The role of advanced glycation end products on sarcoplasmic reticulum calcium handling in diabetic cardiomyopathy

A Thesis

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ABSTRACT

Diabetic heart disease, also called diabetic cardiomyopathy, has been identified as its own clinical identity. Diabetic cardiomyopathy can lead to heart failure, killing ~65% of the patient population, and sudden death. Although we know that impaired calcium (Ca^{2+}) homeostasis is an important factor underlying diabetic cardiomyopathy, the exact mechanisms underlying impaired excitation-contraction are unknown. Our laboratory has previously demonstrated that impaired sarcoplasmic reticulum Ca^{2+}ATPase (SERCA2a) pump underlies diabetic cardiomyopathy. In addition, during diabetes, long-term hyperglycemia leads to the formation of advanced glycation end products (AGE) on sarcoplasmic reticulum (SR) Ca^{2+} regulatory proteins, which could impair their function and induce diastolic dysfunction, which is characterized by an impaired relaxation of the myocardium. Therefore, we investigated whether the formation of AGEs during diabetes impairs SR Ca^{2+} reuptake causing the impaired relaxation. We also investigated whether an AGE cross-link breaker, Alagebrium Chloride (ALT-711), could prevent these alterations in SR Ca^{2+} cycling and restore cardiac function. Groups of low dose streptozotocin (STZ)-induced diabetic rats were treated or not treated with ALT-711 for 8 weeks and compared

to age-matched control rats. Venous blood glucose was measured to assess hyperglycemia at baseline and weekly after injection of STZ. Echocardiography was done at baseline and post 8 weeks to assess cardiac function. After 8 weeks of diabetes, either pressure-volume loops were performed to assess cardiomyopathy or cardiac myocytes were isolated to measure Ca^{2+} transients and SR Ca^{2+} content. SR Ca^{2+} regulatory proteins, SERCA2a and ryanodine receptor (RyR2), expression was also measured by Western blot analysis. As expected, hyperglycemia was seen in diabetic rats compared to controls within 72 hours after the injection of STZ. There was no significant difference in blood glucose concentration between treated and non-treated diabetic rats. SR Ca^{2+} regulatory protein expression, both SERCA2a and RyR2, were significantly decreased in untreated diabetic compared to control groups ($P< 0.05$), but no significant difference was seen with the diabetic treated group compared to controls. Diastolic dysfunction was shown in the diabetic rats through pressure-volume loops and echocardiography proving that diabetic cardiomyopathy does occur in diabetes. Isovolumic relaxation time (IVRT) and myocardial performance index (MPI) improved with treatment of ALT-711 compared to the untreated diabetic rats ($P< 0.05$). Ca^{2+} transient and SR Ca^{2+} load were significantly decreased with diabetes ($P< 0.05$) and an improvement was seen with treatment of ALT-711. These data suggest that in our model of type 1 diabetes, AGE accumulation impairs the SERCA2a pump reuptake and that long-term treatment

with an AGE cross-link breaker will partially repair cardiac function and SR Ca^{2+} handling during diabetic cardiomyopathy.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

STZ……..Streptozotocin

IP……..Intraperitoneally

LV……..Left Ventricle

MPI……..Myocardial Performance Index

EF…..……Ejection Fraction

LVID end diastolic……Left Ventricular Internal Diameter at the End of Diastole

LVID end systolic……..Left Ventricular Internal Diameter at the End of Systole

E/A wave……...Early Diastolic Filling/Atrial Filling

IVRT……Isovolumic Relaxation Time

AGE……..….Advanced Glycation End Product

RAGE…..……...Receptor for Advanced Glycation End Product

SR…..……..……Sacroplasmic Reticulum

SERCA2a……..…Sarcoplasmic Reticulum Calcium ATPase Pump

RyR2……..….Ryanodine Receptor

AG…….Aminoguanidine

ALT-711……..Algebrium Chloride

PTB……...Phenyl Thiazolium Bromide

CICR……..……..Calcium Induced Calcium Release

NCX……...Sodium-Calcium Exchanger

Ca^{2+}……Calcium

CON……..Control Rat

DX……..……Diabetic Rat

DX-ALT……Diabetic Rat Treated with ALT-711

ESPRV……End Systolic Pressure Volume Relationship

EDPRV…..……End Diastolic Pressure Volume Relationship

PRSW……Preload Recruitable Stroke Work

T……..Tau

ECG……Electrocardiography

LITERATURE REVIEW

Diabetes Mellitus

The prevalence of diabetes mellitus is a rapidly growing phenomenon. It not only affects millions of people each year, it also affects animals as well. Diabetes is among the top five causes of death in developed nations and is now considered an epidemic that affects over 3% of the worlds' population.[2] The complications resulting from diabetes are numerous and include coronary artery disease, diabetic heart disease, heart failure, renal failure, neuropathy, blindness, leg injuries and stroke. Because of these complications, the life expectancy of a diabetic patient is greatly reduced from that of a healthy individual with the leading cause of mortality due to cardiovascular complications.[2,11,23] Diabetes is the result of hyperglycemia and glucose intolerance.[2,23] Hyperglycemia is the trademark of diabetes and also plays a major role in the development of diabetic cardiovascular problems. Hyperglycemia increases the number of free fatty acids, contributes to abnormalities in calcium homeostasis and lipid metabolism, and promotes the production of reactive oxygen species, which promotes apoptosis in cardiac myocytes.[74] People and animals that have hyperglycemia

experience polyuria, polydipsia, hunger, fatigue, weight loss, and blurry vision.[23]

There are two forms of diabetes mellitus, type 1 diabetes also known as insulin-dependent diabetes mellitus and type 2 diabetes also known as non-insulin-dependent diabetes mellitus. Insulin is the hormone produced by the pancreas that is responsible for controlling body blood glucose levels. It does this by moving glucose into insulin sensitive tissues like muscle, fat and liver so it can be used as energy.[11,23] Type 1 diabetes mellitus results from an autoimmune destruction of the insulin producing pancreatic beta cells. This causes decreased or absent glucose mobilization in insulin sensitive tissues, such as striated muscle, adipose tissue and smooth muscle. Type 2 diabetes mellitus results from insulin resistance, followed by insulin deficiency due to exhausted pancreatic beta cells that are trying to compensate for the insulin deficiency initially.[11,42]

It is possible to determine whether a patient has diabetes by assessing glucose concentrations in the urine, evaluating glucose concentrations in the blood, or administering a glucose tolerance test. However, there is currently no cure for diabetes mellitus only medical management. It is possible that type 2 diabetes can be prevented by an active and healthy lifestyle.[23]

Diabetic Animal Models

In order to study type 1 and type 2 diabetes, several animal models have been created. Rodents are one of the most common animal models used to

study diabetes. There are type 1 and type 2 rat models of diabetes as well as type 1 and type 2 mice models of diabetes. These models use either a diabetic fatty rodent or streptozotocin induced rodent to kill the beta cells of the pancreas.[9,10,14,17,19,25,29,39,42,47,48,64,67,75,76,87,88] Another diabetic animal model is the diabetic dog. Mongrel dogs, a term used to describe mixed breed dogs, are used as a diabetic dog model and they are injected with varying doses of alloxan monohydrate intravenously to induce diabetes.[4,25,51] Horses are also used as an insulin resistant, pre diabetic animal model.[45,84] Insulin resistance is the impaired ability for insulin to stimulate glucose into insulin sensitive tissues.[84] When glucose cannot be transported into tissues, hyperglycemia occurs and a pre-diabetic state is formed. In recent years, other diabetic animal models have been created using molecular biology including knock-out and knock-in mice.[25,65] It is also possible to induce diabetes in any animal model by a surgical method and completely removing the pancreas, thus removing the animals ability to produce insulin and regulate its glucose.[25]

One model for type 1 diabetes that is used frequently is the diabetic rat model. For this model, rats are injected once with various amounts of a drug called streptozotocin. Streptozotocin (STZ) is a glucosamine-nitrosourea compound that has a cytotoxic effect on the beta cells of the pancreas through DNA damage, mainly alkylation.[76] DNA alkylation leads to activation of poly ADP ribosylation, which leads to decrease ATP production and a decreased insulin synthesis and secretion.[67,76] This impaired insulin synthesis and secretion is

accompanied by an impairment in glucose oxidation, which leads to diabetes in the animal. The amount in a single injection of STZ used in the rodent animal model to mimic insulin-dependent type 1 diabetes that requires insulin injections can go up to 70 mg/kg, while the low dose STZ model used for type 1 diabetes that does not require insulin is 50 mg/kg.[9,10,14,17,19,47,48,76,87,88] The low dose STZ model shows similar clinical manifestations to that of human diabetes. It has a lower mortality rate and does not require insulin injections to survive.[42,47]

One model for type 2 diabetes that is used frequently is the diabetic fatty rat.[29,39] The diabetic fatty rat resembles the clinical manifestation of type 2 diabetes in humans relating to obesity with signs that include hyperglycemia, hyperinsulinemia, and hyperlipidemia.[29] A second model is used to mimic the end stage of type 2 diabetes where the beta cells of the pancreas are completely exhausted. For this model, rats are treated with 90 mg/kg of STZ to completely destroy the beta cells of the pancrease similar to what happens in type 1 diabetes.[29,39]

