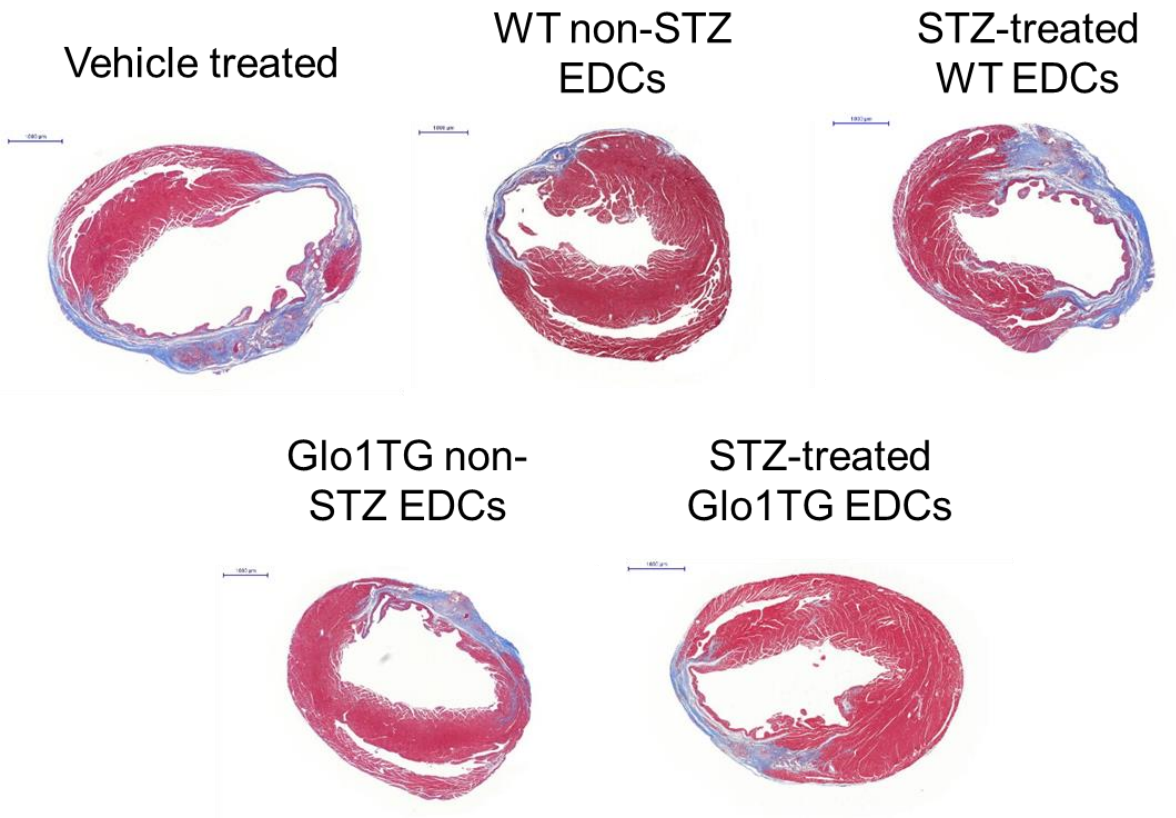
Chronic hyperglycemia decreased the regenerative ability of EDCs to provide therapeutic benefits through impaired stimulation of new myocyte and vessel formation. Constitutively over-expressing Glo1 within hyperglycemic EDCs reduced dicarbonyl

stress and rescued EDCs from the adverse effects of hyperglycemia on EDC-mediated cardiac repair.

