UNIVERSITY OF CALGARY

ENDURANCE EXERCISE TRAINING ATTENUATES FIBROSIS AND COLLAGEN CROSS-LINKING IN MYOCARDIUM OF AGED RATS

by

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Abstract

It has been postulated that endurance exercise training (ET) could attenuate the age-related increase in fibrosis and collagen cross-linking in the myocardium of the heart, which is thought to be a cause of the age-related decline in diastolic function. Fisher 344 x Brown Norway F1 rats underwent ET from late middle age into senescence. Genes for collagen synthesis, degradation, enzymatic cross-linking, and the renin-angiotensin-aldosterone system were assessed by polymerase chain reaction. Matrix metalloproteinase (MMP) activity was assessed and collagen cross-linking was determined indirectly by immunohistochemical staining for advanced glycation end-products. Tissue inhibitor of matrix metalloproteinase-1 (TIMP1) gene expression and MMP activity increased with age but with no ET effect. ET attenuated the age-related increase in fibrosis and collagen cross-linking, while increasing survival rate. This could have attenuated the age-related decline in myocardial compliance which could have helped attenuate the decrease in diastolic function generally seen with aging.

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List of Symbols, Abbreviations and Nomenclature

Symbol	Definition
ACE	Angiotensin converting enzyme
AGEs	Advanced glycation endproducts
ANF	Atrial natriuretic factor
AngII	Angiotensin II
Angtr1	Angiotensin receptor 1
ANOVA	Analysis of variance
%BF	Percent body fat
Col1	Type I collagen
Col3	Type III collagen
DEXA	Dual energy x-ray absorptiometry
ECM	Extracellular matrix
F344BNF1	Fischer 344 Brown Norway F1 hybrid rat
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HF	Heart failure
HRPT	Hypoxanthine-guanine phosphoribosyl transferase
Irf1	Interferon regulatory factor 1
Lox	Lysyl oxidase
Loxl3	Lysyl oxidase-like 3
LV	Left ventricle
LVEDP	Left ventricle end diastolic pressure
LVEDV	Left ventricle end diastolic volume
MMP	Matrix metalloproteinase
MT-MMP	Membrane-type matrix metalloproteinase
P-V	Pressure-volume
ROS	Reactive oxygen species
RT-PCR	Reverse transcription polymerase chain reaction
SV	Stroke volume
RAAS	Renin angiotensin aldosterone system
SERCA	Sarco/endoplasmic reticulum Ca^{2+}-ATPase
TGFβ1	Transforming growth factor β1
TIMP	Tissue inhibitor of matrix metalloproteinase

Epigraph

"It is a rare mind indeed that can render the hitherto non-existent blindingly obvious. The cry 'I could have thought of that' is a very popular and misleading one, for the fact is that they didn't, and a very significant and revealing fact it is too."

- Douglas Adams

Chapter One: Literature review

1.1 Modern Aspects of Aging

Life expectancy has increased significantly over the past two centuries (Arking, 2006). As the actual age at which point the population reaches middle age (~70% survival) and senescence (<50% survival) is higher, there is a higher prevalence of health problems and illnesses that are associated with advanced age. In centuries past, humans were more likely to die from acute diseases and environmental hazards. Indeed, the prominence of deaths attributed to covert disease was so great that the prior medical model of aging postulated that if all diseases were removed, then life expectancy would increase. During the medical revolution, many diseases were removed from society as a consequence of gains made in hygiene practices, sanitization procedures, and medical advancements. As a result, the mean life span greatly improved, but not the maximal life span of humans. This evidence, combined with the observation that even apparently healthy and disease-free organisms aged, led to the development of the biological theory of aging. This theory states that aging is not the summation of pathologies, but the evolutionary result of organisms losing the plasticity to adapt to stressors with age after reproductive maturity is reached (Arking, 2006). Disease and aging in this model are related, but are considered to be separate processes (i.e. biogerontology versus geriatrics). A consequence of the medical revolution is that mean life span of humans has increased, and as such humans are surviving long enough to develop chronic diseases. Therefore humans have seen an increase in the occurrence of chronic disease states such as cardiovascular diseases, including diastolic heart failure. The slough of detrimental health

implications associated with chronic diseases have become an increasing burden on healthcare systems while decreasing quality of life of those affected.

Although the biological theory of aging states that aging and pathologies are two different processes, in practicality it is difficult to distinguish the changes in the heart seen in truly normal healthy aging with those observed in disease states, as they are often dual processes occurring simultaneously and integrating with one another. Disease states, especially those of the cardiovascular system, appear with greater frequency with increasing age to the point where they are almost ubiquitous in elderly individuals, and therefore are difficult to distinguish from normal healthy aging. In addition, the move from an Agrarian to Urban lifestyle (sedentary) in the past century has created a trend in our current society whereby people become more sedentary with increasing age, and thus detrimental changes and complications associated with a sedentary lifestyle are also compounding factors, making it also difficult to distinguish changes to the heart as a result of aging from those due to a sedentary lifestyle.

Heart failure is strongly associated with advanced age (M. A. Chen, 2009; S. Thomas & Rich, 2007) and represents the most common cause of hospitalization for patients older than 65 years of age (DeFrances, Cullen, & Kozak, 2007). In contrast to systolic function (ventricular contraction), which is comparatively well preserved with age, diastolic function (ventricular relaxation) declines with age from 30 years old and onwards in humans (Benjamin et al., 1992; Kitzman, 2002). This occurs even in the absence of cardiovascular diseases. This decline in diastolic function is thought to be a

key cause of the exponentially increased incidence of diastolic heart failure with age (Benjamin et al., 1992; Brenner, Apstein, & Saupe, 2001; Downes, Nomeir, Smith, Stewart, & Little, 1989; Grossman, 1991; Lakatta & Yin, 1982; Lakatta, 1987). With increasing age in normal healthy hearts, there is predominantly an impairment in ventricular relaxation which is mostly observed as a decrease in the rate and volume of early (passive) diastolic filling (Andren, Lind, Hedenstierna, & Lithell, 1995; Lye & Wisniacki, 2000; Mantero et al., 1995). As a result, the late (active) portion of diastolic filling, involving the contraction of the atria to push more blood into the ventricle, plays a more prominent role in ventricular filling. Thus left ventriclular end diastolic pressure (LVEDP) is increased for a given left ventriclular end diastolic volume (LVEDV) with age. Systole occurs when an action potential causes the release of Ca^{2+} from the sarcoplasmic reticulum into the cytoplasm of the cardiomyocytes. Ca^{2+} binds to the troponin-tropomyosin complex of the actin filament which induces a morphological change in the troponin-tropomyosin complex resulting in the exposure of the myosin binding site. Once exposed, cross-bridges adhere and movement occurs with the use of energy from ATP. Removal of the Ca^{2+} from the cytoplasm results in diastole as the troponin-tropomyosin complex returns to its initial form that covers the actin binding site. This allows for cross-bridge dissociation and actin and myosin to remain in the relaxed state. Thus the removal of Ca^{2+} from the cytoplasm is a major contributor diastolic relaxation, which is accomplished by the sarco(endo)plasmic reticulum Ca^{2+} ATPase (SERCA) 2a pump (Mcardle, Katch, & Katch, 2007). Aging is also associated with fibrotic cardiac remodeling with increased accumulation of collagen (fibrosis) likely contributing to diastolic dysfunction in the aged heart. This would cause an increase in

ventricular stiffness which would result in decreased ventricular compliance, and impair the ventricles' ability to passively relax. Indeed, increased LV diastolic compliance is reported in modifications in collagen such as fibrosis, type of collagen, and collagen cross-linking (Kass, Bronzwaer, & Paulus, 2004; Yamamoto et al., 2002). Evolving evidence suggests that age-associated alterations in inflammatory and fibrogenic pathways may be critically involved in the pathogenesis of heart failure in elderly subjects (W. Chen & Frangogiannis, 2010; de Souza, 2002). As mentioned above, it should also be noted that the decrease seen in diastolic functioning in the heart could also be due to reduced SERCA 2a expression or function (Pugh & Wei, 2001; Roffe, 1998; Schmidt et al., 2000). Consistent with this possibility, SERCA2a gene therapy to increase protein and activity levels in senescent rats resulted in improved diastolic function (Schmidt et al., 2000).

Pathological remodeling of the ventricle results firstly in the development of ventricular dilation, followed by systolic dysfunction eventually resulting in heart failure (Iwanaga et al., 2002). There is no single definitive cause for the onset of fibrosis in the heart. The loss of regulation that normally exists between stimulators and inhibitors of fibrosis accounts for connective tissue remodeling (Weber, 2000). An excess of stimulators, due either to absolute stimulator overproduction or to their relative overabundance due to decreased production of inhibitors, can promote fibrosis (Weber, 2000). In addition, it is known that the heart adapts to chronic increases in physiological demand of work by remodeling. Cardiac remodeling is a slow progressive process that is either irreversible or only slowly reversible (Wakatsuki, Schlessinger, & Elson, 2004).

Mechanical distortion (shear or stretch) has been shown to stimulate remodeling, as has hypertension, notably involving the renin-angiotensin-aldosterone system (RAAS) (W. Chen & Frangogiannis, 2010; Wakatsuki et al., 2004). Remodeling is usually preceded by pathological hypertrophy. As cardiac hypertrophy occurs, chronic cardiac volume over-load may result in collagen accumulation, most notably in the subendocardial layer of the free wall of the left ventricle (Takala et al., 1991). However, it is important to note that it is fibrosis and not myocyte hypertrophy that is the cause of myocardial stiffness.

There are two types of fibrosis that can occur in the heart: reparative and reactive. Reparative fibrosis occurs when trauma is inflicted on the myocardium, such as a myocardial infarct where mass cell death occurs through necrosis, and later apoptosis. Previously, the heart was considered to be comprised of post-mitotic cell types incapable of regeneration. However, in recent years it has been found that the heart is capable of growing new cardiomyocytes (Bergmann et al., 2009). Cardiomyocyte regeneration has been shown to contribute to the replacement of adult cardiomyocytes after acute injury but does not contribute significantly to cardiomyocyte renewal during normal aging. The rates of regeneration are too insignificant to have a sufficient impact to completely recover from disease states of the heart such as myocardial infarction, or normal aging *in vivo* (Bergmann et al., 2009; Hsieh et al., 2007). The massive loss of cardiomyocytes due to apoptosis and necrosis in reparative fibrosis induces an inflammatory response that activates fibroblasts to lay down collagen to replace the lost cells, and thus a scar is formed. In reactive fibrosis, no such mass loss of cardiomyocytes occurs. Instead, the interstitial space in the myocardium is increased through increased collagen in the

extracellular matrix (ECM) (Biernacka & Frangogiannis, 2011). With normal healthy aging there is an increase in reactive fibrosis. In a study done by Olivetti et al. (1991) on humans, they showed that there is a progressive loss of cardiomyocytes in the heart (14 and 38 million cardiomyocytes are lost per year in the right and left ventricle, respectively) with a concurrent increase in myocyte cell volume per nucleus (i.e., cardiomyocyte hypertrophy) (Olivetti, Melissari, Capasso, & Anversa, 1991). Indeed, it has been well-characterized in the aging heart that there is a progressive decrease in the number of cardiomyocytes due to apoptosis and necrosis (Kajstura et al., 1996) and subsequent cardiomyocyte hypertrophy (Anversa et al., 1990) accompanied by increased reactive fibrosis (Choi et al., 2009; H. B. Kwak, Song, & Lawler, 2006; H. Kwak et al., 2011; Pugh & Wei, 2001).

1.2 Changes in Extracellular Matrix with Aging

In the myocardium, the collagen in the ECM serves important functions such as a scaffold and support system for the cardiomyocytes, diastolic suction, protection for quiescent myocardial fibroblasts, cardiomyocyte alignment, transduction of contractile forces, electrically separating the atria from the ventricles for proper cardiac cycling, and supporting the vasculature to maintain the architecture and geometry of the heart (Biernacka & Frangogiannis, 2011; de Souza, 2002). Rather than being a static support system, the amount of collagen present in the ECM of the myocardium results from the balance between the rate of collagen synthesis and degradation (Laurent, 1987). The rate of collagen synthesis versus collagen degradation equals the rate of collagen turnover,

which is slow in normal healthy myocardium (Rodriguez-Feo, Sluijter, de Kleijn, & Pasterkamp, 2005). Although the activation of reparative fibrosis has been well-characterized (Biernacka & Frangogiannis, 2011), the activation and mechanism of reactive fibrosis with aging remains unclear. In pathological conditions such as hypertension, it appears that the increase in fibrosis in the myocardium is due to an increase in collagen synthesis. This does not appear to be the case with normal aging (see section "Exercise and Fibrosis"). Nonetheless, cardiac fibroblasts are the key cells that synthesize collagen in the ECM (Rodriguez-Feo et al., 2005).

1.2.1 Collagen Synthesis

One of the most important processes of fibrosis is the activation of fibroblasts (Wakatsuki et al., 2004). This occurs via the phenotypic conversion of fibroblasts to myofibroblasts. This conversion is promoted by both mechanical stimuli exerted on the cells (Wang, Chen, Seth, & McCulloch, 2003) and exposure to transforming growth factor-β (TGFβ1) (Petrov, Fagard, & Lijnen, 2002). In addition, a number of other neurohumoral systems can promote this conversion, specifically the RAAS via angiotensin II (AngII), and β-adrenergic systems, all with the end result of activating fibroblasts to proliferate and differentiate into myofibroblasts (Porter & Turner, 2009; Rosenkranz, 2004). The phenotypic conversion of fibroblasts to myofibroblasts is determined when these cells express α-smooth muscle actin, an important contractile protein that is required for integrin-mediated collagen remodeling (Arora & McCulloch, 1994; Leslie, Taatjes, Schwarz, vonTurkovich, & Low, 1991; Petrov et al., 2002; Porter

& Turner, 2009; Wang et al., 2003; Weber & Brilla, 1991; Weber, Brilla, & Janicki, 1991a; Weber, Brilla, & Janicki, 1991b). Myofibroblasts are capable of producing significantly more collagen than fibroblasts (Petrov et al., 2002). Briefly, collagen is first synthesized intracellularly in the fibroblast or myofibroblast as a soluble procollagen, a large triple-helix precursor molecule, consisting of approximately 100 4-hydroxyproline residues, and propeptides on both the N- and C- terminuses. The procollagen is then secreted into the extracellular space where the N-propeptides are cleaved by a procollagen N-proteinase and the C-propeptides by a procollagen C-proteinase. The insoluble collagen then self-assembles into fibrils in the ECM.