Diabetic Cardiomyopathy

Within the past thirty years diabetic heart disease, the leading killer of diabetic patients, has been identified as its own clinical identity, diabetic cardiomyopathy.[2,11,27,47] Diabetic cardiomyopathy is defined as ventricular dysfunction, myocyte hypertrophy and interstitial fibrosis, that is independent of coronary artery disease, hypertension and atherosclerosis.[26,27,37,63] The cellular

mechanisms that underlie diabetic cardiomyopathy include hyperglycemia, hyperinsulinemia, hyperlipidemia, and an increased production of reactive oxygen species.[37,63] The severity and duration of hyperglycemia has been shown to directly parallel the incidence of diabetic cardiomyopathy in patients with diabetes.[63] These cellular complications lead to the characterized diabetic heart that has an altered structure and function. The diabetic heart has an altered fuel usage that consists of more fatty acids compared to glucose, increased lipid, altered cell signaling and ion homeostasis, and an increase in advanced glycation end products which form cross-links with long lived proteins and alter their function.[9,10,27,42,85] Diabetic cardiomyopathy can be clinical or subclinical depending on the presence of signs and/or symptoms. If diabetic cardiomyopathy is clinical, it can possibly be managed with glycemic control, B-blockers, which decrease heart rate, and calcium (Ca^{2+}) channel blockers.[37] However if left untreated, diabetic cardiomyopathy can lead to arrhythmias, heart failure and sudden death.[17,58]

The first stage of diabetic cardiomyopathy normally consists of an asymptomatic period called diastolic dysfunction.[26,27,47] By virtue of new advanced, noninvasive methods of cardiac evaluation such as Doppler flow and tissue Doppler echocardiography, it is now known that over 50% of the asymptomatic patients with diabetic cardiomyopathy have diastolic dysfunction[17,27,47,74,78] Left ventricular (LV) diastolic dysfunction refers to abnormal left ventricular relaxation that alters the rate and extent of left

ventricular pressure during diastole.[5,78] Left ventricular diastolic dysfunction is also accompanied by left ventricular hypertrophy, myocardial stiffness, and altered structural proteins.[26] Changes in diastolic function are extensively seen in diabetic animals and humans. Diastolic dysfunction has been described through echocardiography and pressure volume loops by an increase in myocardial performance index (MPI), a decreased early diastolic filling with increased atrial filing (E/A wave ratio), an increased isovolumic relaxation time (IVRT), a increased End Diastolic Pressure Volume Relationship (EDPVR) and an increased Tau, the isovolumic relaxation time constant.[11,26,42,74]

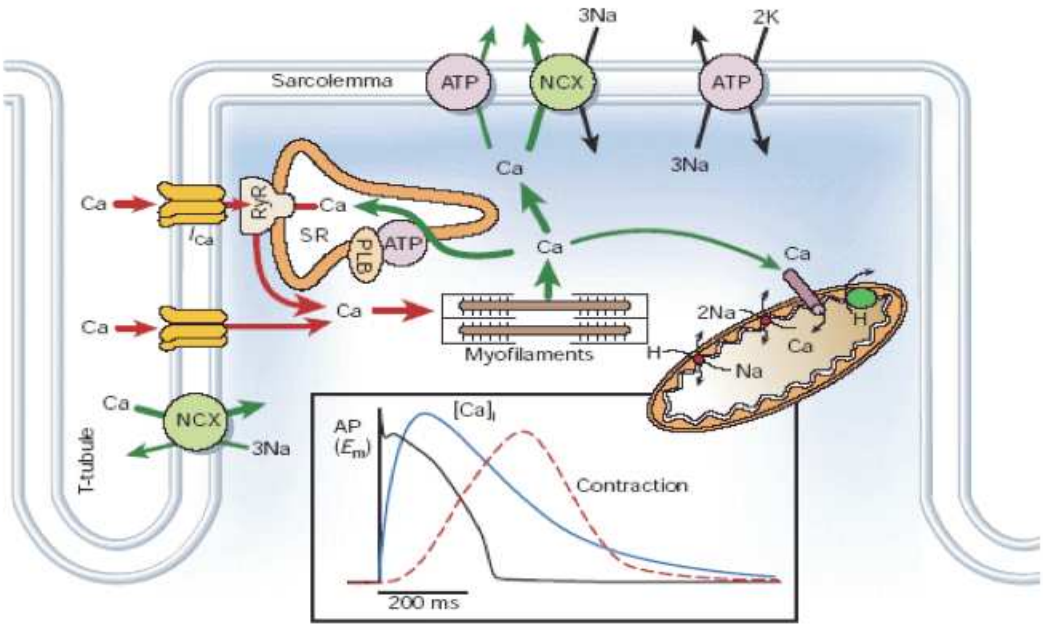
As diabetic cardiomyopathy progresses, systolic dysfunction can be seen following diastolic dysfunction.[11,27,26] Systolic dysfunction is defined as the myocardium's inability to contract properly and eject blood, and can lead to congestive heart failure and sudden death.[17,26,27,37,41,42] However it does take time to develop and be seen clinically. Systolic dysfunction is distinguished by reduced left ventricular ejection fraction. It is also accompanied by bradycardia, reduced systolic blood pressure and fractional shortening.[26,27,41] Many studies have shown diastolic dysfunction in diabetic patients, however systolic dysfunction is seen less frequently than diastolic dysfunction.[26,27] Fang *et al* suggested that this relates to the technique used to study myocardial dysfunction.[27] More sensitive techniques such as echocardiography strain and strain rate, as well tissue Doppler velocity can now detect preclinical systolic abnormalities in diabetic patients.[27] The wide use of these newer techniques is

much needed to better assess systolic function.[26] If diabetic cardiomyopathy progresses to systolic dysfunction, the prognosis for recovery is poor and annual mortality is increased.[26,37]

Calcium Handling

Calcium handling and homeostasis in the heart, an essential aspect to cardiac function, is monitored through a process called excitation-contraction coupling (E-C coupling). Cardiac excitation-contraction coupling is the process of electrical excitation that causes the myocytes in the heart to contract and relax.[8] Ca^{2+} is an essential aspect to E-C coupling and activates the myofilaments in the heart causing contraction. In order for Ca^{2+} to be available for contraction it leaves the interstitium and enters the cell through the L-type Ca^{2+} channel. The entry of Ca^{2+} activates the calcium-induced calcium-release (CICR), which allows Ca^{2+} to leave the SR via the Ca^{2+} release channels, RyR2. After Ca^{2+} leaves the SR via RyR2, it is available to enter the myofilament and bind to troponin, a myosin protein. Following the binding, troponin and tropomyosin, another myosin protein, go through a conformational change allowing myosin and actin to form a cross-link. This cross-linked myosin and actin allows myosin to pull the actin thin filaments together, thus causing muscle contraction. When Ca^{2+} is moved out of the cell via mitochondrial Ca^{2+} export and the Na^{+}/Ca^{2+} exchanger (NCX) or back into the SR via SERCA2a, ATP removes the cross-linked myosin and actin. This

then allows troponin and tropomyosin to revert back to their original conformation causing the heart to relax (Figure 1).[8,9,10,16,31,42,47,60,88]



Bers DM. Cardiac excitation-contraction coupling.
Nature. 415: 198-205, 2002.

Figure 1. Calcium handling in the cardiac myocyte

The time course of the action potential, calcium transient, and muscle contraction in a cardiac myocyte is shown along with sarcoplasmic reticulum calcium ATPase pump (SERCA2a), phospholamban (PLB), mitochondrial export of calcium, and the sodium-calcium exchanger (NCX).

The specific Ca^{2+} release channel in the heart is called type 2 ryanodine receptor calcium release channel. RyR2 is responsible for releasing Ca^{2+} from the SR to allow the myocardium to contract.[8,9,88] RyR2 is a large complex involving multiple regulatory proteins including FK-506 binding protein, calmodulin, PKA, phosphatases 1 and 2A, and sorcin. It is also coupled to other proteins including triadin, junctin and calsequestrin. All the various regulatory proteins that are combined with RyR assist in the regulation of RyR2 and the release of Ca^{2+}. In the myocardium there are several thousand RyR2, which are located at the edge of the sarcroplasmic reticulum and in the T-tubules. When Ca^{2+} is released, the cluster of RyRs all release Ca^{2+} based on the rise in action potential. If the action potential is sufficient, the Ca^{2+} will activate the myofilaments and cause contraction, until the SR's Ca^{2+} load is depleted passed its threshold.[8]

Sarcoplasmic reticulum Ca^{2+}-ATPase Pump is a member of a large family of P-type ATPase enzymes that is responsible for resequestering Ca^{2+} back into the SR. SERCA2a pump utilizes ATP, formed from the hydrolysis of the terminal phosphate bond, to pump Ca^{2+} against its electrochemical gradient in the myocardium.[10,88] SERCA2a pump consists of 10 transmembrane helixes, M1-M10, and 3 cytoplasmic domains; N, the nucleotide binding domain; P, the phosphorylation domain; and A, the actuator domain. It is also regulated by phospholamban (PLB), which must first be phosphorylated and dissociated from SERCA2a to activate SERCA2a. In order for Ca^{2+} to move from the lumen back

into the SR, Ca^{2+} must bind to SERCA2a causing the cytoplasmic domains to separate and phosphorylation of SERCA2a to occur.[8,10,60] Once phosphorylation occurs, SERCA2a can move Ca^{2+} back into the SR and restore the Ca^{2+} load needed for the next wave of contractions.