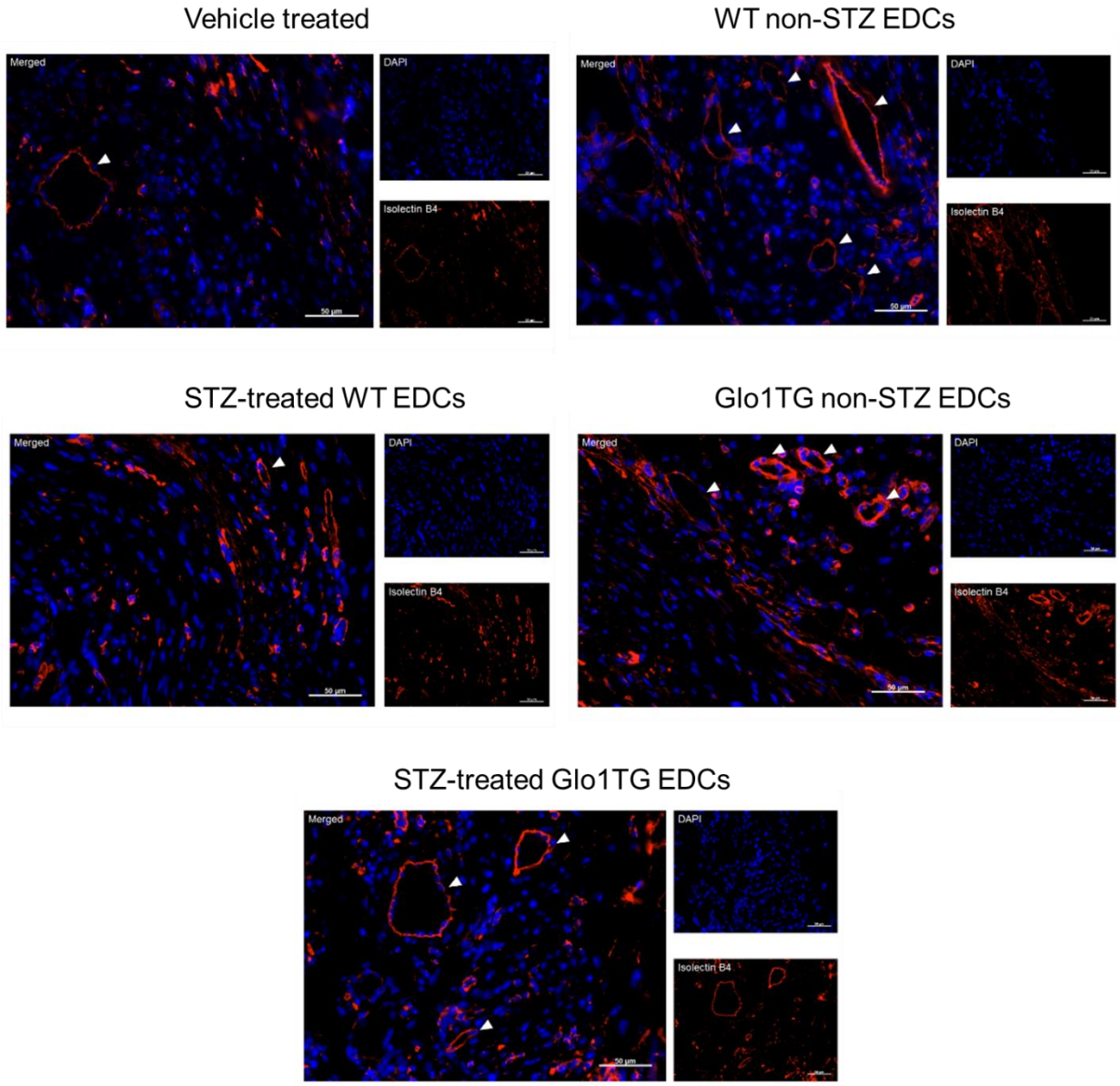
7.0 Supplemental Figures & Tables



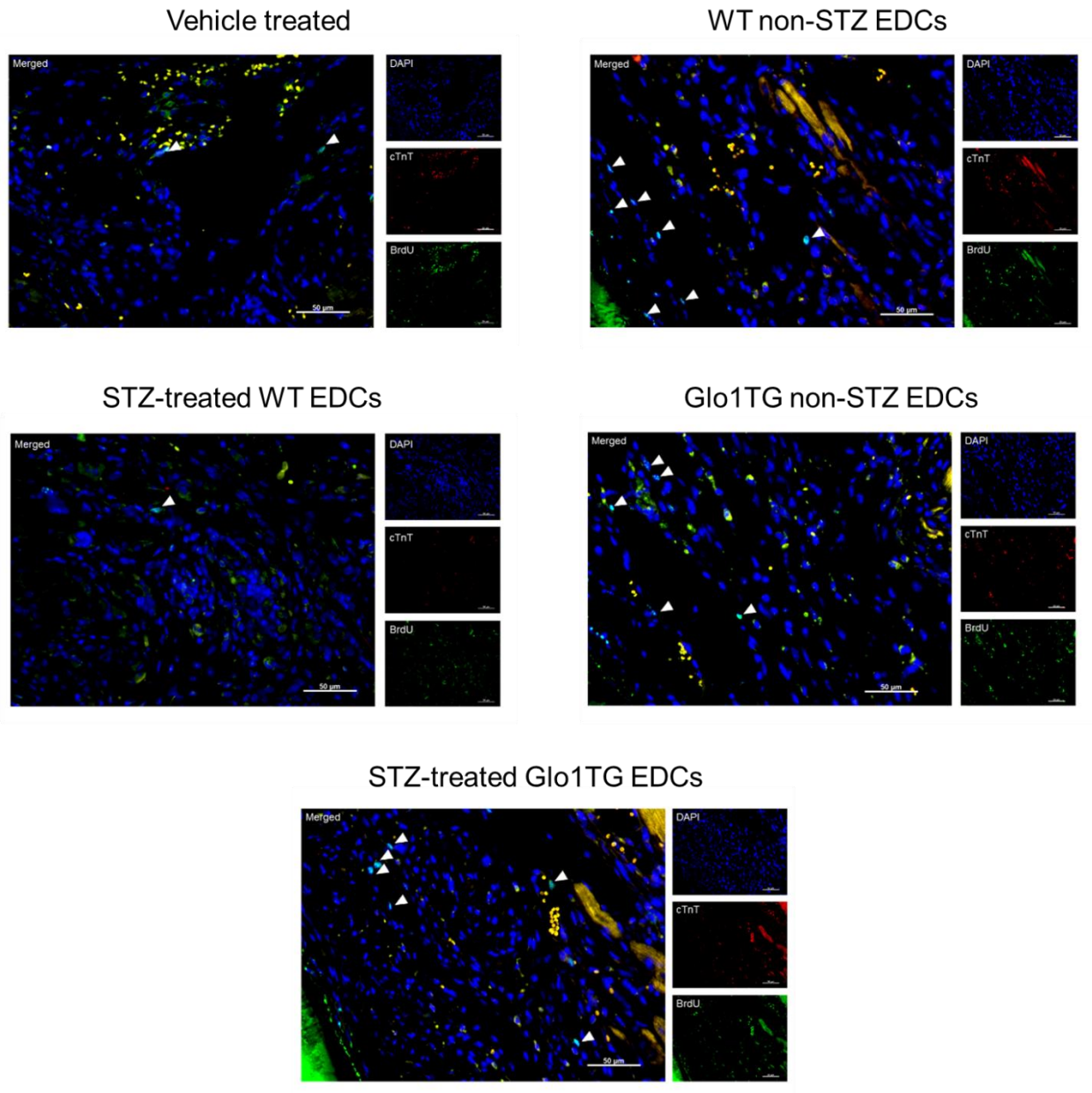
Supplement Figure S1. Representative images of β-galactosidase+ cells (arrow) within EDCs sourced from STZ treated and untreated WT and Glo1TG mice. Scale bar = 200 µm.