As mentioned above, exposure to TGFβ1 is a potent stimulator of collagen synthesis via fibroblast activation through phenotypic conversion to myofibroblasts. TGFβ1 is a biologically active peptide that is present in normal cells, including fibroblasts. It has been demonstrated that TGFβ1 stimulates the expression of collagen and its appearance in the ECM (Ignotz & Massague, 1986). Preliminary studies indicate that TGFβ1 mRNA increases with pressure overload, which could result in more collagen deposition in the aging heart (de Souza, 2002). TGFβ1 may also play an essential role in cardiac aging by inducing myofibroblast differentiation (Desmouliere, Geinoz, Gabbiani, & Gabbiani, 1993) and by enhancing matrix protein synthesis by cardiac fibroblasts (Bujak & Frangogiannis, 2007; W. Chen & Frangogiannis, 2010). Activation of TGFβ1 increases the degree of collagen deposition by myofibroblasts via Smads (intracellular proteins that transduce extracellular signals from TGFβ-ligands) or TGFβ-activated kinase 1, indicating the progression of fibrosis (Imanishi et al., 2004; Wakatsuki et al.,

2004). Both reactive oxygen species (ROS) and AngII may activate TGFβ1 signaling pathways in the senescent heart. In addition, AngII markedly upregulates TGFβ1 synthesis by cardiac fibroblasts, however, the dependence of the pro-fibrotic actions of AngII on TGFβ1 has not been established (W. Chen & Frangogiannis, 2010). Brooks and Conrad (2000) demonstrated that TGFβ1 heterozygous mutant mice had less age associated myocardial fibrosis and improved left ventricle compliance which may have contributed to the increased survival observed over the life span of the mice (Brooks & Conrad, 2000). Also of note, mechanical stretch has been shown to stimulate type III collagen protein and mRNA synthesis rates in cyclically stretched fibroblasts. Collagen synthesis could be caused by the interaction of chemicals such as hormones, neurotransmitters and growth factors that either directly or indirectly interfere with fibroblasts (de Souza, 2002). It has been shown that protein levels of TGFβ1 increase in the heart with aging, which could help to explain the age-related increase in fibrosis in the myocardium (H. Kwak et al., 2011). A recent study by Kwak et al. (2011) demonstrated that a 12 week endurance exercise training program in 31 month old Fischer 344 Brown Norway F1 hybrid (F344BNF1) rats resulted in the attenuation of the age-related increase in protein levels of TGFβ1. Nonetheless, the mechanisms of the effects of aging including fibroblast growth and expansion of the collagen network in the myocardium are still poorly understood. However, it is known that fibrotic remodeling of the aging ventricle plays an important role in the pathogenesis of heart failure (W. Chen & Frangogiannis, 2010).

1.2.2 Collagen Degradation

Given the stability of the collagen concentration in the normal, healthy myocardium, collagen degradation is equivalent to its synthesis. Cardiac fibroblasts are likely to be the cells responsible for collagen degradation (de Souza, 2002). However, the collagen degradation process and how it is affected by normal aging in the myocardium is not known. Whether cardiac fibrosis can be reversed remains controversial (W. Chen & Frangogiannis, 2010). As previously stated, collagen is extremely stable. It is highly resistant to degradation by all proteinases except a specific family of proteolytic zinc-dependent enzymes that are capable of catabolizing collagen. These collagenases are known as Zn^{2+}-dependent matrix metalloproteinases (MMPs). There are over 20 different MMPs that have been identified to date. MMPs contribute to remodeling of the heart through remodeling of the ECM. MMPs are essential in such processes as wound healing and tissue morphogenesis (Spinale, 2002). The family of MMPs can be divided into two groups, the larger being those that are secreted into the extracellular space, and the remainder being MMPs bound to cellular membranes, known as membrane-type MMPs (MT-MMPs). The former group exists in a latent form known as proMMPs that are often docked to the ECM proteins where they exert their action. ProMMPs require activation to function proteolytically. Activation occurs through cleavage of the propeptide domain by various factors such as other MMPs and serine proteases (Spinale, 2002), resulting in a conformational change of the MMP to give substrates access to the catalytic domain. Normal healthy rat myocardium contains mostly proMMPs awaiting activation (Tyagi, Kumar, Banks, & Fortson, 1995). Due to their close proximity to their substrates, once

activated the MMPs have a rapid effect on the ECM. The group of six known MT-MMPs are inactivated during transportation to the cell membrane, and once inserted into the cell membrane, become proteolytically active. MMPs are inhibited by tissue inhibitors of matrix metalloproteases (TIMPs). To date, four TIMPs have been identified. TIMPs are endogenous inhibitors that bind to proMMPs or MMPs in a 1:1 stoichiometric ratio (Spinale, 2002; Tyagi et al., 1995). MMP activity is coordinated in part by TIMPs at both the gene and protein levels, as MMPs and TIMPs have been shown to be co-expressed and co-localized together in the myocardium (Tyagi et al., 1995). It is the balance and magnitude of expression between MMPs and TIMPs that determines the proteolytic activity of MMPs. In addition, a distorted equilibrium between TIMPs and MMPs has been shown to occur in a variety of disease states (Tyagi et al., 1995). Changes in collagen content in cardiovascular syndromes have been shown to be a direct consequence of an increase in the expression and activity of the enzymes that dynamically control the synthesis of collagen (Rodriguez-Feo et al., 2005).

The results of a study by Robert et al. (1997) suggests that depression of the degradative pathway is at least partly responsible for age-associated fibrosis via significant decreases in MMP activity and mRNA (Robert et al., 1997). Therefore decreases in MMP activity as well as an increase in collagen cross-linking (see "Collagen Cross-Linking" section) indicate that a decrease in collagen degradation appears to be an important feature of age-related fibrosis (de Souza, 2002; Robert et al., 1997; Rodriguez-Feo et al., 2005). Thus it has been proposed that the fibrosis that occurs in normal aging is a result of a decrease in collagen degradation with little to no change in collagen

synthesis. Over time, this would result in a net accumulation of collagen in the myocardium. This differs from the traditional view held in pathological states that fibrosis is the result of a disproportional increase in the rate of collagen synthesis compared to collagen degradation (H. Kwak et al., 2011).

1.2.3 Type of Collagen

In the normal adult heart, morphometric assessments indicate that approximately 2-4% of the myocardium is collagen. The amount of collagen in the heart can increase 4-fold with age (P. K. Mays, Bishop, & Laurent, 1988). The normal LV of young (1 mo) F344 rat contains 5.5% collagen, which increases to 12% at early middle age (22 mo) (Eghbali, Eghbali, Robinson, Seifter, & Blumenfeld, 1989). The heart contains multiple types of collagen within its structural matrix. In adult rats, this collagen is comprised of mainly type I, the predominant type (~85%), and III (~11%) collagens, with the remainder consisting of the basement membrane specific collagens IV and V (Chapman et al., 1990; Chapman, Weber, & Eghbali, 1990; de Souza, 2002; de Souza, 2002; Medugorac & Jacob, 1983; Weber et al., 1988). Mays et al. (1988) showed that in male Lewis rats, the proportion of type III collagen in relation to total collagen (types I and III) increased from birth to 6 months of age (54%) and then decreased modestly by 24 months of age (46%), and therefore type I collagen had the inverse trend (P. K. Mays et al., 1988). The tensile strengths of type I and III collagens are different, with collagen type I having a high tensile strength compared to that of steel, while type III has a much lower tensile strength (Burton, 1954). Because of the different tensile strengths of the

types of collagen, one would assume that the balance between types I and III collagens is important in maintaining the normal contractility of the myocardium. It has been proposed that a higher ratio of type I collagen to type III collagen could result in a stiffer and less compliant LV. Although the ratio of type I:III collagens increases in hypertension, dilated cardiomyopathy, and myocardial infarction, the functional consequences of these shifts have not been rigorously determined. A higher ratio of type I:III collagens has been linked to LV end diastolic stiffness (Brower et al., 2006; Burgess et al., 1996; Choi et al., 2009). Burgess et al. (1996), reported a correlation of $r = 0.91$ between LV relaxation and the ratio between type I and III collagens, with an increase in type I:III collagens correlating with a slower rate of LV relaxation. However, in this study as well as in all the aforementioned disease states, total collagen concentration also increased significantly, which is a confounding factor potentially influencing LV relaxation and/or myocardial stiffness. In addition, Linehan et al. (1998) showed that with hypertension, a significant increase in the collagen type I:III ratio was not associated with an increase in diastolic stiffness when overall collagen content was unchanged (Linehan, Seymour, & Williams, 2001). Therefore, due to the unclear nature of the relationship between the ratio between the two collagen types and stiffness of the myocardium, no definitive conclusions can be made on the functional implications of any changes in the type of collagen in the myocardium. Caution must be used when applying changes in type I:III collagen ratios to functional consequences in the myocardium (Brower et al., 2006).

1.2.4 Geometry of the Myocardium

In addition to both the concentration of collagen and, arguably, the relative proportions of the types of collagen, the stiffness of the myocardium may also be dependent on architectural remodeling of the heart. In the myocardium, this is reflected in the geometry of the ECM including an increased diameter of collagen fibrils, decreasing linearity of myocyte sheaths, and increasing disorder of the organization of the myocardium (de Souza, 2002; Debessa, Maifrino, & de Souza, 2001; Masson, Latini, Salio, & Fiordaliso, 2005). This age-associated adverse remodeling has been shown to be primarily due to decreased collagen degradation more so than increased collagen synthesis and proliferation of fibroblasts (Camelliti, Borg, & Kohl, 2005; Masson et al., 2005).

1.3 Types of Collagen Cross-Linking

In addition to the factors previously discussed, the stiffness of the myocardium may also be dependent on the degree of collagen cross-linking (Brower et al., 2006; Norton et al., 1997). Cross-linking is a process whereby collagen fibers are irreversibly covalently linked to one another. This results in increased stiffness of the collagen and also makes the cross-linked collagen extremely stable and resistant to degradation. There is mounting evidence that collagen cross-links are the major determinant of ventricular stiffness in the heart, as opposed to total quantity of collagen or type of collagen (Badenhorst et al., 2003; Brower et al., 2006; Choi et al., 2009; Jyothirmayi, Soni,

Masurekar, Lyons, & Regan, 1998; Kass et al., 2004; Norton et al., 1997). Collagen is not a completely static protein, and changes in the balance between synthesis and degradation may lead to alterations in the composition of the collagen network in the myocardium, including cross-linking (Bishop & Laurent, 1995; Brower et al., 2006). The formation of collagen cross-links occurs naturally with aging (Asif et al., 2000) and the occurrence is also increased in diseases such as hypertension, cardiac ventricular volume overload, and diabetes (Brower et al., 2006).

1.3.1 Cross-Linking

There are two methods of collagen cross-link formation: enzymatic and non-enzymatic. Enzymatic cross-link formation appears to be tissue-specific and occurs naturally during development to physiological maturation. It occurs through a controlled and regulated process involving the enzyme lysyl oxidase, a copper-dependent amine oxidase that catalyzes lysine derived cross-links in collagen (Avery & Bailey, 2005; Choi et al., 2009; Sivakumar, Gupta, Sarkar, & Sen, 2008). The predominant and most extensively researched type of collagen cross-linking is the non-enzymatic process that occurs as a result of the spontaneous modification of proteins by glucose to form advanced glycation end products (AGEs) (Avery & Bailey, 2005; Choi et al., 2009). This type of cross-linking is commonly found in long-lived proteins and aged populations and is the major cause of collagen tissue dysfunction during senescence (Bailey, Paul, & Knott, 1998).

The non-enzymatic process is generally a two-stage process occurring primarily through glucose modifying proteins to form AGEs (Kochakian, Manjula, & Egan, 1996). The non-enzymatic formation of collagen cross-links through glycosylation increases with age (Asif et al., 2000; Choi et al., 2009; Kochakian et al., 1996). The formation of AGEs is a complicated, diverse, and multi-step reaction known collectively as the Maillard reaction (Maillard, 1912), and occurs mainly with the side-chains of lysine and arginine to form additional intermolecular cross-links (Aronson, 2003). Glucose initially rapidly forms reversible early glycosylation products, known as Schiff bases, with proteins. The next step occurs over a period of days where the Schiff base rearranges to form a more stable, but still reversible, type of early glycosylation product known as an Amadori product. Although this reaction is slow, the forward reaction is much faster than the reverse and the net result is an accumulation of Amadori product on their glycated proteins (Aronson, 2003). The Amadori products then undergo a complex series of reactions that may or may not involve oxidation through reaction with ROS to form more reactive species involved in glycoxidation (Avery & Bailey, 2005). The non-oxidative route of the reaction yields the stable end product of AGEs in the first stage of the process which then form irreversible cross-links along collagen molecules in the second stage of the process (see Figure 1) (Aronson, 2003; Avery & Bailey, 2005). The degree to which AGEs accumulate on proteins is determined both by the concentration of glucose and the time of exposure. Thus AGEs accumulate on long-lived proteins with aging and also in conditions of hyperglycemia such as in diabetes and in chronic diseases such as hypertension, to eventually form cross-links (Aronson, 2003; Brower et al., 2006; Brownlee, Cerami, & Vlassara, 1988).

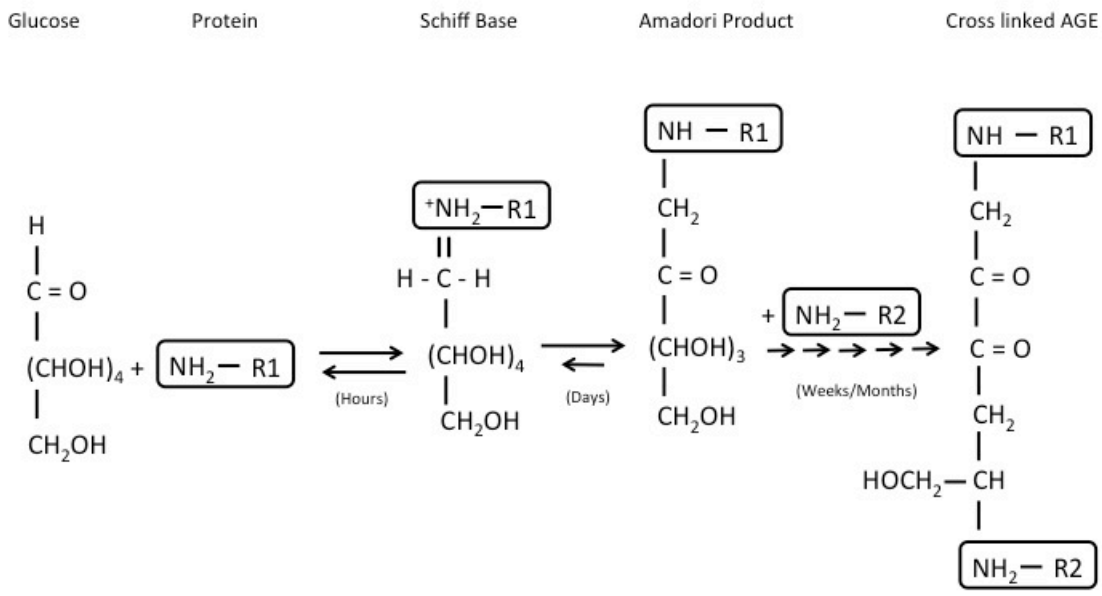

Figure 1: Maillard reaction to form AGE and subsequent cross-linking. Replicated with permission from Aronson, 2003.