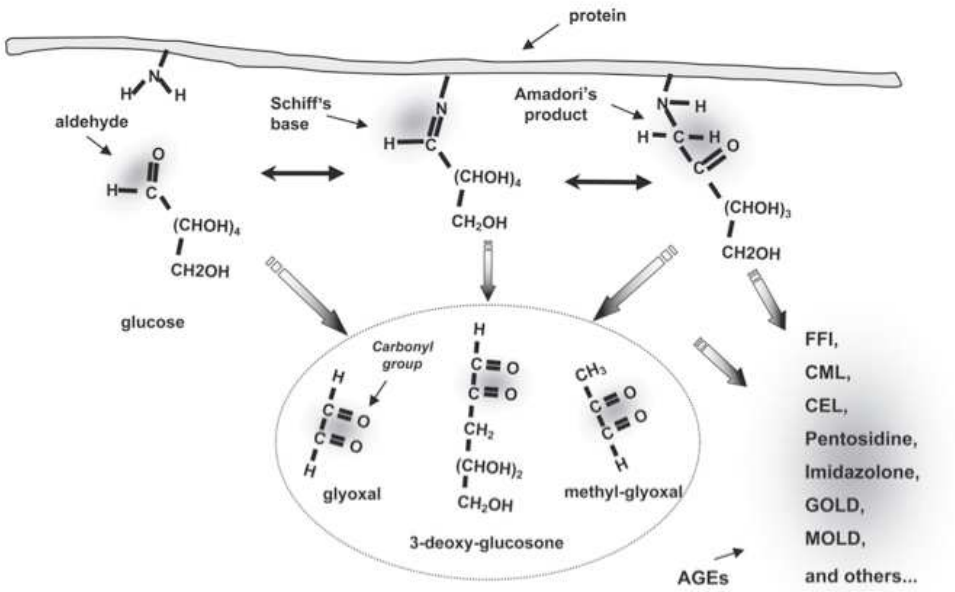
Calcium handling and homeostasis is necessary for the normal physiology of the body. However, when there is altered Ca^{2+} homeostasis, it can lead to various disease states including cardiomyopathy, which is characterized by a decrease in diastolic and systolic function.[42,47,69,88] SERCA2a and RyR2 are very important to the intracellular Ca^{2+} cycling as well as contraction and relaxation of the heart. Thus anything that alters these proteins would affect the pathophysiology of the heart and cause it to not contract or relax properly.[9,10,42,54,88] It has been shown that there is a significant decrease in SERCA2a and RyR2 in failing hearts leading to the prolonged contraction and relaxation due to Ca^{2+} overload in the cytosol.[26,88] However, during diabetic cardiomyopathy there is a significant decrease in diastolic function with only a mild or absent decrease in systolic function.[11,17,26,27,42,47,63,66,74] This could be due to an increase in non-phosphorylated PLB affecting SERCA2a, which would slow the rate of relaxation and not affect the rate of contraction.[88] It could also be due to changes the myofilaments or to advanced glycation end products forming on SERCA2a at an accelerated rate and impairing its function.[9,10,42,66,71] Altered Ca^{2+} handling can also cause arrhythmias that are seen in cardiomyopathy. Under normal circumstances, E-C coupling is regulated by changes in ionic currents

based on action potentials. However, with a decreased ability to move Ca^{2+} through the SR, there can be an imbalance in timing that occurs, thus causing arrhythmias.[21]

Advanced Glycation End Products

Advanced glycation end products (AGE) are molecular alterations of proteins or lipids that become glycated and oxidized after they come in contact with sugars. This modification occurs during the normal aging process and during disease states such as diabetes and renal failure, however they were first discovered in cooked food and called the Maillard reaction.[19,30,36] There are several key components to the formation of AGEs such as the turnover rate of the protein or lipid, the degree of hyperglycemia and thus the extent of diabetes, and the oxidative stress in the surrounding environment.[14,26,30] When one or all of these conditions are met AGEs can begin to form. The initial glycation and oxidation between the protein, nucleic acids and the carbonyl group of glucose, forms Schiff bases, which are compounds that form from the reaction of an aldehyde and a ketone. These Schiff bases then rearrange to form a more stable ketoamine called Amadori products. The reaction between the proteins and the carbonyl group causes a highly reactive intermediate carbonyl group to accumulate. The accumulated carbonyl group can react and form cross-links with the lysine and arginine functional groups on various long lived proteins and form

stable advanced glycation end product compounds.[6,7,13,26,30,33,36,38] Once AGEs are formed, they are irreversible.[6,30] (Figure 2)



Basta G, Schmidt A.M.,De Caterina R. Advanced glycation end products and vascular inflammation: implications for accelerated atherosclerosis in diabetes. *Cardiovasc Res.* 63(4): 582-593, 204.

Figure 2. Formation of advanced glycation end products

The initial glycation and oxidation between the protein, nucleic acids and the carbonyl group of glucose, forms Schiff bases, which rearrange to form Amadori products. In addition, a variety of highly reactive carbonyl intermediates can be formed, which can react again with free amino groups to form AGE products such as imidazolone, N-ε-carboxy-methyl-lysine (CML), N-ε-carboxy-ethyl-lysine (CEL), glyoxal-lysine dimer (GOLD) and methyl-glyoxal-lysine dimer (MOLD).

Advanced glycation end products have pathophysiological roles in various disease states such as diabetes, renal disease, heart failure, and coronary artery disease, as well as during aging. [6,19,26,36,42,49,52,61] During these disease states, AGEs accumulate at an accelerated rate, producing multiple organ dysfunction within weeks.[42] In order to initiate the extracellular signaling that causes alterations in cellular structure during disease processes and aging, AGEs accumulate in the extracellular matrix of long lived proteins, such as collagen. As AGEs accumulate, they form cross-links with eachother.[6,19,32,41] These cross-links lead to the increased vascular stiffness seen in cardiovascular diseases prior to the formation of fibrosis.[3,4,6,14,51,52,57,79,80] Also, the oxidative stress that occurs during AGE formation, leads to increased effects of aging.[49] AGEs can also initiate alterations in cellular function by binding to a specific receptors known as receptor for advanced glycation end products (RAGE). RAGE is a member of the immunoglobin superfamily and is found on macrophages, epithelial cells, mesangial cells, endothelial cells, and smooth muscle cells.[6,13,28,38,41] AGEs that interact with RAGEs on endothelial cells activate cytokines and activate monocyte migration, which increases permeability to macromolecules.[6,30] However the opposite effect happens with AGEs on macrophages causing apoptaxis, the process of reducing monocyte migration.[7] AGEs are also known to decrease the bioavailability of nitric oxide, which is needed to inhibit vascular complications like atherosclerosis.[28,30]

Advanced glycation end products play multiple important roles in the pathogenesis of myocardial injury. The formation of AGEs and their cross-links lead to increased collagen and myocardial stiffness.[3,4,6,14,19,51,52,57,79,80] This myocardial stiffness can contribute to the diastolic and systolic dysfunction seen in diabetes and heart failure. With the increase in AGEs, there is an increase in hypertension, incidence of coronary artery disease and atherosclerosis, as well as an impaired left ventricular function.[6,14,26,28,36,38,42] The impaired left ventricular function could be due to the stiffness itself from the AGE cross-linking, or other cellular aspects such as impaired sarcoplasmic reticulum Ca^{2+} regulatory proteins.[9,10,14,26,36,42,47] The SR Ca^{2+} regulatory proteins, such SERCA2a and RyR2, are responsible for pumping calcium into and out of the SR.[8,42,47] When calcium is pumped into or out of the SR, it causes the heart to contract and relax, thus these proteins are very important in the contraction and relaxation of the myocardium. Because these proteins are long lived proteins, AGEs can form on them, change the molecular structure of the protein, and interrupt their function causing relaxation and calcium homeostasis problems seen in diastolic dysfunction.[9,10,42,47] Other cardiac problems associated with AGE accumulation include vascular dysfunction, oxidative stress, and cellular damage.[26,36,52] The vascular dysfunction is related to the degree of AGE accumulation on collagen as well as the reduction in nitric oxide through AGE accumulation on endothelial cells.[36,52]

Experimental Therapies for Advanced Glycation End Products

Advanced glycation end products have been shown to cause multiple organ dysfunctions through their cross-linking with long-lived proteins.[6,19,26,36,42,49,52,61] Over the past few decades, a novel mechanism of removing AGEs from the body through therapeutic intervention has been studied and various pharmaceuticals have been created. Aminoguanidine (AG) was the first compound designed that was meant to inhibit the formation of advanced glycation end products and made it to phase III human clinical trials, but was stopped due to decreased funding.[15] ALT-946 is another inhibitor of advanced glycation end products and has been shown to prevent diabetic nephropathy.[19] Algebrium chloride (ALT-711) has a different pharmaceutical approach than AG and has been used to alter advanced glycation end products by chemically breaking the cross-linking.[4,6,15,19,32,42,87] Another cross-link breaker is called phenyl thiazolium bromide (PTB), and it has been shown to reduce AGE accumulation on blood vessels and reduce diabetic hypertrophy.[81]

Aminoguanidine, the first therapeutic compound made to reduce AGEs, pharmaceutically inhibits advanced glycation end products. It does this by blocking the intermediate reactive carbonyls on the glycation products.[12,19] AG has been proven to reduce the amount of AGEs that form. *Brownlee et al* helped verify that AG inhibits AGE formation by studying the effects of AG on the advanced glycation end products formed on collagen with the addition of

glucose. They did this by analyzing fluorescence specific for collagen on an SDS-PAGE gel. The collagen and glucose that were analyzed without AG had a significantly increased amount of high molecular weight cross-linked peptides, as opposed to the collagen that had AG added to it, suggesting that AG prevented the cross-linked peptides from forming.[12] AG has also been shown to improve diabetes, decrease albuminuria and proteinuria, improve the effects of aging and improve nitric oxide bioavailabilty.[15,50] Renal and cardiovascular complications, such as cardiac stiffness and hypertrophy, that are linked with AGE accumulation have also demonstrated improvement with AG treatment.[15,19,50] Li *et al* assisted in this discovery by proving that AG reduced the effects of aging in cardiac and renal tissue by decreasing hypertrophy, vasodilatory impairment, nephron loss and glomerular sclerosis.[50] Even though AG does have many positive attributes, it does not have the ability to break the cross-linked advanced glycation end products once they are already formed, which is why other therapeutic agents such as ALT-711 were created.