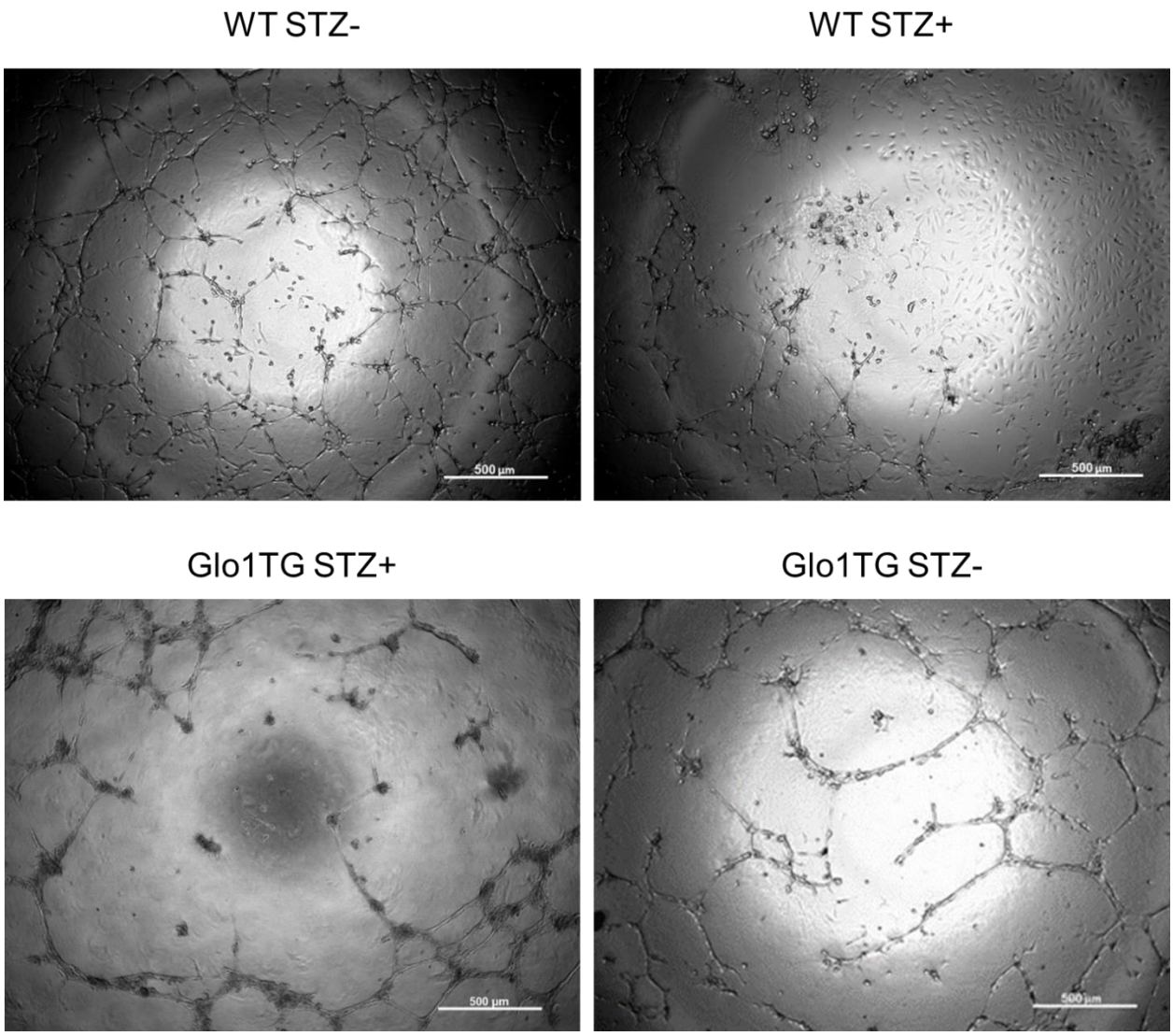
Supplement Figure S2. Representative Masson's trichrome images of each cell therapy 3 weeks after myocardial infarction. Scale bar = 1000 µm.



Supplement Figure S3. Representative isolectin B4+ images of each cell therapy 3 weeks after myocardial infarction. DAPI (blue). Isolectin B4 (red). Arrows indicate examples of vessels used for quantification. Scale bar = 50 µm.



Supplement Figure S4. Representative BrdU+ images of each cell therapy 3 weeks after myocardial infarction. DAPI (blue). Cardiac troponin T (red). BrdU (green). Scale bar = 50 µm.



Supplement Figure S5. Representative images of tubule formation of HUVECs after 16 hours of exposure to conditioned media prepared from EDCs. Scale bar = 500 µm.

	Placebo		WT STZ-		WT STZ+		Glo STZ-		Glo STZ+	
Weeks post MI	**1 week**	**4 weeks**	**1 week**	**4 weeks**	**1 week**	**4 weeks**	**1 week**	**4 weeks**	**1 week**	**4 weeks**
EDV (µl)	63.9 ± 4.5	55.9 ± 4.8	49.8 ± 3.8	56.9 ± 3.8	58.7 ± 4.3	68.7 ± 3.8	58.1 ± 2.7	67.6 ± 4.0	58.2 ± 4.2	67.2 ± 4.6
ESV (µl)	42.6 ± 4.5	36.1 ± 3.2	33.3 ± 2.8	34.1 ± 2.5	39.9 ± 3.5	48.5 ± 3.4	41.5 ± 2.8	43.3 ± 3.4	42.6 ± 3.3	45.6 ± 4.0
SV (µl)	21.3 ± 1.4	18.6 ± 1.4	16.5 ± 1.5	22.8 ± 1.8	18.9 ± 1.1	20.2 ± 0.8	16.7 ± 0.5	24.3 ± 1.2	15.6 ± 1.3	21.6 ± 1.2
FAC (%)	20.8 ± 2.0	18.1 ± 1.6	20.8 ± 1.3	24.8 ± 1.3	18.7 ± 1.2	18.0 ± 1.1	18.2 ± 0.9	21.6 ± 0.8	15.9 ± 0.8	20.2 ± 1.4
CO (ml/min)	8.3 ± 0.7	6.9 ± 0.5	6.5 ± 0.6	9.2 ± 0.7	7.2 ± 0.5	7.7 ± 0.2	6.7 ± 0.3	9.4 ± 0.5	6.6 ± 0.6	9.1 ± 0.6