1.3.2 Consequences of Non-Enzymatic Collagen Cross-linking

The formation of AGEs increases with increasing age, and is seen as a true mechanism of aging (Avery & Bailey, 2005; Kochakian et al., 1996). As mentioned above, collagen cross-links not only make the collagen extremely stable and resistant to degradation, but also greatly increase the tensile strength of the collagen (Avery & Bailey, 2005). Given the evidence that these cross-links play a central role in the determination of the stiffness of a tissue, the attenuation or reduction in the formation of collagen cross-links would result in an increase in the compliance of the tissue. This has significant implications in the aging myocardium due to the age related decline in diastolic functioning that could be attributed to a stiffer myocardium. Reducing the accumulation of collagen cross-links would potentially result in a less stiff and more compliant myocardium which could improve passive diastolic filling (Arbab-Zadeh et al., 2004; Takemoto et al., 1992).

Aminoguanidine prevents the formation of non-enzymatic cross-linking by blocking the reactive carbonyl group of the Amadori product formed by glycation, thus preventing later cross-linking steps from occurring (Lewis & Harding, 1990). In a study by Avendano et al. (1999), the administration of aminoguanidine prevented collagen cross-link formation and associated LV stiffness in diabetic dogs. This was accomplished without preventing the diabetes-induced increase in fibrosis (Avendano et al., 1999). Other pharmacological studies aimed at inhibiting cross-link formation via administration of aminoguanidine have shown that it can attenuate cardiac hypertrophy, increase the

vasodilatiory response, and attenuate the increase in aortic impedance seen with aging (Corman et al., 1998; Li et al., 1996). Breaking cross-links via administration of thiazolium derivatives (ALT-711) reduces vascular stiffness and improves cardiac function as indicated by an increase in stroke volume index (borderline significant) (Asif et al., 2000; Masson et al., 2005). A study done by Vaitkevicius et al. (2001) showed that in aged but not senescent 21 year old rhesus monkeys, a 3 week treatment of ALT-711 was able to increase atrial and LV compliance as well as increase cardiac output (Tigges, Gordon, Mcclure, Hall, & Peters, 1988; Vaitkevicius et al., 2001). In late middle aged humans, ALT-711 was able to increase arterial compliance while reducing pulse pressure (Kass et al., 2001). Thus interventions that reduce collagen cross-linking pose a promising therapy to attenuate the effects of aging on the myocardium and vasculature.

The significance of cross-linking to myocardial functioning has been probed by groups such as Norton et al. (1997), who found that increased myocardial stiffness secondary to hypertension in spontaneous hypertensive rats is the result of increased collagen cross-linking rather than increased collagen concentration or type I:III collagen ratio (Norton et al., 1997). Woodiwiss et al. (2001) also demonstrated that a decrease in collagen cross-linking results in reduced myocardial stiffness and ventricular dilatation, irrespective of collagen concentration or type, in two rat models of pressure-overload induced heart failure (Woodiwiss et al., 2001). Therefore, it is strongly suggested that cross-linking has a significant impact on myocardial stiffness and thus functioning irrespective of total collagen concentration. However, it must be remembered that myocardial and specifically LV stiffness is affected by both collagen quantity and quality

(cross-linking), with the effects of changes in collagen quantity being modified by changes in collagen quality (Badenhorst et al., 2003).

1.4 Exercise and Diastolic Function

Existing evidence supports the hypothesis that exercise training may improve heart diastolic, and to a degree, systolic function (Arbab-Zadeh et al., 2004; Choi et al., 2009; Takemoto et al., 1992). Although some controversy remains in humans, most studies agree that endurance exercise training can improve or even reverse the age-associated decline in passive diastolic function (Fleg et al., 1995; Levy, Cerqueira, Abrass, Schwartz, & Stratton, 1993; Takemoto et al., 1992). As one example of a study which did not find a benefit of exercise training, long-term endurance exercise training in older human male subjects did not correspond with improved passive diastolic filling (Fleg et al., 1995). Other studies have shown the opposite. Takemoto et al. (1992) found that late middle aged subjects who had been aerobically training for years had better passive diastolic filling than their sedentary counterparts (Takemoto et al., 1992), while Levy et al. (1993) found that 6 months of endurance exercise training also yielded a significant improvement in passive diastolic filling during rest and during exercise (Levy et al., 1993). In animal studies, Brenner found similar results in 24 month old F344BNF1 rats after 12 weeks of endurance exercise training, with a complete reversal of the age-associated decline in passive diastolic filling (Brenner et al., 2001). In contrast, Choi et al. (2009) found that a 12-week endurance exercise training program in 25 month old F344 rats resulted in an improvement in myocardial contractility, but not relaxation (Choi

et al., 2009). However, in that study the early diastolic filling phase was not differentiated from active relaxation, thus improvements in passive stiffness could have been masked. Although the molecular mechanisms by which improvements in passive diastolic filling could occur are currently unknown, one idea is that it could be by decreasing the stiffness of the myocardium through alterations of the ECM, secondary to alterations to the factors regulating ECM collagen synthesis, breakdown, and cross-linking as discussed above.

1.5 Exercise Training and Fibrosis

One characteristic of the ECM that could have a significant impact on early diastolic functioning is the quantity of collagen in the myocardium. As mentioned above, collagen quantity in rat heart increases significantly during life span (Anversa et al., 1990; Eghbali et al., 1989; P. K. Mays et al., 1988; D. P. Thomas, Mccormick, Zimmerman, Vadlamudi, & Gosselin, 1992). The literature presents controversy in regard to whether exercise training is able to affect the accumulation of collagen in the myocardium. Changes in the quantity of collagen with endurance exercise training have been shown in some (H. B. Kwak et al., 2006; H. Kwak et al., 2011) but not all (Choi et al., 2009; D. P. Thomas et al., 1992; D. P. Thomas, Cotter, Li, McCormick, & Gosselin, 2001) studies, while most studies show that the relative ratio of type I to type III collagen is not significantly altered with training in aged animals (D. P. Thomas, Zimmerman, Hansen, Martin, & McCormick, 2000; Woodiwiss, Oosthuyse, & Norton, 1998). In addition, Kwak et al. (2011) found that endurance exercise training provided protection

against age-associated adverse geometric changes such as alinearity and increased diameter of collagen of the ECM in the LV of rat hearts (H. Kwak et al., 2011).

In the studies that saw an exercise training effect in the attenuation of fibrosis, this result must either be due to a decreased synthesis or increased degradation of collagen in the ECM of the myocardium. Current evidence favours the latter mechanism (de Souza, 2002; Kim, Rodriguez-Enriquez, & Lemasters, 2007; H. Kwak et al., 2011; Robert et al., 1997). Kwak et al. (2011) found no difference in type I collagen mRNA with aging into senescence in F344BNF1 rats, while Thomas et al. (2000) found that type I and III collagen mRNAs decreased with aging in F344 rats (H. Kwak et al., 2011; D. P. Thomas et al., 2000). These data do not support the concept of age-related fibrosis occurring due to increased synthesis. Kwak et al. (2011) also found a marked increase in TIMP1 protein expression with aging, which is in agreement with the theory that the fibrosis that occurs in pathological conditions of aging differs from that of healthy normal aging where there appears to be either no change or a decrease in the rate of collagen synthesis coupled with a significant decrease in the rate of collagen degradation (Lakhan & Harle, 2008; Masson et al., 2005). In addition, this may occur predominantly in certain regions of the myocardium. Derumeaux et al. (2008) showed that exercise training significantly decreased collagen content in the endocardium, but not the epicardium (Derumeaux et al., 2008). It is currently unknown if this training effect can be observed if training is commenced later in life and continued into senescence, the latter representing an age with a much higher incidence of diastolic dysfunction.

1.6 Exercise and AGE Cross-Link Formation in the ECM of the Myocardium

Although to date no studies exist looking at the effect of endurance exercise training on collagen cross-linking in the human heart, it has been shown that endurance exercise training increases arterial compliance and can attenuate age-related decreases in arterial compliance in humans (Tanaka et al., 2000). It has been shown that endurance exercise training can significantly attenuate the amount of collagen cross-linking in the myocardium of rat hearts (Choi et al., 2009; D. P. Thomas et al., 1992; D. P. Thomas et al., 2000). Choi et al. (2009) were not able to fully elucidate the influence of long-term exercise training on the AGEs formation and collagen cross-link formation and cardiac performance. In their study, they endurance exercise trained 25 month old rats until 28 months of age. They found that endurance exercise training significantly improved contractility (systolic performance) of the heart and reduced myocardial stiffness, with a borderline significant improvement in passive diastolic filling. Choi did find that although training did not influence the total amount of collagen in the myocardium, it did significantly increase the quality of the collagen in the myocardium by decreasing myocardial collagen solubility as an index of AGEs cross-linked collagen. Thomas et al. (2000) showed that endurance exercise training attenuated the age-associated increase in collagen cross-linking without affecting the overall increase in collagen concentration (D. P. Thomas et al., 2000). Based upon these studies, endurance exercise training has been hypothesized to be able to favourably change the characteristics of myocardial collagen, and thus affect the performance of the heart, despite the ratio between types of collagen being similar or if the quantity of collagen remains the same (Choi et al., 2009; Norton et

al., 1997; D. P. Thomas et al., 2000). Again, it is unknown if this effect can occur if training is commenced later in life and into senescence.

1.6.1 Collagen Turnover and Cross-Linking

As mentioned above, the balance between collagen synthesis and degradation in the myocardium determines the quantity of collagen in the ECM. The rate of collagen synthesis and degradation determines collagen turnover. The quantity of collagen cross-links that accumulate in non-diabetic individuals in normal healthy aging would depend on the rate of collagen turnover in the ECM. Higher rates of turnover would limit the formation of AGEs and thus limit collagen cross-linking by reducing the accumulation of old and damaged collagen proteins that are susceptible to forming AGEs. Low rates of protein turnover would be detrimental to the ECM of the myocardium, as it would allow old damaged collagen proteins to accumulate. The accumulation of old damaged proteins increases the likelihood of them reacting with glucose to eventually form AGEs and subsequently cross-link. In relation to the pharmacologically based therapies discussed above, it may be possible to physiologically manipulate collagen cross-linking through endurance exercise, but not resistance training. While endurance exercise has been shown to have beneficial effects on the heart with aging, resistance training has been shown to have detrimental effects and possibly exacerbate age related changes (such as decreasing arterial compliance) and is associated with LV hypertrophy in healthy middle aged men (Miyachi et al., 2003; Miyachi et al., 2004). Therefore the mode of exercise applied is crucial. Thus, the mechanism responsible for decreasing AGEs cross-linking within

collagen in aged trained rats via endurance exercise may be the result of altered AGEs collagen formation and/or collagen degradation (Choi et al., 2009). Increases in the rate of collagen turn-over could result in newly synthesized, less mature collagen and lower concentrations of collagen cross-linking (Takala et al., 1991).

Exercise training may induce positive changes in collagen turnover and thus cross-link formation in the heart by favorably changing pressure, volume, and the neurohormonal condition of the heart (Choi et al., 2009; Takala et al., 1991; D. P. Thomas et al., 2000). It has also been proposed that endurance exercise training induces an increase in antioxidative defenses that could inhibit collagen cross-link formation. Chelators, sulfhydryl compounds and certain antioxidants that have been suggested to act as inhibitors of AGEs-formation by uncoupling glycation from subsequent glycation and cross-linking reactions, and thus could prevent further glycation reactions during the process of AGE formation (Choi et al., 2009; Fu et al., 1994; Kochakian et al., 1996). Thus, it may be hypothesized that exercise induced anti-oxidative effects could inhibit AGEs-formation. Unfortunately, the detection of individual AGEs is difficult, and thus this hypothesis has yet to be tested (Choi et al., 2009).

1.7 Current Study

The current study addresses the effects of long-term endurance exercise training initiated in late middle age (28 months) into senescence (34-36 months) on collagen quantity, quality in AGE formation, genetic expression of relevant collagens, MMPs,

TIMPs, RAAS factors, enzymatic factors, and collagenase activity in hearts of F344BNF1 rats. The 50% survival rate for this rat strain is 33 months old (Turturro et al., 1999), meaning that the 34-36 month old animals were within the period of senescence, an age associated with prominent diastolic dysfunction (Burlew, 2004; Hacker, McKiernan, Douglas, Wanagat, & Aiken, 2006). This strain of rat has also been shown to live long enough to experience age-related changes similar to humans (Turturro et al., 1999). Specifically, this strain of rat has been shown to experience age-related alterations in cardiovascular (heart and aortic) structure and function similar to that of humans (Walker et al., 2006). As mentioned previously, due to the severely advanced ages of the rats (senescence), they are inclined towards conditions that commonly occur in parallel with aging. Aging and a sedentary lifestyle such as that which a caged rat would experience, would result in increased body fat accumulation and perhaps in turn insulin resistance. All four factors are risk factors for metabolic syndrome as well as hypertension (Grundy et al., 2004). The implications of this could include endurance exercise training exerting its' main beneficial effect on these conditions, and any measured result could be a secondary effect. The majority of studies focus on younger ages that are pre-senescent, but very little is known about the effects of endurance exercise training on hearts that have already experienced significant detrimental age-related changes. It is hypothesized that endurance exercise training initiated in late middle age, after significant detrimental age-related changes in the hearts had already manifested themselves, into senescence would attenuate the accumulation of collagen and collagen cross-linking in the myocardium of male F344BNF1 rats. It was postulated that this could occur by increasing protein turnover by attenuating the age-related decrease in

collagen degradation by decreasing TIMP gene expression while increasing MMP gene expression and activity, with no change or a decrease in signaling factors such as TGFβ1 for collagen synthesis.

Chapter Two: Methods

2.1 Animals

Male F344BNF1 rats aged 7 mo (young adult) and 29 mo (late middle aged) were obtained from the National Institute of Aging colony maintained by Harlan (Indianapolis, IN). All rats were allowed free access to food and water and were housed 2-3 per cage in the same pathogen free room. Twenty-four 29 mo rats were randomly assigned to the training group (35T), with the remaining thirty-three 29 mo (35C) and ten 7 mo adult rats acting as a sedentary control group (7C) and having only normal cage activity. Aged animals were 34-36 mo at the time of sacrifice. The 50% survival rate for this rat strain is 33 mo (Turturro et al., 1999), meaning that the older animals were within the senescent period at the time of sacrifice. All experimental protocols were approved by the University of Calgary Animal Care Committee (BI 09R-11).