Alagebrium chloride, also known as dimethyl-3-phenacylthiazolium chloride and ALT-711, is an antiglycation therapeutic agent that has the ability to break the glucose derived cross-links between proteins.[4,6,14,15,19,32,42,87] ALT-711 was created so it was possible to remove AGEs once they were created, as opposed to just inhibiting them like AG. With the treatment of ALT-711, it is possible to reverse the cardiovascular and aging effects of advanced glycation end products by reducing the amount of advanced glycation end products. ALT-

711 has been proven to have an antioxidative effect on the myocardium as well as the whole body by reducing the proinflamatory mechanisms, reactive oxygen species and oxidative stress on the mitochondria, produced by AGEs. Guo *et al* showed that the oxidative modifications, known as AGEs, occur on cardiac proteins, as well as vascular collagen, in the aging rat heart. They found that there was an increase in mitochondrial DNA depletion and a decrease in antioxidant enzymes such as superoxide dismutase and glutathione peroxidase in the aging heart, which decreased with treatment of ALT-711.[32] ALT-711 has also been shown to reverse the myocardial stiffness, reduced left ventricular hypertrophy, increase the cardiac output, and reverse the diastolic dysfunction seen in diabetes, and other cardiovascular diseases such as atherosclerosis and heart failure.[3,4,6,14,15,19,20,32,40,42,81,87] Candido *et al* used diabetic rats to show an increase in AGE accumulation on collagen via collagen fluorescence, an increase in RAGE expression via RT-PCR and immunohistochemistry, and a decreased collagen solubility correlating to an increase collagen cross-linking during diabetes. However, all were reversed with the treatment of ALT-711 and cardiac function was improved.[14] The improvement in cardiac function due to decreased AGE accumulation seen by treatment of ALT-711 has been proven to have beneficial effects related to diastolic dysfunction, myocardial stiffness, left ventricular hypertrophy, increased collagen but its beneficial effect on Ca^{2+} handling was yet to be discussed.[4,14,15,19,32,87] For this reason, my research

group has chosen to study the effects of ALT-711 on AGE accumulation with regards to Ca^{2+} handling and homeostasis during diabetic cardiomyopathy.

INTRODUCTION

Diabetes is a significant health problem in the world today. Diabetes has become an epidemic disease and it is estimated that by the year 2025, it will affect over 300 million people.[11] In the United States alone, about 8 % of people suffer from diabetes and about one million of those people have chronic insulin-dependent (type 1) diabetes.[2,17] Type 1 diabetes is characterized by sustained hyperglycemia resulting from the destruction of pancreatic beta cells. Diabetes is also a common cause of cardiovascular disease. Within the past thirty years, diabetic cardiomyopathy has been identified as its own clinical unit, independent of coronary artery disease and atherosclerosis.[27,63] Ventricular diastolic dysfunction, the first stage of diabetic cardiomyopathy, has also been found in about 50% of asymptomatic patients.[27,47] Diabetic cardiomyopathy is known to trigger arrhythmias and progress not only to heart failure, but also sudden death.[17,58]

Patients with diastolic dysfunction have either an increase in myocardial stiffness due to an increase in collagen content or an abnormal filling and relaxation of the left ventricle, leading to heart failure and sudden death.[5,17,58] Abnormal filling and relaxation of the left ventricle is due to an altered Ca^{2+} homeostasis.[22,27] Altered Ca^{2+} homeostasis, an essential part for excitation-

contraction coupling and the cardiac cycle, has been associated with chronic diabetes due to the impaired cardiac contractility and relaxation during diabetes. However, the exact mechanism for this impaired Ca^{2+} homeostasis and the specific therapeutic strategies for this patient population, are not well known.[17,18,47,48,62]

Intracellular Ca^{2+} flux is very important in the myocardium and is regulated by a process called excitation-contraction coupling. Calcium enters through the L-type Ca channel and activates Ca^{2+} release from the SR.[8] The SR functions as the main regulator of intracellular Ca^{2+} and is a major determinant of cardiac contraction and relaxation.[8,27] The SR Ca^{2+} release channels, RyR2, release about 90% of the Ca^{2+} needed for contraction, the last 10% Ca^{2+} comes from the L-Type Ca^{2+} channel. The SR Ca^{2+} ATPase pump reuptakes the majority of Ca^{2+} during relaxation when it is not inhibited by or bound to PLB. The remaining Ca^{2+} not brought back into the SR is removed from the myocardium by NCX.[8] Changes in the critical processes that regulate intracellular Ca^{2+} are trademarks for diabetic cardiomyopathy.[17,58] Decreased expression of the SR Ca^{2+} regulatory proteins such as SERCA2a and RyR2 have been recognized in diabetic rats with contractile dysfunction.[47,55,88] Also, impaired cardiac function due to diastolic abnormalities, characterized by slow Ca^{2+} transient decays and diastolic cytosolic Ca^{2+} overload, have been found to be associated with decreased SERCA2a expression in diabetic myocytes.[1,47,62,86] However, the specific mechanism by which SR Ca^{2+} cycling is impaired during

diabetic cardiomyopathy is not well known.

In order to fully understand the complications associated with diabetes, researchers have begun to focus their attention on the effects that chronic hyperglycemia, a problem associated with diabetes, has on the body. In particular, hyperglycemia accelerates the formation of highly bonded cross-links between glucose and proteins. These cross-links form products known as advanced glycation end products, which interact with long lived proteins and change their form and function.[9,10,19,36,51] AGEs are formed naturally in the body during the aging process, however this process is accelerated to days or weeks during the diabetic process.[19,36] AGEs contribute to cardiac dysfunction by inducing myocardial fibrosis and stiffness.[3,4,6,14,19,36,57,79,80,87] Bidasee and colleagues have demonstrated that AGEs are formed on intracellular cardiac long-lived SR proteins such as SERCA2a and RyR2 after just a few weeks of diabetes. Therefore, it is possible that the modification of the SR proteins by AGEs could lead to altered Ca^{2+} homeostasis. However, the functional significance of AGEs and SR Ca^{2+} regulatory proteins has not been determined by Bidasee and colleagues.[9,10] Therefore, our goal was to investigate the mechanism by which AGE accumulation impairs SR Ca^{2+} regulatory proteins.

We investigated this problem by using an antiglycation therapeutic agent named alagebrium chloride, also known as dimethyl-3-phenacylthiazolium chloride and ALT-711, which chemically breaks AGE cross-links. ALT-711 is the first in its class of drugs to chemically break these cross-links. Other

compounds such as Aminoguanidine, prevent AGE cross-links, but cannot reverse the cross-link process once it has already begun.[19] ALT-711 has been tested in several pre-clinical animal studies and has been shown to reverse the disease processes in the heart and kidney.[3,19,20,40,81] Candido *et al* also showed that 10 mg/kg of ALT-711 delivered in the drinking water of rats breaks the cross-linked AGEs during diabetes.[14] Our hypothesis was that the formation of advanced glycation end products during diabetes impairs sarcoplasmic reticulum Ca^{2+} reuptake in the cardiac myocyte and that the treatment of Alagebrium Chloride will normalize SR Ca^{2+} handling, and therefore improve diastolic dysfunction in diabetes.

MATERIALS AND METHODS

Animal Model

Eight-week-old male Wistar rats, at 250 grams, were randomly divided into 3 groups (n=11 rats/group): untreated age-matched control group (CON); untreated diabetic group (DX); and a diabetic ALT-711 (Shanghai Inc, China) treated group (DX-ALT). Diabetes was induced at 10 weeks of age in DX and DX-ALT by a single injection of streptozotocin, using a low dose (50 mg/kg IP diluted in 1mL citrate buffer) to induce a mild form of diabetic cardiomyopathy that mimics insulin-dependent diabetes in humans. The DX-ALT group received the AGE crosslink breaker at 10 mg/kg per day PO from 11 to 19 weeks of age.[14,28] The volume of ALT-711 administered in the drinking water was calculated every other day based on the rats daily individual water consumption. To confirm diabetes, hyperglycemia was assessed by monitoring venous blood glucose concentration prior to treatment (baseline) and weekly after injection of STZ for 8 weeks. Blood samples were obtained from the tail vein and measurements were made using a glucometer (BD Logic). Animals were also weighed once a week to monitor clinical condition. This animal protocol was

approved by The Ohio State University Institutional Animal Care and Use Committee.