Supplement Table S1. Echocardiographic measurements of left ventricular function over the 4-week follow-up period. EDV= end diastolic volume, ESV = end systolic volume, SV = stroke volume, FAC = fractional area shortening, CO = cardiac output. Data are mean ± SEM

	SW (mmHg*uL)	Vmax (uL)	Vmin (uL)	Ves (uL)	Ved (uL)	Pmax (mmHg)	Pmin (mmHg)	Pmean (mmHg)	Pdev (mmHg)	Pes (mmHg)	Ped (mmHg)	HR (bpm)
Placebo	1237 ± 107	43 ± 4	21 ± 4	25 ± 4	38 ±4	83 ± 2	5 ± 0.7	34 ± 1	78 ± 2	76 ± 3	9 ± 1	501 ± 13
WT STZ-	2108 ± 230	49 ± 3	21 ± 2	24 ± 2	45 ± 3	93 ± 5	4 ± 1	38 ± 2	89 ± 4	89 ± 5	7 ± 1	568 ± 10
WT STZ+	1961 ± 206	54 ± 5	22 ± 4	25 ± 4	50 ± 5	81 ± 1	0.5 ± 1	30 ± 2	81 ± 1	77 ± 1	4 ± 1	490 ± 13
Glo1 STZ-	2177 ± 770	55 ± 20	26 ± 9	28 ± 10	52 ± 18	91 ± 32	2 ± 0.5	36 ± 13	89 ± 32	87 ± 31	5 ± 2	519 ± 183
Glo1 STZ+	1646 ± 182	48 ± 4	23 ± 3	26 ± 3	43 ± 3	86 ± 0.9	0.7 ± 0.5	34 ± 2	85 ± 0.8	83 ± 0.9	4 ± 0.5	538 ± 16

	dP/dt max (mmHg/s)	dP/dt min (mmHg/s)	dV/dt max (uL/s)	dV/dt min (uL/s)	P@dV/dt max (mmHg)	P@dP/dt max (mmHg)	V@dP/dt max (uL)	V@dP/dt min (uL)	PVA (mmHg*uL)	PE (mmHg*uL)	CE	Tau (ms)
Placebo	5682 ± 325	-5151 ± 306	952 ± 87	-1034 ± 80	24 ± 6	45 ± 2	40 ± 4	23 ± 4	1800 ± 179	562 ± 104	0.6 ± 0.1	8 ± 0.5
WT STZ-	8457 ± 199	-6895 ± 449	1175 ± 133	-1570 ± 245	21 ± 7	52 ± 2	46 ± 3	21 ± 2	2707 ± 190	599 ± 55	0.8 ± 0.04	7 ± 0.4
WT STZ+	6574 ± 71	-5774 ± 121	1154 ± 170	-1520 ± 277	9 ± 3	43 ± 1	49 ± 5	23 ± 3	2562 ± 311	627 ± 139	0.8 ± 0.3	6 ± 0.2
Glo1 STZ-	8347 ± 2951	-6845 ± 2420	1022 ± 361	-1411 ± 499	6 ± 2	50 ± 18	53 ± 19	27 ± 9	3191 ± 1128	1014 ± 358	0.7 ± 0.3	6 ± 2
Glo1 STZ+	7829 ± 226	-6242 ± 187	1074 ± 123	-1226 ± 163	21 ± 5	47 ± 1	45 ± 4	25 ± 3	2377 ± 299	730 ± 167	0.7 ± 0.05	6 ± 0.3

Supplement Table S2. Hemodynamic measurements of left ventricular function over the 4-week follow-up period. SW = stroke work, HR = heart rate, PVA = pressure-volume area, CE = cardiac events. Data are mean ± SEM

8.0 References

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