2.2 Endurance Training Exercise Protocol

The endurance exercise training group initially ran on the treadmill (Columbus Instruments, Columbus, OH) 5 days per week as described in a previous study (Betik & Hepple, 2008). The exercise protocol was designed to gradually acclimate the rats to exercising on the treadmill. Duration of exercise was increased progressively so that by the third week of training, the animals were running for 60 minutes per day at a grade of 10%, as this was shown to yield significant benefits for skeletal muscle and whole body function in late middle aged animals (Betik & Hepple, 2008). Each training session

consisted of six repetitions of 10 minutes of continuous running with 2 minutes of rest in between repetitions. Within the 10 minute segments, 8 minutes was performed at a base velocity, and a higher velocity was used for the last 2 minutes. The base velocity was slowly increased from 5 m/min to 7 m/min at the third week, with increments of 0.5 m/min each week until the animals could not tolerate an increase in velocity (which coincided with the ninth week of training). The 2 minute interval at the end of the 10 minute segment was performed at 2.0 m/min faster than the base velocity. After the eighth week, the training sessions were reduced to 4 days per week with the goal being to maintain any adaptations that occurred during the initial training sessions and to allow more recovery time as the animals aged into senescence (Betik, Thomas, Wright, Riel, & Hepple, 2009). The last training session occurred at least 48 hrs before the rats were sacrificed to avoid the acute effects of exercise.

2.3 Tissue Collection

Rats were weighed and anaesthetized with sodium-pentobarbitol. Percent body fat (%BF) was determined via dual energy x-ray absorptiometry (DEXA). Hearts were removed and trimmed of excess fat and vessels. The hearts were then weighed and mid-belly cross sections were taken from the heart for histochemistry, mounted on cork, and snap-frozen in liquid nitrogen cooled isopentane to avoid the formation of gaseous nitrogen around the specimen and resulting slower heat transfer that occurs in direct liquid nitrogen snap-freezing. Samples were stored at -80°C until sectioned for staining.

The remainder of the heart tissue was snap-frozen in liquid nitrogen and then ground into a powder using a liquid nitrogen cooled ceramic mortar and pestle and stored at -80°C.

2.4 Van Geison's Stain for Collagen

To stain for collagen, 10 μm thick cross-sections were sliced using a Microme HM 550 MVP cryostat at -20°C from the mid-belly of the heart and put onto glass slides which were kept frozen at -80°C until use. Before staining, slides were allowed to air-dry at room temperature for less than 10 minutes until the moisture on the slides had evaporated. Tissue sections were stained with Van Geison's stain as described by Bancroft (1982). Slides were immersed in working Weigert's iron-haematoxylin solution (half solution containing 5g Haematoxylin in 500 mL 95% ethanol, other half solution containing 5.8g ferric chloride ($FeCl_3 \bullet 6H_2O$) and 5 mL HCL in 495 mL distilled water) for 3 minutes. Slides were then rinsed in tap water for 1 minute to promote blueing of the nuclei before immersing in Van Geison's staining solution (0.5 g acid fuchsine in 500 mL of saturated aqueous picric acid) for 2 minutes. Slides were then briefly washed in two changes of acid-alcohol (140 mL of 95% ethanol and 1 mL HCl in 59 mL of distilled water), and then dehydrated in 3 changes of 100% alcohol (Bancroft & Stevens, 1982). Slides were then allowed to dry and were mounted with a coverslip. 100x images were taken with a Nikon Coolpix 990 digital camera mounted on a Nikon Eclipse E400 stage (Nikon, Mississauga, ON) at the anterior, lateral, and posterior portions of the subendocardium of the free wall of the LV (see Figure 2). Analysis was done by overlaying a 100-point grid on each image and

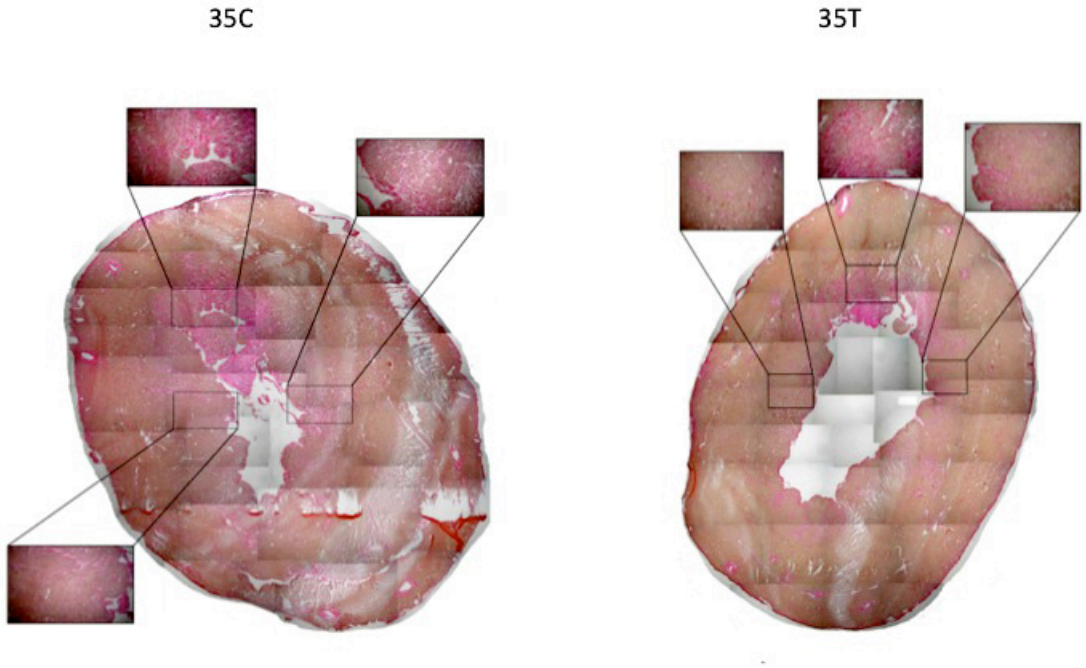

Figure 2: Cross-section of a 35C (left) and 35T (right) heart. Highlighted regions on each sample (clockwise, starting from lower-left) are posterior, lateral, and anterior regions. Image was modified by enhancing contrast and saturation of colours to highlight the collagen.

counting which points fell on cardiomyocyte, collagen, nothing, or other (see Figure 3). The relative proportions of each tissue type were calculated.

2.5 RNA Isolation

RNA from the powdered heart samples was extracted using TRIzol® (Invitrogen, Carlsbad, CA). Approximately 90 mg of the powdered samples were weighed out and homogenized with 1 mL of TRIzol®. Samples were allowed to sit at room temperature for 5 minutes before 200 µL of chloroform (Sigma-Aldrich, St. Louis, MO) was added to samples which were immediately shaken for 15 seconds. Samples were then allowed to rest for 2 minutes before being centrifuged at 14 000 rpm for 15 minutes at 4°C. The clear supernatant was pipetted into fresh tubes where 1 mL of isopropanol was added and to each sample and incorporated via inversion. Samples were then allowed to rest for 10 minutes at room temperature before being centrifuged again at 14 000 rpm for 15 minutes at 4°C. Centrifugation produced a small pellet of RNA. The supernatant was carefully poured out of the tubes and 1 mL of 75% ethanol was added to wash the RNA. Tubes were gently shaken to dislodge the pellet from the wall of the tube. Samples were then centrifuged at 14 000 rpm for 5 minutes at 4°C, after which the ethanol supernatant was carefully poured out of the tubes and tubes were allowed to air-dry for up to 10 minutes, to remove the remaining ethanol without permitting the pellet to go completely dry. The pellet was then re-dissolved in 100 µL of UltraPure™ DNase/RNase-Free Distilled Water (Invitrogen, Carlsbad, CA) by vortexing.

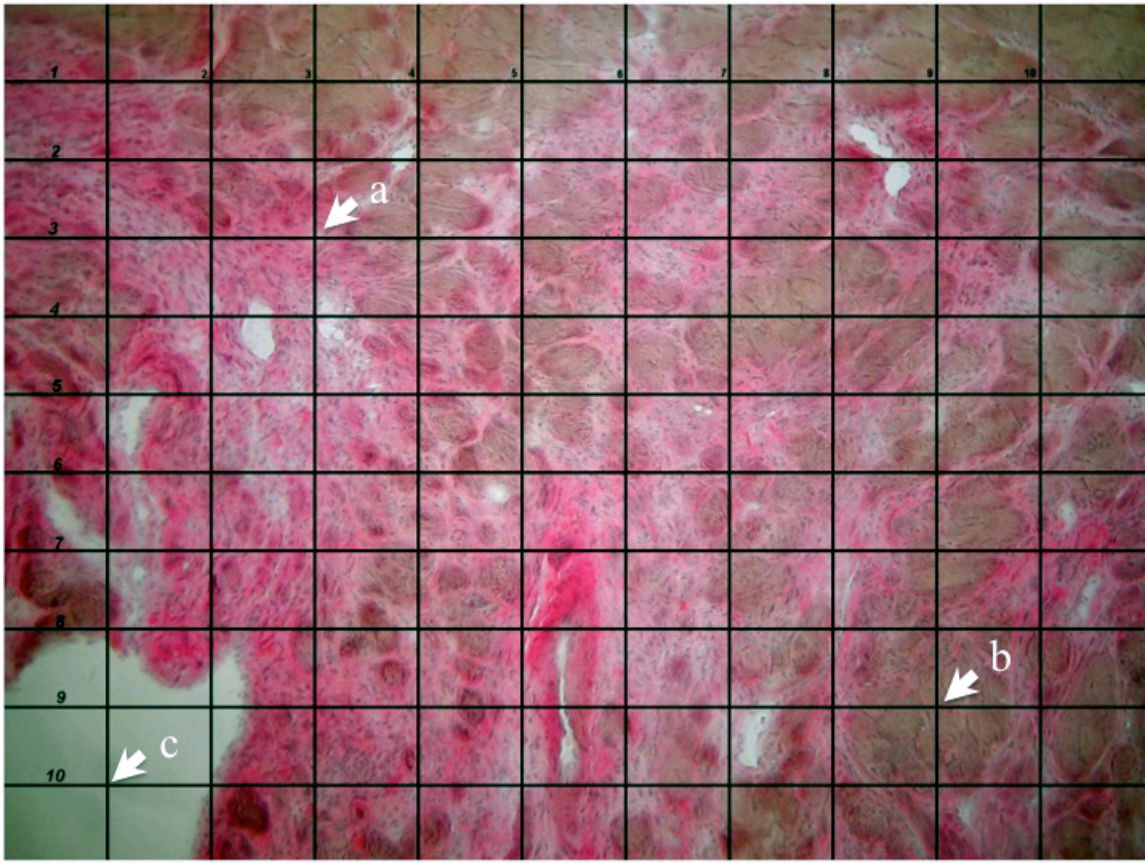

Figure 3: 200x image of lateral region of Van Geison's stain for collagen, overlaid with grid used for analysis. 'a' denotes collagen, 'b' denotes cardiomyocyte, and 'c' denotes empty space. Image was modified by enhancing contrast and saturation of colours to highlight the collagen.

2.6 Quality of RNA

The isolated RNA was assessed for quality through gel electrophoresis and subsequent imaging. The agarose gel was made by mixing 50 mL 1x TBE buffer and 0.5 g agarose together and microwaving the solution for approximately 30 seconds until the agarose was completely dissolved. The mixture was allowed to cool to slightly above room temperature to be able to handle and to prevent the gel from hardening before 2.5 µL of SYBR®Safe DNA gel stain (10 000x concentrate in DMSO) was added. The solution was then poured into the gel mold apparatus and let set until solidified. The gel was then removed from the molding apparatus and set in an electrophoresis tank. 1x TBE buffer was poured into the tank until the gel was covered. For each sample, 3 µL loading dye and 10 µL 1:10 RNA in UltraPure™ DNase⁄RNase-Free Distilled Water (Invitrogen, Carlsbad, CA) were combined. 10 µL of the resulting solution was carefully pipetted into a well in the gel. The electrophoresis lid was then placed on the tank and run for 30 minutes at 100V followed by 50V for 45 minutes. The gel was removed from the electrophoresis tank and images were taken using ChemiGenius^2 Bio-Imaging System (Syngene, Frederick, MD) in conjunction with the software program GeneSnap 6.05 (Syngene, Frederick, MD). Images were taken under EtBr/UV transillumination and quality of the RNA was assessed by qualitatively observing the presence of clear bands with a solid rectangular shape (see Figure 4).

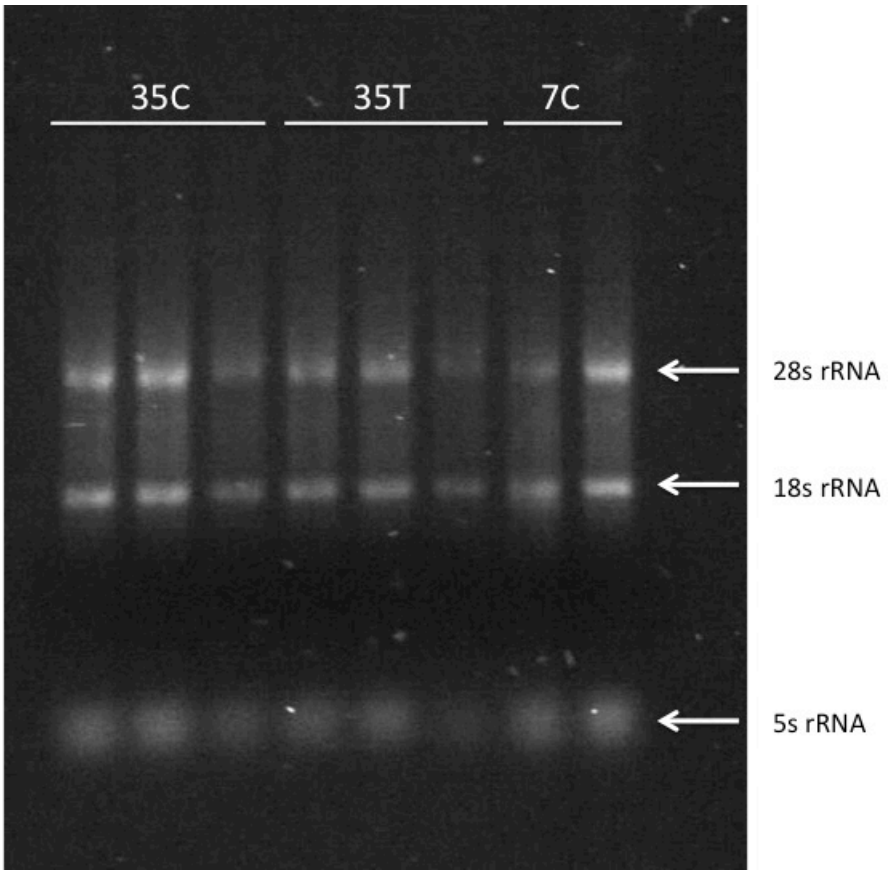

Figure 4: Image of an agarose gel containing samples from each group. Bands of interest are highlighted with arrows.