Echocardiography:

Transthoracic echocardiography was done to assess systolic and diastolic function at baseline and post 8 weeks of diabetes on all three groups. To evaluate echocardiography we used two-dimensional, m-mode, Doppler flow and tissue Doppler imaging.[22,24] Two-dimensional, M-mode, and pulsed-wave Doppler imaging were obtained in rats lightly anesthetized with isoflurane, using high-resolution high-frequency digital imaging system with a 21 MHz linear array transducer and ECG recording (Vevo 2100, VisualSonics, Toronto, Canada), following techniques previously described by our laboratory.[24,44,47,56] Color flow guided, pulsed-wave Doppler imaging was recorded for pulmonary artery, mitral flow and aortic flow.[47,68] Using these techniques and the apical four-chamber view, early and late diastolic tissue velocities (E/A wave) were recorded as well as myocardial performance index (MPI). MPI was calculated from the sum of the LV isovolumic relaxation time (IVRT) and isovolumic contraction time divided by the aortic ejection time. Ventricular structure and function were assessed by long-axis view, short axis and M-mode at the level of the papillary muscle. Left ventricular ejection fraction (EF), a measurement of systolic function, was calculated by: EF=[LVID end-diastolic – LVID end-systolic/ LVID end-diastolic] X

100%. Echocardiographic image measurements were performed at another time using the Vevo measurement software and by using an average of 3 cardiac cycles.

Pressure-Volume Loops:

Pressure-Volume loops were performed on rats at 18 weeks at QTest laboratories on all three groups. The rats were anesthetized with pentobarbital (IP) and endotracheally intubated and ventilated. Arterial pressure was measured by placing a 2F high-fidelity conductance/micromanometer catheter (Miller Instruments) into the femoral artery advancing to the abdominal aorta. Then left ventricular mechano-energetic measurements (ESPVR, EDPVR, Tau, PRSW) were taken by inserting a 2F high-fidelity conductance/micromanometer catheter into right carotid artery that then advances into the aortic valve into the left ventricular chamber to determine pressure and volume simultaneously. A balloon catheter was placed in the inferior vena cava through the femoral vein to reduce the preload, while an in-dwelling catheter was placed in the jugular vein as a positive control. Electrocardiography (ECG) measurements were also taken to monitor the animal. After instrumentation was stabilized for 10 minutes, data were collected. In order to get a family of pressure-volume loops, left ventricular preload was reduced by slowly increasing the balloon catheter in the inferior vena cava three different times. The resulting left-ventricular pressure and

volume data were analyzed (IOX/ECG Auto; EMKA Technologies). After the experiment was completed, rats were euthanatized and disposed of appropriately.[34,35,59]

Left Ventricular Fibrosis Testing:

Left ventricular fibrosis testing was done on all three groups of rats at post 8 weeks diabetes. Left ventricular cross sections were taken from the heart, placed in the appropriate mold and fixed using optimal cutting temperature (OCT) compound. The samples were then frozen in dry ice, labeled and sent to the Ohio State University's Core Pathology Lab where the fibrosis testing was performed. The molds were stained with Masson Trichrome staining and then analyzed under a microscope by Dr. Amy Gerwitz at The Ohio State University College of Medicine.

In vitro electrophysiologic measurements:

Rats at 18 weeks that were not used for pressure-volume loops were anesthetized using pentobarbital sodium 30 mg/kg IP and left ventricular myocytes were isolated for measurements of Ca^{2+} transients and SR Ca^{2+} content.

Ventricular myocyte isolation:

Single cell electrophysiology was performed using isolated rat ventricular myocytes.[24,44,47,72,73,83] Myocytes were obtained using a Langendorff apparatus by enzymatic perfusion. The hearts were removed within 5 minutes from the anesthetized rats and attached to the Langendorff apparatus. They were then perfused with tyrode buffer with $CaCl_2$ for 6 minutes (37^0C, pH=7.35 and oxygenated with 95% O_2 and 5% CO_2), which contained (in mM) NaCl (135), KCl (5.4), $MgCl_2$ (1), NaH_2PO_4 (0.33), Hepes (10), glucose (10), and $CaCl_2$. Following the initial perfusion, $CaCl_2$ was removed for 7 minutes. Collagenase (type II, Worthington Biochemical, 1 mg/mL) was added next to the calcium free tyrode buffer to digest the heart for 15-20 minutes. When the heart was soft and lolled, the left ventricle was minced and the cells were washed in tyrode containing $CaCl_2$. Only rod-shaped cells with sharp margins and clear striations were included in the study. All recordings were made within 5 hours of isolation.[24,44,47,72,73,83]

Measurement of Ca^{2+} transient and SR Ca^{2+} Load:

Ca^{2+} transient was measured with fluo-3-loaded cardiac myocytes with confocal Ca^{2+} imaging within an hour after myocyte isolation, in Dr. Sandor Gyorke's laboratory, as previously described.[43,47,77,82] A Laser Scanning Confocal System (Olympus Fluoview 1000 confocal microscope interfaced to an IX-70 inverted microscope and equipped with an 60 1.4 NA oil objective) was used to evaluate intracellular Ca^{2+}. The 488-nm beam of an argon-ion laser

excited the Fluo-3 loaded cardiac myocytes, and the fluorescence was acquired at wavelengths greater than 515 nm, at a rate of 2-6 ms per scan, in the line scan mode. The measurements of Ca^{2+} transients and transient decay were calculated by mean area under the curve. In order to measure SR Ca^{2+} content, rapid application of caffeine (10 mM) was applied to the myocytes. The magnitude of the fluorescent signal was then quantified in terms of F/F0, where F0 is considered baseline.[24,43,47,77,82,83] All values were recorded as an average of three myocytes.

Measurements of SR Ca^{2+} regulatory proteins:

Left ventricular myocardium was taken from the rats of all three groups at 8 weeks of diabetes and was prepared using a 25 ug protein target. The muscle preparation that was used for SERCA2a was a whole heart muscle preparation for western blot analysis. The left ventricles of all three groups were individually weighed to 40-60 mg, homogenized with a buffer (50 mM Tris HCl,150 mM NaCl,1 % Triton, Protease Inhibitor Cocktail) and centrifuged at 0.8 rcf at 4 degrees Celsius for 20 minutes. The supernatant was saved for BCA analysis to determine protein content followed by western blot analysis. The muscle preparation used for RyR2 was a crude membrane extract preparation for western blot analysis. The left ventricles of all three groups were individually weighed at 200 mg, homogenized with a buffer (0.3 M Sucrose, 2 mM EGTA, 20

mM Hepes, Protease Inhibitor Cocktail), centrifuged at 110,000g for 1 hour at 4 degrees Celsius. The pellet was then saved and resuspended in a storage buffer (0.3 M Sucrose, 20 mM Hepes) for BCA analysis to determine protein content and western blot analysis.[68] For the western blot analysis, samples from all three groups were then loaded onto the same gel to ensure equal blotting between the groups. Proteins were subjected to SDS-PAGE electrophoresis for 1 hour and then electrophoretically transferred to PVDF membranes for 1.5 hours, except the RyR2 was transferred overnight at 4 degrees Celsius. Following the transfer the membranes were washed with TPBS and blocked for 1 hour using a 10% nonfat milk and TPBS blocking solution (BioRad). They were then subsequently immunobloted.[45,46,47,53] Membrane proteins were incubated with mouse RYR2 for 1 hour or SERCA2a antibodies overnight at 4 degrees Celsius (1:3000 dilution, Affinity Bioreagents; 1:1000, Affinity Bioreagents). Next they were washed, and incubated with the appropriate secondary antibodies (1:50,000, Jackson ImmunoResearch Laboratories; 1:5000, Sigma Aldrich) conjugated to horseradish peroxidase for 1 hour. Both incubations were done at room temperature while shaking. Quantitative determination of protein was performed by autoradiography after revealing the antibody-bound protein by enhanced chemiluminescence reaction for 2 minutes. The data were normalized to actin or calsequestrin, previously quantified by reprobing each membrane with calsequestrin polyclonal IgG (Calbiochem) or Actin monoclonal IgG (Sigma Aldrich).

Statistical analysis:

The number of animals that we decided to use was determined by the variance associated with *in vivo* and *in vitro* measurements. Since we expect a dropout rate of less than 15%, we completed studies with 12 rats per group in order to provide statistical power to permit the detection of significant differences between groups. A two-way ANOVA (treatment and time factors) for the *in vivo* measurements, and a one-way analysis of variance (treatment factor) for the *in vitro* measurements were preformed, as appropriate. Data were reported as means $\pm$ standard error. Statistical significance was defined as $P < 0.05$.

RESULTS

Blood Glucose and Body Weight

As expected, the STZ-treated rats showed hyperglycemia within 72 hours post injection, which continued during the course of the eight-week diabetic period (Figure 3, $P<0.05$). There was no significant difference between diabetic treated rats and the diabetic rats. However, the control rats showed no sign of hyperglycemia throughout the eight-week period. The diabetic rats also had a significantly lower body weight when compared to the control rats (Figure 4, $P<0.05$) and the diabetic treated rats had a tendency to have a higher body weight than the diabetic rats at week four and eight ($P<0.054$, $P<0.074$). As previously seen in our laboratory, even though the STZ treated rats were diabetic, they did not require insulin injections and remained clinically stable throughout the eight-week period. They showed no outward signs of heart failure during this time.[47]

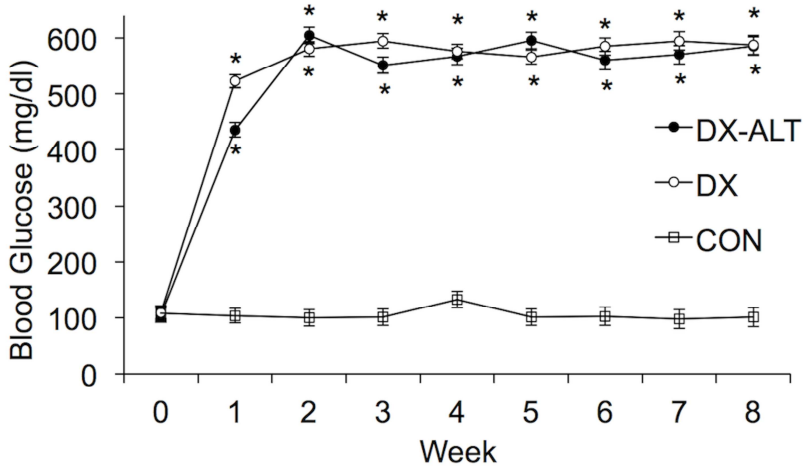


Figure 3. Blood glucose measurements of control (CON), diabetic (DX), and diabetic treated (DX-ALT) groups at baseline through 8 weeks diabetes

*Mean ± SE blood glucose levels for CON, DX, DX-ALT rats at baseline to post 8 weeks diabetes. n= 10-11/group. *P<0.05 when comparing values from age-matched controls.*

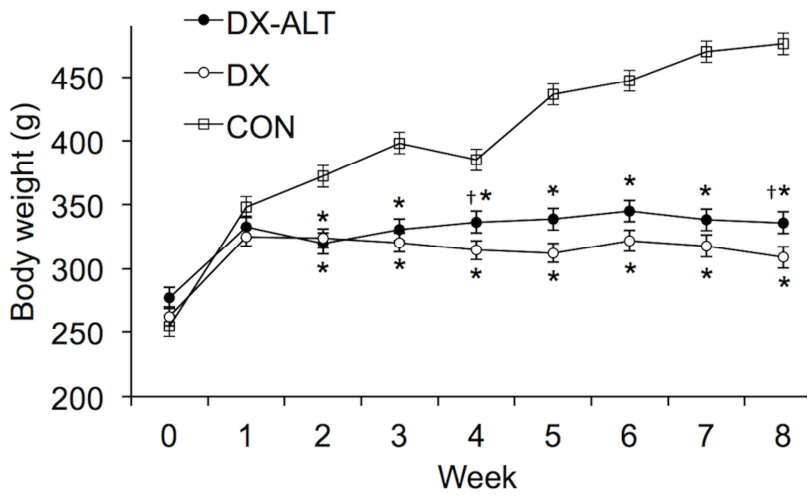


Figure 4. Body weight measurements of control (CON), diabetic (DX), and diabetic treated (DX-ALT) groups at baseline through 8 weeks diabetes

*Mean ± SE body weight values for CON, DX, DX-ALT rats at baseline to post 8 weeks diabetes. n=10-11/group. *P < 0.05 when comparing values from age-matched controls. † P <0.1 when comparing values from DX group.*

Echocardiography:

We then evaluated the effects of diabetes and ALT-711 treatment on systolic and diastolic function by echocardiography. Throughout our examinations we observed a mild decrease in ejection fraction, an indication of systolic function, between the control and diabetic rats (Table 1, Figure 5, $P<0.05$). However, we observed evidence of diastolic dysfunction through multiple parameters. The isovolumic relaxation time obtained by the mitral and aortic Doppler flows as well as the myocardial performance index were significantly increased in diabetic rats compared to controls (Table 2, Figure

5,P<0.05). ALT-711 treatment did not significantly change ejection fraction of the diabetic myocardium, however it did reduce the increase in isovolumic relaxation time and myocardial performance index. This suggests that ALT-711 therapy helped prevent cardiac dysfunction and improved the diastolic dysfunction observed during diabetes (Figure 5).

	CON		DX		DX-ALT	
	Baseline	**8 weeks**	**Baseline**	**8 weeks**	**Baseline**	**8 weeks**
EF (%)	85.7±2.6	84.4±1.8	86.11±2.02	67.8±2.54*#	83. 7 ± 2.08	73.8 ± 0.14*#
FS (%)	57.7±3.11	55.7 ±2.22	57.7±2.50	39.6±2.07*#	54.4±2.4 2	44.5±1.71*#
IVS;d (cm)	1.85±0.07	2.11±0.07	2.05±0.07	1.85±0.10	2.02 ± 0.10	1.84 ± 0.09
IVS;s (cm)	3.42±0.07	3.76±0.14#	3.64±0.11	2.94±0.09 *#	3.54 ± 0.10	3.15 ± 0.09 *#
LVID;d (cm)	6.46±0.22	7.39±0.13#	6.47±0.16	7.74±0.1 7#	6.59 ± 0.26	7.75 ± 0.2 #
LVID;s (cm)	2.79±0.29	3.29±0.21#	2.74±0.19	4.69±0.2 2*#	3.00 ± 0.19	4.32 ± 0.20 *#
LVPW;d (cm)	1.64±0.07	1.84±0.09	1.70±0.08	1.63±0.10	1.63 ± 0.08	1.60 ± 0.08
LVPW;s (cm)	3.17±0.12	3.43±0.1#	3.11±0.12	2.57±0.08 *#	2.95 ± 0.12	2.67 ± 0.14*
LV Mass (mg)	610.1±42.5	891.3±36.2#	676.8±35.8	812. 9±70.4#	663.2 ±35.3	792.6 ± 41.2
LV Vol;d (ul)	216.5±16.5	289.6±11.3#	215.9±11.45	32 2.1±15.9#	226.6 ± 19.6	323.2 ± 18.4 #
HR (b/min)	329.6±9.21	318.24±8.64	344.7±2.00	243.8± 7.95*#	335.4 ±10.5	268.3 ± 6.02*#

Table 1. M-Mode echocardiography parameters for control (CON), diabetic (DX), and diabetic treated (DX-ALT) groups at baseline and 8 weeks diabetes

*Data are mean ± SE for n = 9-11/group. EF: ejection fraction, FS: fractional shortening, IVS;d: Interventricular septal dimension-diastole, IVS;s: Interventricular septal dimension-systole, LVID;d: Left ventricular internal diameter-diastole, LVID;s: left ventricular internal diameter-systole, LVPW;d: left ventricular posterior wall dimension-diastole, LVPW;s: left ventricular posterior wall dimension-systole, LV Mass: Left ventricular mass corrected, LV Vol;d: Left ventricular volume-diastole, HR: heart rate. * P<0.05 when comparing values from age-matched controls for same time point. # P<0.05 when comparing values from baseline.*

	CON		DX		DX-ALT	
	Baseline	**8 weeks**	**Baseline**	**8 Weeks**	**Baseline**	**8 weeks**
MV E/A	1.52±0.06	1.52± 0.06	1.62±0.092	1.64± 0.080	1.59± 0.11	1.82± 0.15
IVRT	22.4±0.83	25.4± 1.07	23.0± 0.95	44.98±1.88*#	24.8±1.14	37.3±2.07*†
MV Dec Acc (mm/s^2)	38647.0± 4166.0	34047.0± 4493.3	39465.7± 3418.6	39593.8± 8829.9	40454.0± 4650.1	26790.0± 2305.4
LV MPI	0.54±0.04	0.57± 0.05	0.46±0.03	0.76± 0.08*#	0.61± 0.04	0.56± 0.08†

Table 2. Doppler parameters of diastolic function for control (CON), diabetic (DX), and diabetic treated (DX-ALT) groups at baseline and 8 weeks diabetes

*Data are mean ± SE for n= 9-11/group. MV E/A: Ratio of maximum velocity of E and maximum velocity of A wave, IVRT: Isovolumic relaxation time, MV Dec Acc: Maximum velocity deceleration acceleration, LV MPI: Left ventricle myocardial performance index. * P<0.05 when comparing values from age-matched controls for same time point. # P<0.05 when comparing values from baseline. † P<0.05 when comparing DX values vs. DX-ALT.*

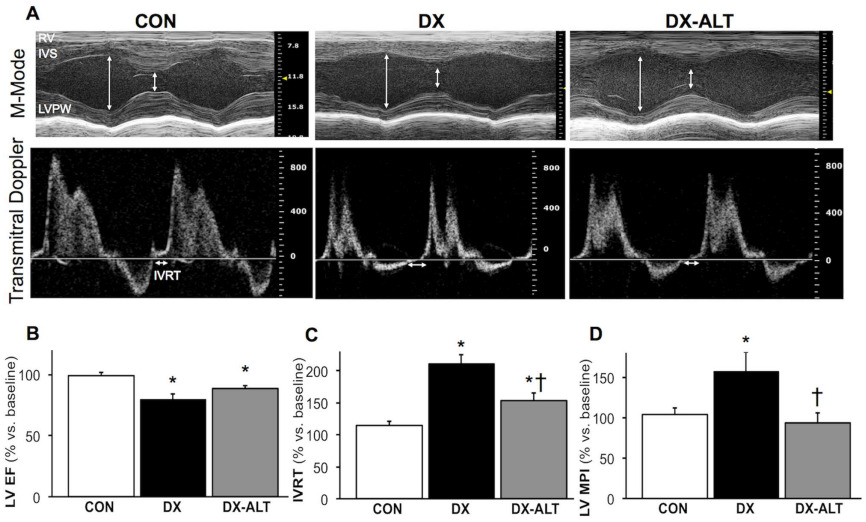


Figure 5. Representative echocardiography figures and data derived from echocardiography for control (CON), diabetic (DX), and diabetic treated (DX-ALT) groups at 8 weeks diabetes