2.7 Quantity of RNA

The quantity of RNA was measured using the Quant-iT™ RiboGreen® RNA Assay Kit (Invitrogen, Carlsbad, CA) on a 96-well micro-reader plate. Ribosomal RNA standard (100 µg/mL in Tris-EDTA (TE) buffer, 10 mM Tris-HCl, 1 mM EDTA, ph 7.5 in DEPC-treated water) (Invitrogen, Carlsbad, CA) was diluted with 1x TE to a 1:100 dilution. Sequential volumes of 0 µL, 1 µL, 5 µL, 25 µL, 50 µL, and 100 µL of the diluted RNA standard was pipetted into wells along with 100 µL, 99 µL, 95 µL, 75 µL, 50 µL, and 0 µL respectively of 1x TE was added to create a standard curve. In non-standard curve wells, 2 µL of 1:10 diluted RNA samples along with 98 µL 1x TE were added to each well in duplicate. 1x TE was added to the Quant-iT™ RiboGreen® RNA Reagent to yield a 1:500 dilution with a final volume enough for 100 µL per well for both the standard and sample wells. 100 µL of the diluted Quant-iT™ RiboGreen® RNA Reagent was added to all wells and the plate was allowed to rest for 2 minutes at room temperature protected from light. A FL_X-800 Microplate Fluorescence Reader (Bio-Tek Instruments, Inc., Winooski, VT) was used in conjunction with the software program KC4™ Kineticalc 3.03 (Bio-Tek Instruments, Inc., Winooski, VT) to read the fluorescence of the samples and standard at wavelengths between 485 nm and 530 nm. The quantity of RNA in each sample (ng/uL) was determined against the standard curve.

2.8 Reverse Transcription of RNA

Reverse transcription was then performed on the RNA samples to transcribe the RNA back into DNA fragments (cDNA) using SuperScript® First-Strand Synthesis

System for RT-PCR (Invitrogen, Carlsbad, CA). For each sample, a volume of UltraPure™ DNase⁄RNase-Free Distilled Water (Invitrogen, Carlsbad, CA) and the RNA sample were combined to yield a final volume of 8 µL and a final concentration of 1 µg RNA per µL. 1 µL of each dNTP and Oligo dt were added to the mixture, and the resulting solution was incubated for 5 minutes at 65°C, and then for 2 minutes on ice. 9 µL of reaction mix was then added to each tube, which consisted of 2 µL of 10x RT buffer, 4µL of 25mM $MgCl_2$, 2 µL of 0.1 M DTT, and 1 µL of 40 U/µL RNAse Out. Samples were then incubated for 2 minutes at 42°C, after which 1 µL of 50 U/µL Superscript II RT was added to each sample. Samples were then placed in an Eppendorf Mastercycler® Gradient Thermal Cycler and incubated for 50 minutes at 42°C and then for 15 minutes at 70°C. Samples were then held at 4°C until removal at which time 180 µL of UltraPure™ DNase⁄RNase-Free Distilled Water (Invitrogen, Carlsbad, CA) was added to each sample to make a 1:10 RT dilution. Samples were stored at a temperature of -30°C or less until use.

2.9 Quantitative RT-PCR

PCR was performed on the cDNA using the following primers from University Core DNA Services (University of Calgary, AB) and QIAGEN (Germantown, MD): collagen type 1 (Col1a1), collagen type III (Col3), transforming growth factor beta (TGFβ1), matrix metalloproteinase 1 (MMP1), matrix metalloproteinase 2 (MMP2), matrix metalloproteinase 9 (MMP9), tissue inhibitor of matrix metalloproteinase 1 (TIMP1), tissue inhibitor of matrix metalloproteinase 4 (TIMP4), atrial natriuretic factor

(ANF), angiotensin receptor 1 (Angtr1), angiotensin converting enzyme (ACE), lysyl oxidase (Lox), lysyl oxidase-like 3 (Loxl3), and interferon regulatory factor 1 (Irf1). Housekeeping genes used were 18s RNA, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), β-Actin, and hypoxanthine-guanine phosphoribosyltransferase (HRPT) (University Core DNA Services (University of Calgary) and QIAGEN (Germantown, MD)) (see Table 1). Primer pairs were designed using Primer3plus (A. Untergasser et al. 2007. Nucl. Acids Res. 35(Web Server issue): W71-W74) to create a PCR product of approximately 200bp. Primer pairs were "BLASTED" (Basic Local Alignment Search Tool) against the rat gene sequence bank hosted by the NIH:NCBI to check for sequence specificity to the target gene. Primers were diluted to obtain a 10 μM solution. Equal volumes of both the forward and reverse primers were combined and stored at -80°C until use. QuantiTect SYBR Green PCR Master Mix and diluted primer of interest were combined in a 5:1 ratio to get a final volume sufficient for 15 μL per sample. UltraPure™ DNase/RNase-Free Distilled Water (Invitrogen, Carlsbad, CA) was added to each cDNA sample to yield a 1:10 dilution, with a final volume sufficient for 10 μL per well with sample done in duplicate for both the housekeeping gene and gene of interest. Into each well of a 96-well iCycler iQ™ PCR plate (Bio-Rad, Mississauga, ON) (0.2 mL Certified, DNase & RNase Free), 15 μL of housekeeping gene primer in QuantiTect SYBR Green PCR Master Mix solution was pipetted into one-half of the plate, and 15 μL of gene of interest primer in QuantiTect SYBR Green PCR Master Mix solution was pipetted into the other half of the plate, which was enough for 23 samples and one negative control in duplicate for each housekeeping gene and gene of interest. For each sample, 10 μL of diluted cDNA was pipetted into a well in duplicate and was mixed by pipetting for both

Table 1: Forward and reverse primers designed by Primer3plus and synthesized by University Core DNA Services (University of Calgary)

Gene	Forward	Reverse
Col1a1	tgctgccttttctgttcctt	aaggtgctgggtagggaagt
TGFB1	atacgcctgagtggctgtct	tgggactgatcccattgatt
MMP1	acagtttccccgtgtttcag	cccacacctaggtttcctca
MMP2	agctcccggaaaagattgat	tccagttaaaggcagcgtct
MMP9	ccaccgagctatccactcat	gtccggtttcagcatgtttt
TIMP4	acctccggaaggagtacgtt	tgacaggttgtgagctggag
ANF	gggggtaggattgacaggat	ctccaggagggtattcacca
Angtr1	accaggtcaagtggatttcg	atcaccaccaagctgtttcc
ACE	gagccatccttccctttttc	ggctgcagctcctggtatag
Lox	gctgtgagacgagagggaac	ccatgtcccacaggagaaat
Loxl3	caggaccaggactctgcttc	ccatactggagctgcacaga
Irf1	cgacgaaggagtaggacgag	tgcatctctagccagggtct
HRPT	gcagactttgctttccttgg	ccgctgtcttttaggctttg

* Col3, TIMP1, B-actin, GAPDH and 18s RNA designed and supplied by QIAGEN

the housekeeping gene and the gene of interest. The same was done for the negative control, with 10 µL of RNase-free water added in duplicate. iCycler iQ™ Optical Tape (Bio-Rad, Mississauga, ON) was then applied to the plate to seal each well and the plate was placed into an iCycler (version 2.039, Bio-Rad) for measurement. The plate was analyzed using iCycler™iQ Optical System Software version 3.0a with a protocol of 15 minutes at 95.0°C followed by 50 cycles of 15 seconds at 94.0°C, 30 seconds at 52.8°C, and 30 seconds at 72.0°C. The plate then underwent 1 minute at 95.0°C, followed by 100 cycles of 10 seconds of decreasing temperature increments starting at 95.0°C and decreasing by 0.5°C per cycle. The plate was then held at 12.0°C until removal. RNA expression levels were calculated using the delta-delta-CT method and measured against the housekeeping gene.

2.10 Immunohistochemistry for Advanced Glycation Endproducts (AGEs)

7µm thick cross-sections were sliced at the mid-belly of heart and put onto glass slides which were kept frozen at -80°C until use. Before staining, slides were allowed to air-dry at room temperature for less than 10 minutes until the moisture on the slides had evaporated. Slides were then fixed in acetone (Sigma, St. Louis, MO) at 4°C for 10 minutes then allowed to air-dry for less than 10 minutes until all residual liquid had evaporated. Slides were then washed in 1x PBS solution for 5 minutes at room temperature, and then placed in the permeabilization solution (0.1% Triton® X-100, Sigma, St. Louis, MO; in 1x PBS) for 15 minutes at room temperature while rocking

slowly. Slides were then washed in 3 changes of 1x PBS (8 g NaCl, 2 g KCl, 2.16 g $Na_2HPO_4 \bullet 7H_2O$, 0.2 g KH_2PO_4, in 1 L of dH_2O, pH 7.4) for 5 minutes in each wash at room temperature. Slides were removed from solution, excess moisture was carefully dried from the slide using a Kimwipe and care was taken to not touch the tissue, then a circle was drawn around the tissue using a PAP pen to provide a hydrophobic barrier to solutions applied to the tissue on the slide when laid flat. Blocking solution (10% goat serum, Sigma, St. Louis, MO; 1% BSA Sigma, St. Louis, MO; in 1x PBS) was then pipetted onto the tissue and incubated at room temperature for 30 minutes, after which the solution was aspirated off of the tissue. Primary antibody (AGE antibody, ab23722, Abcam, MA) at a 1:200 dilution in blocking solution was then pipetted onto the tissue and incubated overnight at 4°C. A negative control slide was added which had no primary antibody and was incubated with blocking solution overnight at 4°C to check for non-specific staining of the secondary antibody. The following day the primary antibody solution was aspirated off of the tissue and 3 changes of 1x PBS was pipetted on to the tissue for 5 minutes in each wash at room temperature. Blocking solution was then pipetted onto the tissue and incubated at room temperature for 30 minutes, after which the solution was aspirated off of the tissue. Secondary antibody (Alexa Fluor® 488 goat anti-rabbit IgG (H+L), Invitrogen, Carlsbad, CA) at a 1:1000 dilution in blocking solution was then pipetted onto the tissue and incubated at room temperature in the dark for 60 minutes. From this point onwards, slides were kept in the dark as the secondary antibody and remaining labeling chemicals were light-sensitive. The secondary antibody solution was then aspirated off of the tissue and 2 changes of 1x PBS was pipetted on to the tissue for 5 minutes in each wash at room temperature. DAPI (300 nM in 1x PBS,

Invitrogen, Carlsbad, CA) was then pipetted onto the tissue and incubated for 5 minutes at room temperature, then aspirated off of the tissue and 3 changes of 1x PBS was pipetted on to the tissue for 5 minutes in each wash at room temperature. Wheat germ agglutinin, Alexa Fluor® 594 conjugate (Invitrogen, Carlsbad, CA) 1:200 diluted in 1x PBS was then pipetted onto the tissue and incubated for 10 minutes at room temperature, then aspirated off of the tissue and 3 changes of 1x PBS was pipetted on to the tissue for 5 minutes in each wash at room temperature. Slides were then carefully dried using a Kimwipe and care was taken to not touch the tissue, and a coverslip was applied to each slide over the tissue mounted with ProLong® Gold antifade reagent (Invitrogen, Carlsbad, CA).

Images were taken of the anterior, lateral, and posterior regions of the subendocardium and myocardium of the free wall of the LV with an Arcturus^{XT}™ Laser Capture Microdissection System (Life Technologies Corporation, Carlsbad, CA) at a 100x magnification. An image of each WGA and AGE signals were taken at each location (see Figure 5). Images were analyzed using ImageJ 1.44p (NIH, USA) software. Briefly, for each given region, the image stained for WGA that identified the ECM was converted from colour to a black and white image. The threshold was adjusted to clearly identify the cardiomyocytes. This image was then inverted so that the back regions represented the cardiomyocytes and the white regions represented the ECM. The positions of the cardiomyocytes were recorded by the software, and a mask was created of the positions. This mask was overlaid onto the image stained for AGEs, so that only the ECM portion was analyzed for AGE fluorescence (intensity of colour signal).

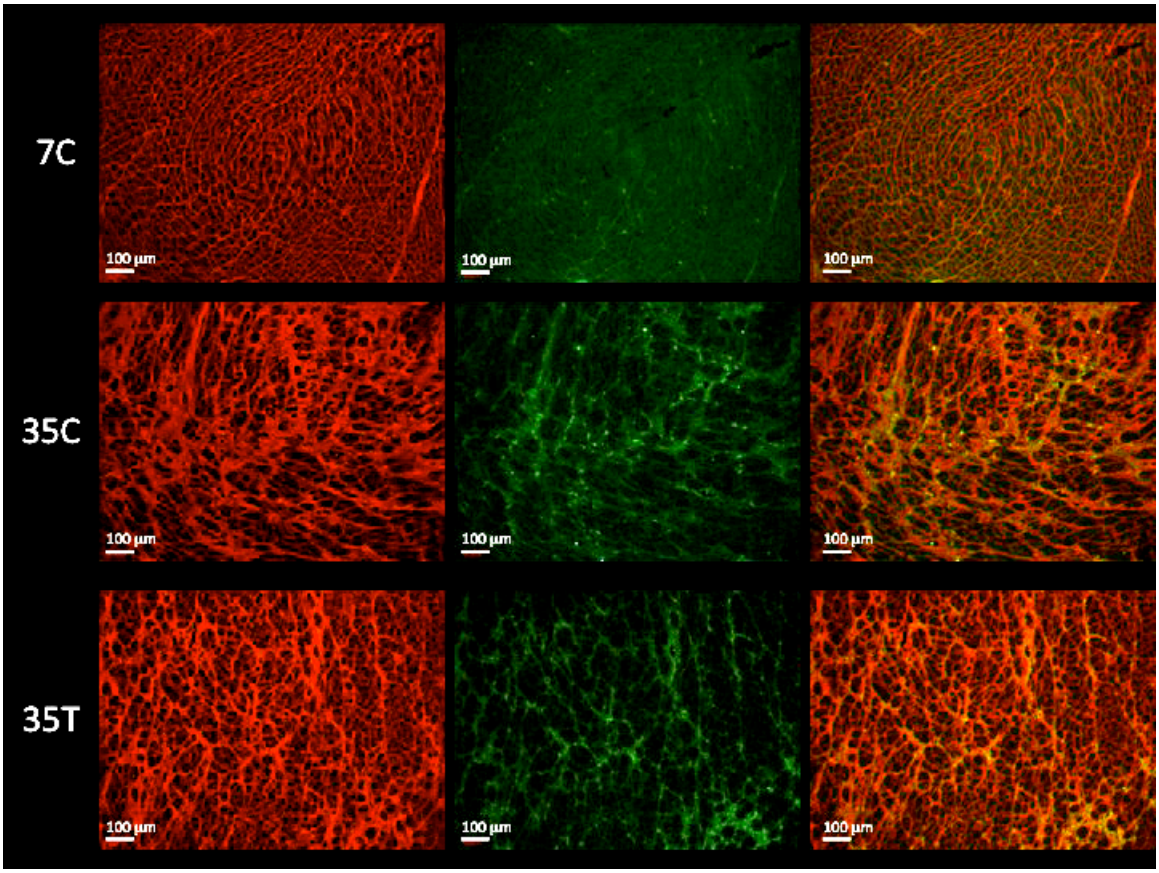

Figure 5: A Sample image each from 7C, 35T, and 35C groups. First column is WGA signal, second column is AGE signal, third column is a merged image of the first and second columns. Image was modified by increasing overall brightness to highlight the signaling.