*(**A**) A: representative paired M-mode echocardiograms (top panel) and transmitral Doppler flow (bottom panel) at 8 weeks of treatment in CON, DX, DX-ALT groups. IVS, interventricular septum; LVPW, left ventricular posterior wall; RV, right ventricle; IVRT: isovolumic relaxation time. (**B**) Mean ± SE of percentage change after 8 weeks of treatment compared to baseline value for ejection fraction (EF) of the left ventricle (LV) in CON, DX, DX-ALT groups. (**C**) Mean ± SE of percentage change at 8 weeks compared to baseline value for isovolumic relaxation time (IVRT) of the left ventricle in CON, DX, and DX- ALT groups. (**D**) Mean ± SE of percentage change at 8 weeks compared to baseline value for myocardial performance index (MPI) in control CON, DX, and DX-ALT groups. n = 9 -11/group, * P < 0.05 when comparing values from age-matched controls. † P <0.05 when comparing values from DX group.*

<u>Pressure-Volume Loops:</u>

We also assessed the systolic and diastolic dysfunction through pressure-volume loops. Systolic function was decreased in the diabetic rats compared to

control as evident by the decrease in End Systolic Pressure Volume Relationship (ESPVR,Table 3, $P<0.001$). However, the Preload Recruitable Stroke Work (PRSW), an indicator of myocardial contractility that is independent of preload and afterload, was not significantly changed proving that the systolic function is only mildly decreased in the diabetic rats compared to control. The diastolic function was also significantly decreased in untreated diabetic rats when compared to control rats, as evident by the increase in the End Diastolic Pressure Volume Relationship (EDPVR) and Tau (τ), the isovolumetric relaxation constant. This suggests an increased relaxation impairment of the diabetic myocardium (Table 3,$P<0.05$). However, ALT-711 therapy did not significantly change the ESPVR in the diabetic heart, but it did show a tendency to improve the EDPVR and Tau.

	CON	DX	DX-ALT
ESPVR (mmHg/s)	30.4±3.4	15.5±2.8*	16.4 ± 3.5*
EDPVR (mmHg/s)	1.9±0.4	3.3±0.3*	3.1 ± 0.4
τ (s)	8.3 ± 0.9	10.8± 0.4*	10.4 ± 1.2
PRSW	0.41 ± 0.09	0.28 ± 0.06	0.29 ± 0.10

Table 3. Load-independent parameters of systolic and diastolic function obtained by pressure volume loop showing diastolic function for control (CON), diabetic (DX), and diabetic treated (DX-ALT) groups at 8 weeks diabetes

*Data are mean ± SE for n= 4/group. ESPVR: End systolic pressure volume relationship, EDPVR: End diastolic pressure volume relationship, Tau: Isovolumic relaxation constant, PRSW: Preload Recruitable Stroke Work. * P<0.05 when comparing values from age-matched controls.*

Left Ventricular Fibrosis Testing:

Fibrosis testing was done to insure that the diastolic dysfunction seen in diabetic rat hearts was not due to fibrosis, but rather due to reduced sequestration of Ca^{2+}. After Masson Trichrome staining left ventricular rat hearts, it was evident that there was no significant difference in fibrosis of the control, diabetic or the diabetic treated rat hearts (Figure 6). This suggests that cardiac dysfunction is primarily due to impaired Ca^{2+} handling and not myocardial stiffness.

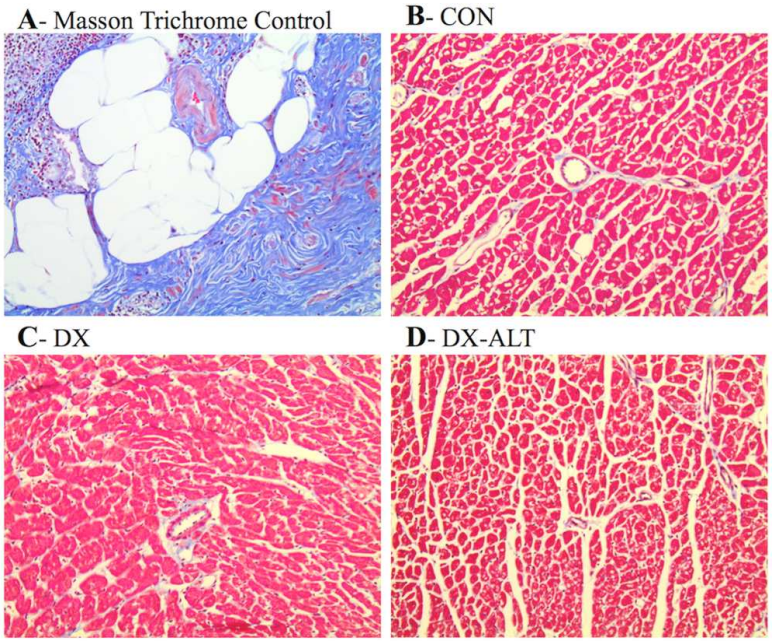


Figure 6. Masson Trichrome staining to assess fibrosis of control (CON), diabetic (DX), and diabetic treated (DX-ALT) groups at 8 weeks diabetes

Images B-D are cross-sections of left ventricular rat heart tissue stained with Masson Trichrome and viewed under a 20X microscope to assess the level of fibrosis in CON, DX, DX-ALT. N=2/group.

In vitro electrophysiological measurements:

Because we know that advanced glycation end products accumulate on SR Ca^{2+} regulatory proteins such as SERCA2a and alter their function, we measured the Ca^{2+} transient and SR Ca^{2+} load to determine if calcium handling was altered during diabetic cardiomyopathy and whether treatment with an AGE

cross-link breaker improved SR Ca^{2+} handling. There was a significant decrease in Ca^{2+} transient amplitude in untreated diabetic and diabetic treated rats when compared to control (Figure 7,$P<0.001$). Also, the Ca^{2+} transient decay was significantly prolonged in the diabetic rats when compared to control (Figure 7,$P<0.05$). This was improved with the treatment of ALT-711 suggesting an improvement in SR Ca^{2+} reuptake (Figure 7,$P<0.05$). When caffeine was applied to the myocytes, SR Ca^{2+} load was assessed. SR Ca^{2+} load was significantly decreased in the diabetic rats and was not significantly decreased in the diabetic treated rats proving its improvement of SR Ca^{2+} handling (Figure 7,$P<0.05$).

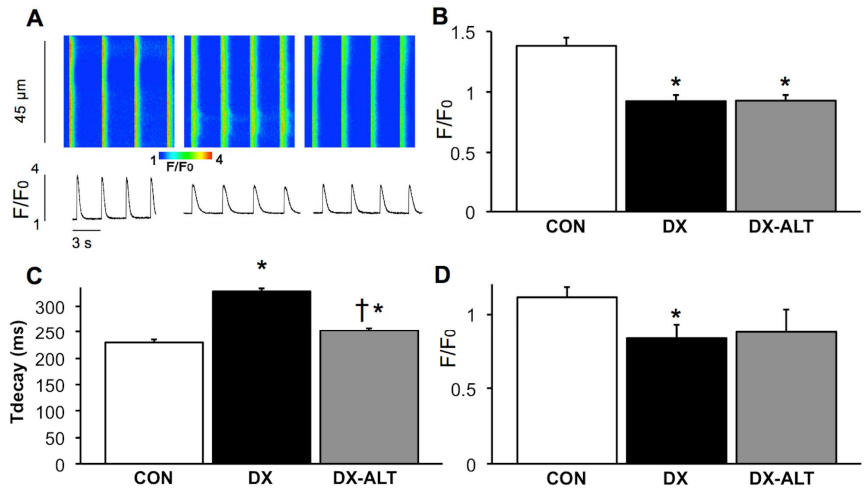


Figure 7. Representative confocal imagining figure of control (CON), diabetic (DX), and diabetic treated (DX-ALT) groups at 8 weeks diabetes

*(**A**) Representative confocal line scan images of Ca^{2+} transient along with their spatial averages in myocytes from age-matched CON (left), DX (middle) and DX-ALT (right) rats. F0, diastolic fluorescence. (**B**) Mean ± SE of Ca^{2+} transient amplitude (F/F0) for CON, DX and DX-ALT rats, n= 43 -44/group. (**C**) Mean ± SE of the time constant (τ) of Ca^{2+} transient decay in CON, DX, and DX-ALT rats. n= 40 ± 4/group (**D**) Mean ± SE for caffeine-induced Ca^{2+} transient amplitudes. n = 4-5/group, *P <0.05 when comparing values from age-matched control myocytes. † P <0.05 when comparing values from DX group.*

Measurement of SR Ca^{2+} regulatory proteins:

To determine if the expression of SR Ca^{2+} proteins were altered due to accumulation of AGE, we performed quantitative immunoblot analysis on SERCA2a and RyR2. SERCA2a expression was significantly decreased in diabetic rat hearts and was not significantly changed between diabetic treated and controls (Figure 8,P<0.05). In contrast, we did not detect any changes in

calsequestrin (CASQ) protein expression, the internal loading control. We also observed a decrease in RyR2 expression in the diabetic myocardium when compared to control, with no evidence of a decrease in diabetic treated rat hearts (Figure 8,$P<0.05$). We did not detect any changes in actin protein expression, the internal control.

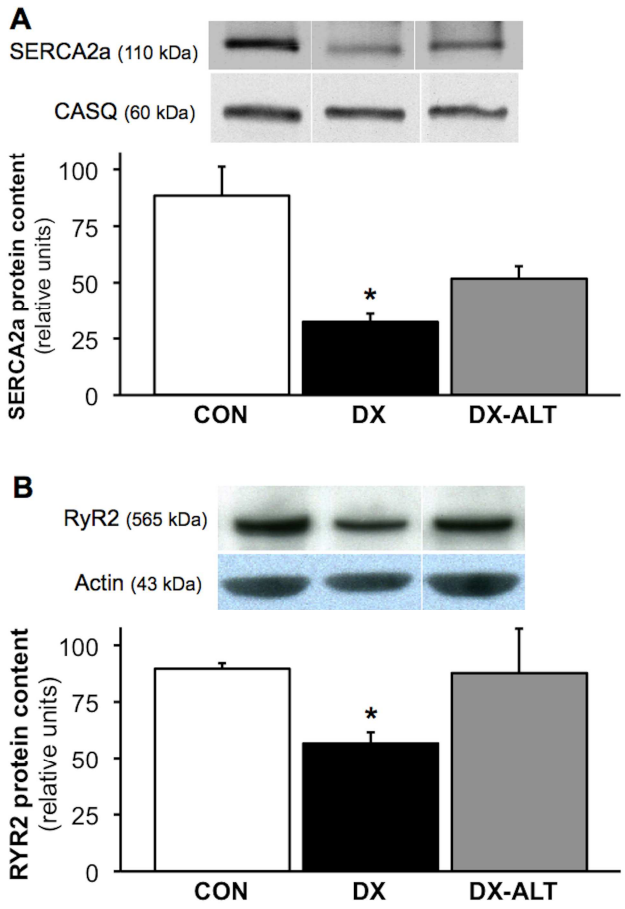


Figure 8. SERCA2a and RYR2 protein expression of control (CON), diabetic (DX), and diabetic treated (DX-ALT) groups at 8 weeks diabetes

(**A**) *Top panel:* representative immunoblot of SERCA2a and calsequestrin (loading control) expression in the myocardium of CON, DX, DX-ALT, *Bottom panel:* normalized optical density (OD; relative to calsequestrin). Data are mean ± SE for n= 4-5/group. (**B**) *Top panel:* representative immunoblot of RYR2 and actin (loading control) protein expression in the myocardium of CON, DX, DX-ALT, *Bottom panel:* normalized OD (relative to actin). Data are mean ± SE for n=5/group. *P <0.05 when comparing values from age-matched control heart.

DISCUSSION

This study suggested that long term treatment with ALT-711, an AGE cross-link breaker, does help reduce the AGE accumulation on SR Ca^{2+} regulatory proteins, such as SERCA2a and RyR2. This in turn ameliorates the SR Ca^{2+} handling abnormalities causing diastolic dysfunction seen in the diabetic heart. The treatment with ALT-711 then partially prevents the diastolic dysfunction seen in the type 1 diabetic cardiomyopathy rodent model.

Left Ventricular Diastolic Dysfunction:

The low dose streptozotocin diabetic rat model is a well-established model of type 1 diabetes. Streptozotocin kills the beta cells of the pancreas that are responsible for producing insulin and results in hyperglycemia within 72 hours after injection (Figure 3). Our laboratory established the low dose streptozotocin model to mimic the clinical signs of human diabetic disease, such as hyperglycemia, polyuria, polydipsia, glucosuria, decreased body weight and cardiovascular dysfunction (Figure 4). This model has a 3% mortality rate and the rats maintain diabetes for at least 8 weeks with no need for insulin injection (Figure 3).[44,47] The diabetic ALT-711 treated rats also have a tendency to have

an increased body weight compared to the diabetic rats showing clinical improvement due to the treatment of ALT-711(Figure 4).

The cardiac dysfunction during diabetes, also know as diabetic cardiomyopathy, is a major cause of cardiovascular disease in the United States today. It can lead to heart failure and sudden death, killing approximately 65% of the patient population.[17,58] Left ventricular diastolic dysfunction is the first stage of diabetic cardiomyopathy.[27,47] Diastolic dysfunction refers to the mechanical and functional abnormalities of the ventricles that causes the myocardium to not relax properly.[5,78] Our laboratory has previously described that diastolic dysfunction occurs during diabetic cardiomyopathy using a decreased E/A wave ratio seen on echocardiography.[47] This study confirmed this diagnosis with a significant increased isovolumic relaxation time and increased myocardial performance index on echocardiography (Table 2, Figure 5). Then the diastolic dysfunction was further proven through pressure volume loops with a significant increased end diastolic pressure volume relationship and Tau (Table 3). There was also only a mild form of systolic dysfunction in the diabetic rats seen with a decrease in ejection fraction on echocardiography and a decreased end systolic pressure volume relationship shown through pressure volume loops (Table 1, Table 3, Figure 5). The significant decreased isovolumetric relaxation time and myocardial performance increase seen in the ALT-711 treated diabetic rats, as well as a trend toward a decrease in end diastolic pressure volume relationship change in treated vs. untreated diabetic treated rats demonstrates that the AGE

cross-link breaker improves diastolic function in diabetic rats. Because of this, we speculate that the diastolic dysfunction seen in diabetic rats is triggered by advanced glycation end products, which was also suggested by Bidasee *et al*.[10] The advanced glycation end products are forming cross-links on long lived proteins in the heart, such as SERCA2a and RyR2, contributing to diastolic dysfunction.[3,4,6,14,19,51,52,57,79,80] We also proved that the diastolic dysfunction we observed was not due to fibrosis by examining sections of left ventricular rats hearts using Masson Trichrome staining (Figure 6). The lack of fibrosis was seen in the diabetic rats and in the ALT-711 treated diabetic rats. This is in agreement with previous studies done by Dent *et al* that showed a lack of fibrosis in the diabetic rat myocardium with mild diastolic dysfunction.[22] Because the mild diastolic dysfunction seen in our treated and untreated diabetic rats was not due to excess collagen, and studies done by Norton *et al*[57] suggested that diastolic dysfunction can happen prior to the formation of fibrosis due to the development of advanced glycation end products, we concluded that the diastolic dysfunction we observed was due to impaired excitation-contraction coupling caused by excess accumulation of AGES on SR Ca^{2+} regulatory protein, SERCA2a.[10,27,57]

Sarcoplasmic Reticulum Calcium Handling and Protein Expression:

Sarcoplasmic reticulum Ca^{2+} homeostasis is critical for proper contraction and relaxation of the myocardium.[8] During diastolic dysfunction, the ventricle does not relax properly. In this study, as well as previous studies done in our

laboratory, we observed prolonged Ca^{2+} transient decays, a decreased Ca^{2+} transient amplitude, a decreased SR Ca^{2+} load and decreased SERCA2a protein content (Figure 7, Figure 8).[47] These factors are all characteristic of an abnormal Ca^{2+} homeostasis and we previously demonstrated that this is associated with the improper relaxation of the left ventricle and decreased SERCA2a activity.[22] Since we hypothesize that the diastolic dysfunction is caused by AGEs accumulating on SERCA2a and a decreased SERCA2a activity is leading to an improper Ca^{2+} handling, we investigated whether treatment with ALT-711 would partially prevent diabetic cardiomyopathy. When comparing untreated diabetic and ALT-711 diabetic treated rats, we observed a significant decrease in Ca^{2+} transient decay, partial restoration of SR Ca^{2+} stores, and an increase in SERCA2a protein content with the rats that were treated with ALT-711 (Figure 7, Figure 8). These data suggested that Ca^{2+} is able to be reuptaken back into the SR at a nearly normal rate, proving that SERCA2a function is improved after ALT-711 therapy. The improvement in SERCA2a pump activity by ALT-711 confirms that the advanced glycation end products accumulating on SERCA2a are broken down by ALT-711 repairing SERCA2a's function.

The decrease in Ca^{2+} transient decay persisted even though RyR2 protein expression in the diabetic myocardium was partially restored by treatment of ALT-711 (Figure 8). In correlation with these findings, we did observe a mild reduction in cardiac contractility, as evident by the mild decrease in ejection fraction in treated and untreated diabetic rats, along with the decrease in cardiac

relaxation. Because it has been reported that there is an abnormal SR Ca^{2+} release during diastole by Shao *et al*, these findings would support the theory that AGE accumulation may impair RyR2 function leading to SR Ca^{2+} leak during diastole as well as SERCA2a function and that ALT-711 therapy could also improve SR Ca^{2+} release during the relaxation phase.[70] These data further support the concept that AGE accumulation may play a more important role during diastolic dysfunction than systolic dysfunction.[36]

CONCLUSIONS

In the STZ-induced diabetic rodent model we observed diabetic cardiomyopathy with a decrease in diastolic function. We observed a decrease in SR Ca^{2+} regulatory protein function and expression in untreated diabetic group compared to the controls with an increase in function and expression in ALT-711 treated diabetic rats. We conclude that treatment with an AGE cross-link breaker partially normalizes excitation-contraction coupling in the diabetic myocardium by reducing the advanced glycation end products that accumulated on the SR regulatory proteins and thus by partially repairing their function. Therefore, this study provides novel mechanistic insight into the mechanism of SR Ca^{2+} impairment in the diabetic myocardium by AGE accumulation. Finally, because there are minimal therapeutic strategies to improve myocardial relaxation, this study could assist with the development of a new strategy to benefit patients with diabetic cardiomyopathy.

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