2.11 Bradfords/EnzChek

MMP activity was measured using EnzChek® Gelatinase/Collagenase Assay Kit (Molecular Probes®, Invitrogen, Carlsbad, CA). Samples were homogenized in a buffer containing 100 mM Tris (hydroxymethyl) aminomethane (OmniPur, EMD Chemicals Inc., Gibbstown, NJ); 200 mM NaCl (Fisher Scientific, Fair Lawn, NJ); 0.1% Triton X-100 (BDH Inc., Toronto, ON); pH 7.4 (Cha & Purslow, 2010). Samples were centrifuged for 20 minutes at 10000g at 4°C, with the resulting supernatant aspirated off and stored. The protein content of a 1:10 dilution of the supernatant was determined in duplicate via a Bradford assay. 20 uL of the 1:10 diluted supernatant was added to 1 mL of Bradford reagent (100 mg Coomassie Brilliant Blue G-250 (Sigma-Aldrich, St. Louis, MO); 100 mL 85% phosphoric acid (Fisher Scientific, Toronto, ON); 50 mL 95% ethanol; to make a final volume of 1 L in double-distilled water and filtered), and was compared against a standard of BSA Fraction 5 (2.0 mg/mL in 0.9% NaCl containing sodium azide. Rockford, IL). Protein concentration was calculated as ug/uL.

The supernatant of the samples were used in the EnzChek® kit as per manufactures' instructions. Briefly, 40 uL of Reaction Buffer was added to each well of a Microfluor®2 plate, except for the negative control where 90 uL of Reaction Buffer was added. 10 uL of DQ™ gelatin was added to each well followed by 50 uL of sample supernatant, or Clostridium collagenase for the positive control. Samples, positive, and negative controls were all done in triplicate. The plate was left to incubate at room

temperature and protected from light. The plate was measured after 10 minutes, 2, 19, 24, 50, 72, 145.5, 168, 192, 240, and 312 hours in a VICTOR^{3}™ V Multilabel Counter model 1420 (PerkinElmer®, Waltham, MA) with the Wallac 1420 Workstation Version 3.00 Revision 3 (PerkinElmer®, Waltham, MA). Excitation was set at 485/14 nm and emission at 535/25 nm. Negative control values were subtracted from sample values to account for background fluorescence. Values were normalized to the amount of protein in each sample, and plotted against time to yield the rate of MMP activity.

2.12 Statistics

For analysis of MMP activity, a two-way Analysis of Variance (ANOVA) was used with group (7C, 35C, and 35T) and time (hours) as the two factors. For analysis of survival curves, a Kaplan-Meier Survival Analysis Log Rank test was used to compare the 35C and 35T groups. For all other tests, a one-way ANOVA was used to compare all three groups (7C, 35C and 35T), with a Holm-Sidak post hoc multiple comparison test. The significance level was set at 0.05 ($p < 0.05$). Values are expressed as means ± standard error of the mean. Where equal variance tests failed, the non-parametric Kruskal-Wallis ANOVA on Ranks test was employed using the Dunn's Test as the post-hoc if a significant difference was found. When only two groups were compared, a Students t-test was applied. Statistical analysis was done using SigmaPlot version 11 (Systat Software Inc. Chicago, IL).

Chapter Three: Results

3.1 Physiological Characteristics

There was a significantly greater rate of survival between 29 mo and 35 mo of age in 35T (75%) verses 35C animals (63%) as described by Betik et al. (2009) ($p<0.05$). This is reflected in Figure 6, where the training effect is seen by the shift in the survival curve of the 35T group to the right relative to their age-matched controls.

Body mass was significantly greater in 35C compared to 35T and 7C (see Table 2) ($p<0.05$). Body masses between 35T and 7C were not different. In addition to body mass, the %BF was significantly greater in 35C compared to 35T and 7C ($p<0.05$), with no difference between 35T and 7C groups. Figure 7 shows the changes in body mass of the 35T and 35C groups over the duration of the training program (Betik, 2010). The 35C groups' body masses had a slow rate of body mass decline while the 35T groups' body mass had a greater and more linear rate of decline.

The heart mass of 7C was significantly less than 35T, which was significantly less than 35C (see Table 2) ($p>0.05$).

Qualitatively, the 35T animals appeared healthier as reflected in the quality of their coats, which were sleeker and shinier, and shed less easily than the 35C animals.

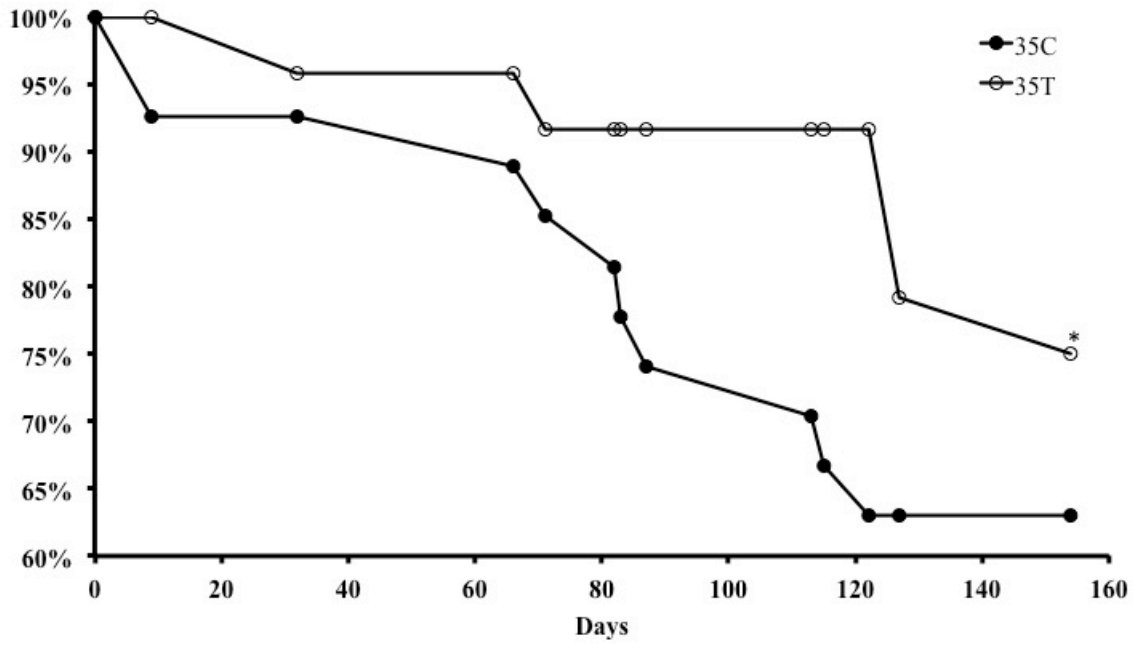

Figure 6: Percentage of aged animals surviving in each group. Day zero indicates the beginning of the experiment when animals were 29 mo of age. Asterisk denotes significant difference in 35T compared to 35C ($p<0.05$).

Table 2: Mean body mass, percent body fat, and heart mass of 7C, 35T, and 35C groups. Values are ± SEM

	YA	SEN-TR	SEN-SED
Body Mass (g)	435 ± 6.9	437 ± 11.8	514 ± 11.9 *
% BF	14 ± 0.5	15 ± 1.4	25 ± 0.9 *
Heart mass (ug)	909 ± 28.2 $^{\Psi}$	1150 ± 26.0 $^{\sharp}$	1260 ± 34.1 *

Values are ± SEM

* denotes significant difference between 35C and other groups

$^{\sharp}$ denotes significant difference between 35T and other groups

$^{\Psi}$ denotes significant difference between 7C and other groups

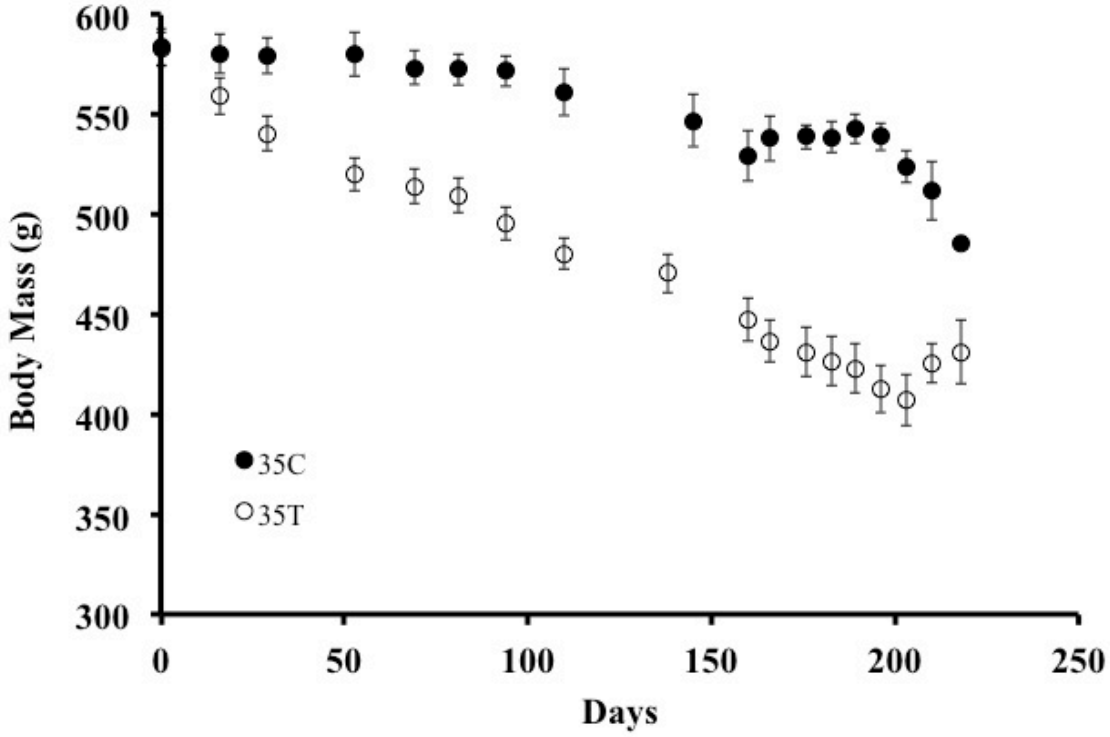

Figure 7: Mean body masses of each group. Day zero indicates the beginning of the experiment when animals were 29 mo of age. Data points are ± SEM.

3.2 Collagen Quantity

Point counting in tissue cross-sections revealed a significantly greater percent of connective tissue as determined by Van Geison's staining of all three regions (anterior, lateral, and posterior) combined in hearts of the sedentary control animals 55 ± 4% versus 5 ± 1% in 35C versus 7C, respectively (see Figure 8). This increased connective tissue content with aging was attenuated by regular endurance exercise to 43 ± 5% in 35T. This benefit of exercise was greatest for the anterior region where there was 40% less connective tissue in the 35T group than the 35C group ($p<0.05$).

3.3 Immunohistochemistry

35T animals had significantly less AGE fluorescence than 35C animals in all regions of the myocardium analyzed, combined (see Figure 9) ($p<0.05$). There were no differences between the aged groups in any one specific region of the free wall of the LV. Significance occurred when observing all three regions of the myocardium combined. Because the amount of AGEs is proportional to the amount of cross-linking in the ECM, a higher amount of AGEs corresponds to a higher amount of cross-linking, and a lower amount of AGEs corresponds to a lower amount of cross-linking. Therefore the 35T animals had less cross-linking than their sedentary counterparts.

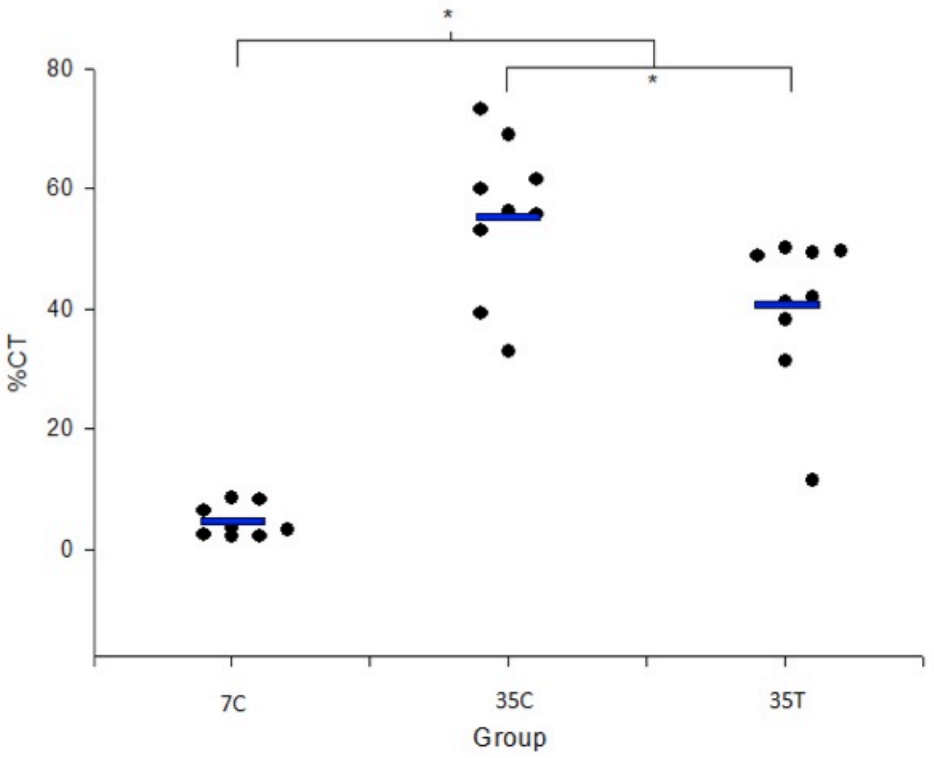

Figure 8: Mean percent connective tissue of combined anterior, lateral, and posterior regions of free wall of left ventricle. Dots denote individual animal values, bar denotes mean of group.

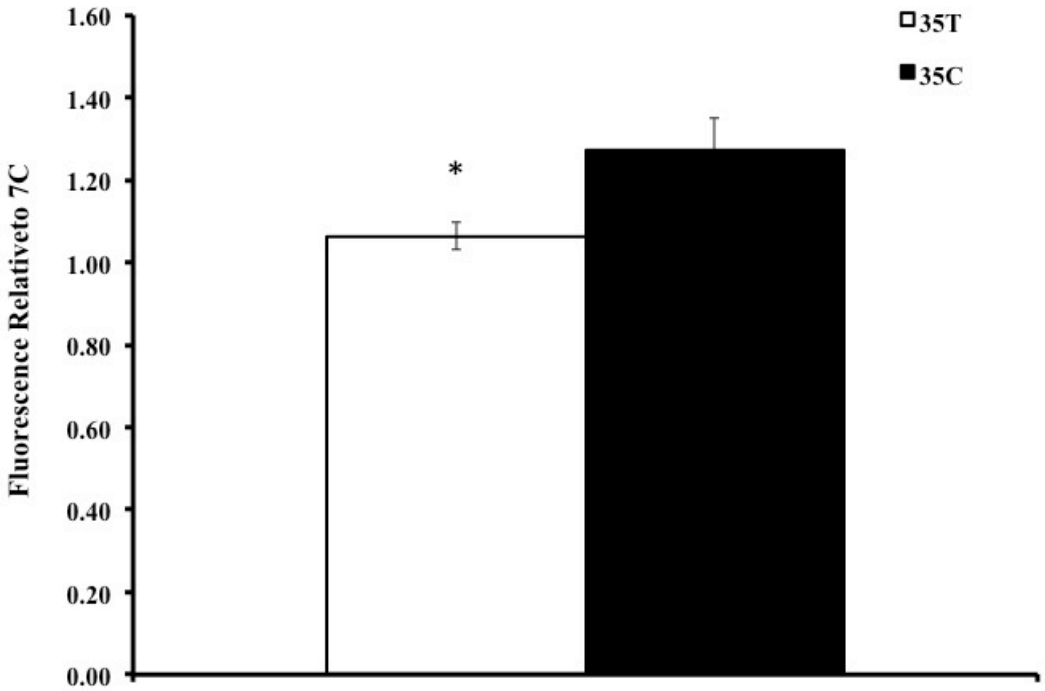

Figure 9: AGE fluorescence of 35T and 35C expressed relative to the 7C group. Asterisk denotes significant difference between 35T and 35C groups ($p<0.05$).

Qualitatively, the 35C animals' myocardium was observed to be adversely altered in that the ECM appeared more web-like with increased space between myocytes that were comprised of thick collagen "veins", as well as the myocardium appearing very disorganized as compared to that of the 7C animals (see Figure 5). The 35T animals exhibited an ECM pattern that more closely resembled that of the 7C animals. The myocardium appeared more organized and less web-like than that of the 35C animals.

3.4 MMP Activity

The rate of MMP activity was significantly higher in both aged groups compared to the 7C group ($p<0.05$). There was no difference in the rate of MMP activity between 35T and 35C (see Figure 10).

3.5 Gene Expression

TIMP1 gene expression was significantly greater in both aged groups compared to 7C ($p<0.05$) (see Figure 11). ACE gene expression trended to be higher in old groups compared to 7C ($p=0.06$-0.09). There were no other significant differences found for genes associated with RAAS (i.e. Angtr1), enzymatic cross-linking (i.e. Lox, Loxl3, and Irf1), ANF, or SERCA2a (see Figure 11).

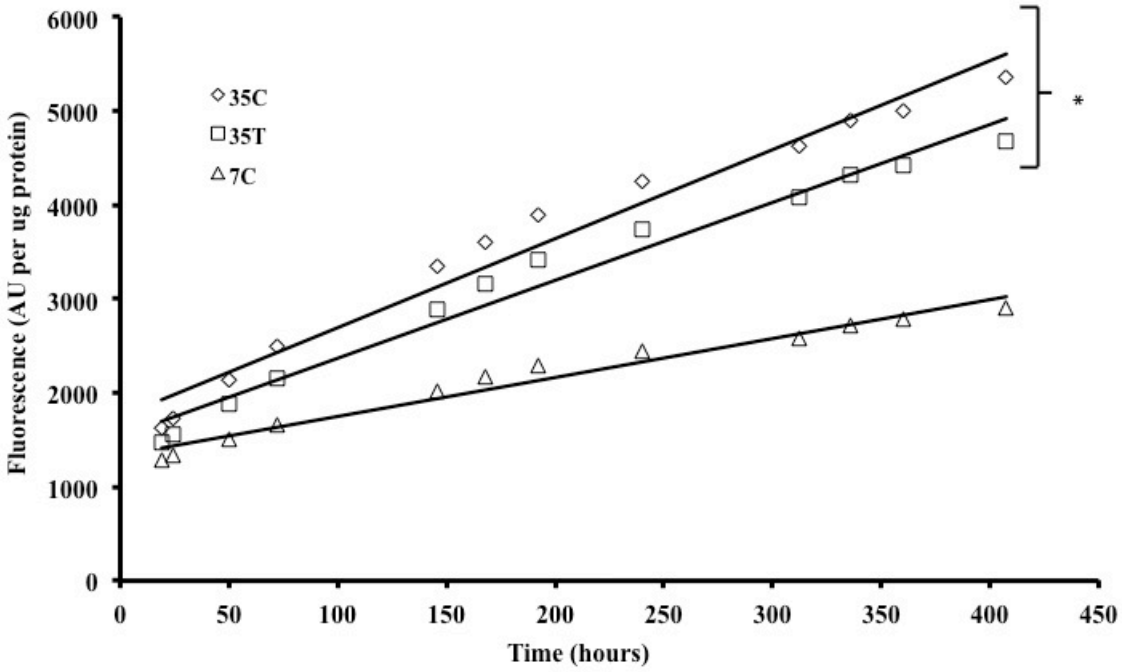

Figure 10: MMP activity of three groups. Asterisk denotes significant difference between aged groups and 7C ($p<0.05$).

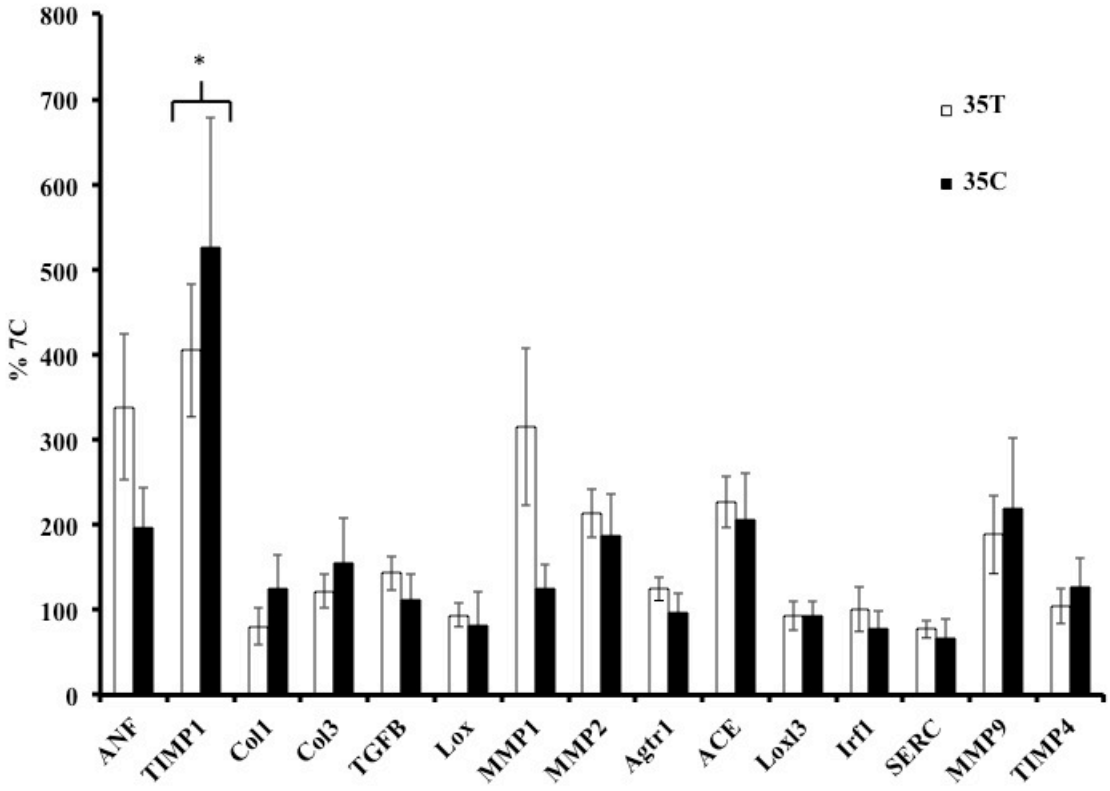

Figure 11: Gene expression of 35T and 35C expressed as a percentage of 7C group. Asterisk denotes significant difference between aged groups and 7C ($p<0.05$).

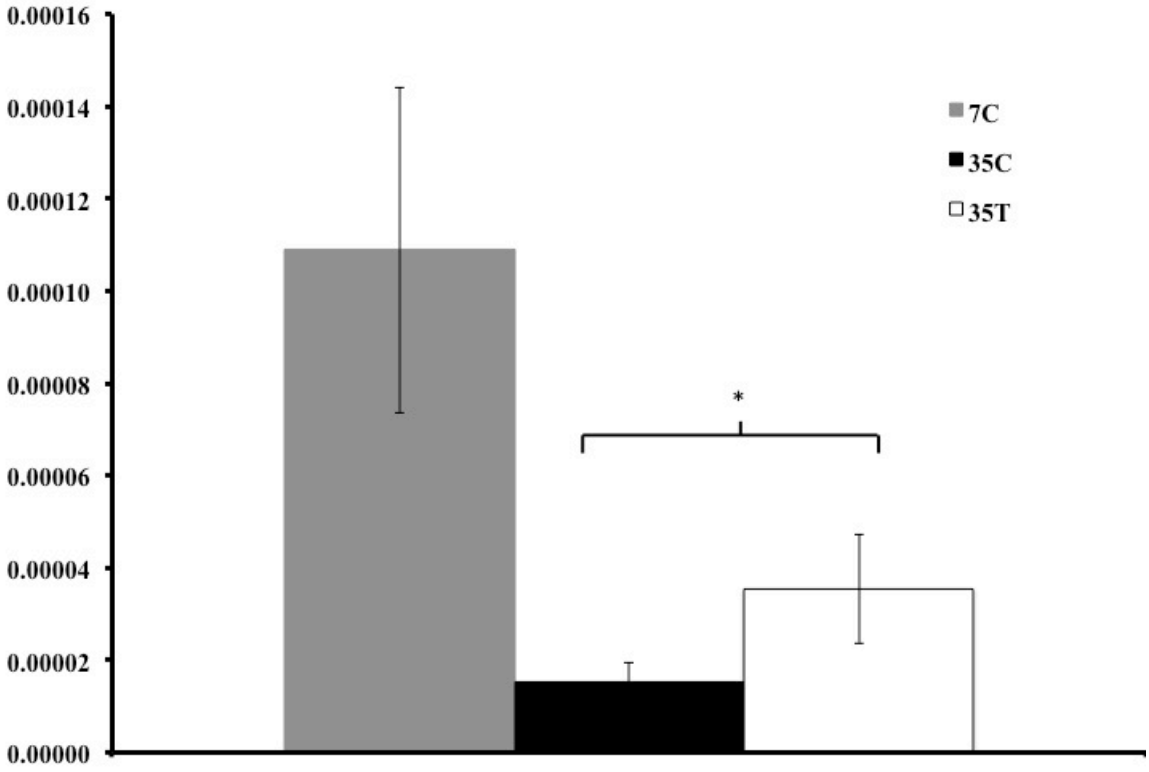

Figure 12: Ratio of gene expression of MMP1 to TIMP1. Asterisk denotes significant difference between aged groups and 7C ($p<0.05$).

The ratio between MMP1 and TIMP1 gene expression was significantly higher in 7C compared to aged groups ($p<0.05$), with 35T animals trending to have a greater ratio than 35C animals ($p=0.13$) (see Figure 12). In addition, MMP2 gene expression trended to be higher in the aged groups compared to 7C ($p=0.05$-0.08). When expressed relative to 7C gene expression, 35T MMP1 expression trended to be higher than 35C MMP1 expression ($p=0.09$) (see Figure 11, Figure 13). There were no other significant differences found for genes associated with collagen degradation (i.e. MMP9, TIMP4).

The ratio between Col1 and Col3 gene expression trended to be lower in 35C animals compared to other groups ($p=0.07$) (see Figure 14). There were no other significant differences found for genes associated with collagen synthesis (i.e. TGFβ1).

The non-parametric test yielded results that were in agreement with parametric tests.

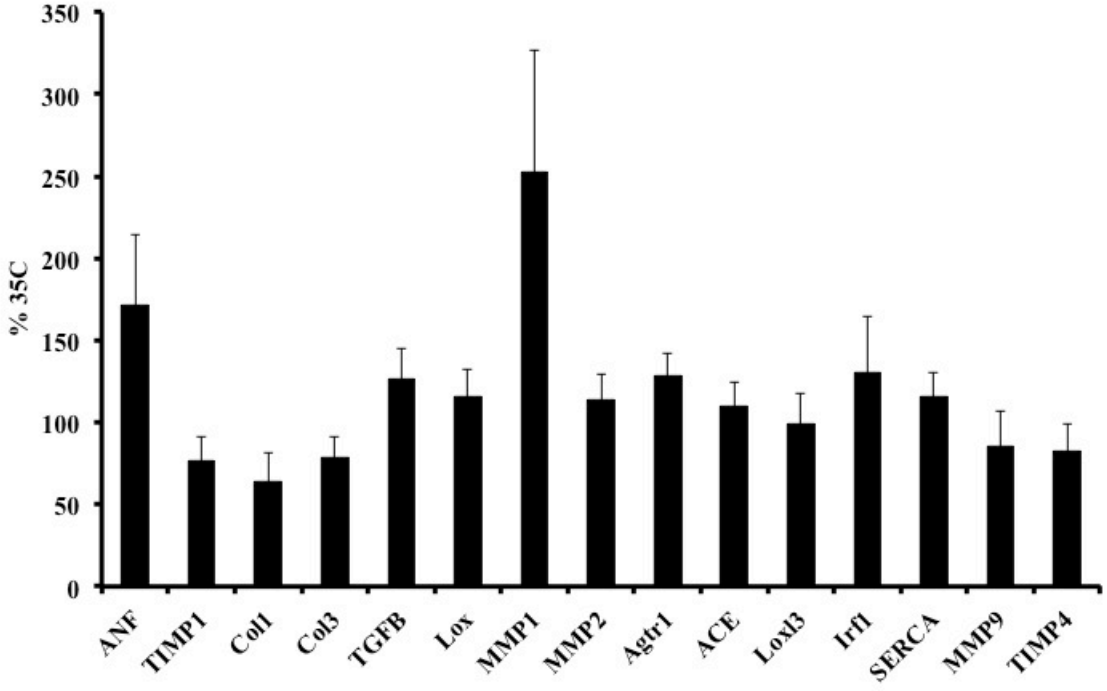

Figure 13: Gene expression of 35T expressed as a percentage of 35C group.

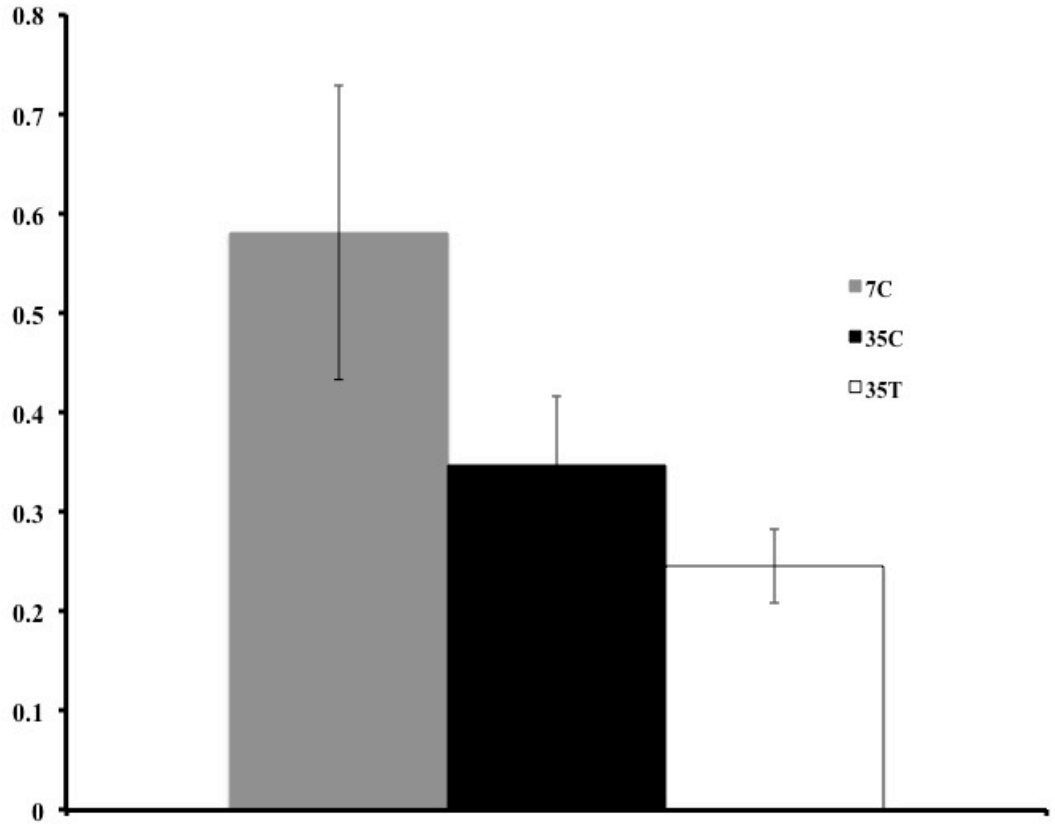

Figure 14: Ratio of gene expression of Col1 to Col3.

Chapter Four: Discussion

4.1 Aging

The Biological Theory of aging postulates that aging is a natural phenomenon distinct from the Medical Theory of aging, which views aging and death as a consequence of disease. In general, pathological disease states cause a relatively sudden and sizeable change in homeostasis, which triggers an equally large response from the body in an attempt to counteract the pathology and restore homeostasis. From this perspective, aging in general (and specifically the heart) cannot be considered a disease as there is no sudden insult to the body or heart as seen in pathological disease states. Aging itself appears to be the result of a compounded accumulation of small changes to the body over a prolonged period of time. Therefore while studying disease states, one would be able to detect significantly large changes in the body when taking "snapshot" measurements, or one-time only measurements. As a result, relatively small changes in gene expression, which when measured alone may not appear significant, may in fact yield significant consequences when applied over a sufficient period of time. Because aging is a slow process as a result of small changes over a prolonged period of time, detecting these small changes that yield significant changes with age are less likely to be measurable with "snapshot" measurements, as this kind of measurement does not have sufficient resolution to detect these more subtle changes.

4.2 Physiological Characteristics

As reported previously (Betik et al., 2009; Betik, 2010; M. M. Thomas, Vigna, Betik, Tupling, & Hepple, 2011; M. M. Thomas, Khan, Betik, Wright, & Hepple, 2010), in the current study, the body masses of the 35C animals were significantly greater than the 7C, but the body masses of the 35T animals were not different from the 7C animals (see Table 2). This indicates that the 35T animals experienced a training effect. The intensity of the training was sufficient to increase their caloric expenditure despite access to an *ad libitum* diet, so that they maintained a lower body weight with age than their sedentary age-matched counterparts.

The average heart mass of the 35C group was greater than that of the 35T group, both of which exhibited a greater heart mass than that of the younger 7C group. This indicates that cardiac hypertrophy occurred in the aged groups, while exercise attenuated this effect in the endurance exercise trained group. Because body masses differed significantly between the aged groups, calculated heart-to-body mass ratios would be skewed in favour of showing an exaggerated hypertrophied heart in the 35T group due to their lower body mass as compared to the 35C group. Although no tibial length measurements were obtained to provide a growth constant from which to measure relative changes in heart masses at the time of sacrifice, it is safe to assume that the tibial lengths would not be affected by aging or training status (LaMothe, Hepple, & Zernicke, 2003; Yin, Spurgeon, Rakusan, Weisfeldt, & Lakatta, 1982). Similarly, all groups used in this study had obtained adulthood and thus absolute heart masses could be appropriate

alternatives instead of relative changes in heart mass to a measured constant. From these observations, it can be inferred that although both aged groups experienced cardiac hypertrophy, the 35C group experienced hypertrophy to a significantly greater extent than the 35T group. Thus a training effect can be seen with the 35T group, with training attenuating the degree of cardiac hypertrophy as compared to the 35C group. However, the form of cardiac hypertrophy (pathological versus physiological) is unknown and the extent of each type of hypertrophy is also unknown. McMullan and Jennings (2007) identified that physiological hypertrophy in response to exercise training stimuli differs in its structural and molecular profile to pathological hypertrophy. Pathological hypertrophy usually presents at the cellular level, an upregulation of fetal genes; at the tissue level, fibrosis; at the organ level, cardiac dysfunction; and at the organism level, increased mortality. In contrast, physiological hypertrophy is associated with a normal organization of the myocardium in conjunction with a normal or enhanced cardiac function (McMullen & Jennings, 2007). With this in mind and with the current data, the 35C group fits the profile of pathological hypertrophy, with the highest quantity of fibrosis coupled with a high degree of disorganization of the myocardium, and a lower survival rate compared to the 35T group. The 35T group tended to fit the physiological hypertrophy profile with having a noticeably more normal and organized myocardium. Their significantly increased survival rate in comparison to the 35C group could be due to a more normal cardiac function.

Although the current understandings on the signaling pathways leading to both physiological and pathological hypertrophy are incomplete, a signaling pathway for each

has been proposed (McMullen & Jennings, 2007). Briefly, endurance exercise training stimulates growth factors such as IGF-1, which acts through PI3-K (p110α) and then Akt and further downstream factors to signal physiological hypertrophy. Pathological stimulus such as AngII and ET-1 activate the G-protein-coupled receptor (GPCR) leading to the dissociation of Gαq and activation of further downstream factors to signal pathological hypertrophy. Interestingly, it has been shown that the IGF-PI3-K pathway has been reported to play an important role in maintaining or improving function of the heart and reducing fibrosis in heart failure (Davis et al., 2006; McMullen et al., 2004; McMullen & Jennings, 2007; McMullen et al., 2007; Welch et al., 2002). It would be of interest to investigate if endurance exercise training exerts these beneficial effects on the aging heart through this pathway. In addition, it has been suggested that the IGF-PI3-K pathway may exert its' beneficial effect also by inhibiting pathological hypertrophy signaling molecules downstream of GPCR (DeBosch et al., 2006; McMullen & Jennings, 2007; McMullen et al., 2007). This is important as it suggests that the two hypertrophy processes can occur together and interact with each other. Thus the 35T group could have less pathological hypertrophy and more physiological hypertrophy due to the above mentioned pathways, and warrants further investigation.

4.3 Survival Rate

The 35T group had a significantly greater survival rate than their sedentary age-matched controls (see Figure 6). The shift in the survival curve of the 35T group to the right relative to their age-matched controls indicates that a greater percentage of the

endurance exercise trained animals reached advanced ages than the sedentary age-matched controls, most notably after 32 months of age. Thus a greater percentage of 35T animals than 35C animals attained and survived into biological senescence. This is in agreement with other studies demonstrating that voluntary exercise with rats running in wheels results in an increased rate of survival (Holloszy, Smith, Vining, & Adams, 1985; Holloszy & Schechtman, 1991; Holloszy, 1993). To the best of our knowledge, this is the first study to report survival rate in enforced treadmill endurance exercise trained rats. Although this increase in survival rate could be due to a number of different factors, with regard to the heart, the lesser survival of the 35C group compared to the 35T group could be due to a decrease in diastolic functioning that occurs with aging. With age, it has been shown that the early phase of LV filling during diastole decreases (Brenner et al., 2001; Palka, Lange, & Nihoyannopoulos, 1999). This could be explained by the increase in collagen concentration and cross-linking seen with aging. The fact that the collagen concentration in the myocardium doubles over the lifespan of a rat in conjunction with the five fold increase in cross-linking observed in the LV with aging (Anversa et al., 1990; Eghbali et al., 1989; P. K. Mays et al., 1988; D. P. Thomas et al., 1992), provides an attractive explanation for the decrease in diastolic functioning in the heart with age. These physiological changes in the myocardium could result in a stiffer, less-compliant heart that has a decreased ability to relax and a greater limitation to filling during diastole. The significant decrease in quantity and increase in quality of collagen in the aged hearts of the 35T group could decrease the stiffness of the myocardium and increase the compliance of the LV, and thus help restore early LV filling during diastole to some degree. A heart that is better able to function (more compliant) is also better able to adapt

to stressors and survive to a more advanced age than a heart that has a lesser degree of health and function.

4.4 Impact of Exercise on Age-Related Changes in the Heart

The effects of endurance exercise training, including preservation of cardiac geometry and attenuation of adverse remodeling including fibrosis and collagen cross-linking, may increase the capacity of the heart to pump blood while reducing internal work (Jin et al., 2000; H. B. Kwak et al., 2006). Evidence in support of this notion in humans include that 6 months of endurance exercise training in late middle aged to older healthy untrained men improved VO_{2max} related to improvements in SV and ejection fraction, while previously trained older men who underwent detraining showed the opposite effect (Schulman et al., 1996). Long-term endurance trained older men and women (Master athletes) were found to have enhanced early diastolic filling which either more closely resembled, or was indistinguishable from that of young men (Arbab-Zadeh et al., 2004; Forman et al., 1992). Indeed, it appears that Master athletes are able to maintain high levels of cardiovascular functioning such as SV and ventricular compliance until the 7^{th} decade of life (Tanaka & Seals, 2008). Interestingly, in a model of senescent rat as well as elderly patients, exercise training attenuated age-associated myocardial diastolic dysfunction and restored the cardiac protective effect of preconditioning otherwise lost as a result of aging (Brenner et al., 2001; Derumeaux et al., 2008; Derumeaux et al., 2008; Palka et al., 1999; Spurgeon, Steinbach, & Lakatta, 1983; Tate et al., 1990). These findings are consistent with the notion that the attenuation of age-related

fibrosis and collagen cross-linking caused by endurance exercise training, as seen in the current study, provides some alleviation against reduced elasticity and ventricular compliance and elevated internal work (Centurione et al., 2003).

4.4.1 Fibrosis

It has been shown in previous studies that endurance exercise training can attenuate the decline in myocardial contractility and the increase in myocardial stiffness in aged (25 months, F344) rats (Choi et al., 2009; D. P. Thomas et al., 1992). It has also been shown that collagen concentration in rat heart increases significantly during the life span (Anversa et al., 1990; Eghbali et al., 1989; P. K. Mays et al., 1988; D. P. Thomas et al., 1992). Although some studies demonstrate that endurance exercise training can result in an attenuation of the increase in collagen quantity with age up to 34 months in the F344BNF1 rat (H. B. Kwak et al., 2006; H. Kwak et al., 2011), other studies did not see significant change in collagen content of the LV in endurance trained aged animals (Choi et al., 2009; D. P. Thomas et al., 1992; D. P. Thomas et al., 2001). The observations of the current study support the theory that endurance exercise training can attenuate the age-related increase in fibrosis. Indeed, we found a 21.4% reduction in collagen content with endurance exercise training as compared to the sedentary controls in fibrosis in the subendocardium of the free wall of the LV of the heart. Because stiffness of the myocardium is determined by increases in collagen quantity mediated by changes in collagen quality, one caveat which could influence the discrepancy observed in previous studies is the added complication of collagen cross-linking. All studies investigating

changes in collagen cross-linking in aged endurance exercise trained animals did observe a decrease the amount of cross-linked collagen in trained animals compared to sedentary age-matched controls (Choi et al., 2009; D. P. Thomas et al., 2001). (See "AGE Fluorescence" section).

In the current study, endurance exercise training commencing in late middle age and continuing into senescence was able to significantly attenuate the age associated increase in collagen in the ECM of the subendocardium of the free wall of the LV of the heart (43.8%), as compared to the sedentary age-matched controls (55.7%) (see Figure 8). Interstitial collagen residing in the ECM of the myocardium is not a static protein. Changes in the balance between synthesis and degradation can in turn change the quantity and composition of the ECM in the heart (Bishop & Laurent, 1995). Thus the decrease in the percentage of connective tissue seen in the 35T group must either be due to a decreased synthesis or increased degradation of collagen in the ECM of the myocardium. Current evidence favours the latter mechanism (de Souza, 2002; H. Kwak et al., 2011; Robert et al., 1997; Rodriguez-Feo et al., 2005). Increased or maintained levels of collagen degradation would be the result of increased or maintained levels of MMP enzymes and activity, and perhaps coupled with maintained or reduced levels of TIMPs. This was the rationale given to investigate MMP and TIMP expression.

4.4.2 Collagen Degradation: MMP and TIMP Expression

An imbalance between collagen synthesis and degradation will unavoidably alter the collagen content in the ECM. It is believed that dysregulation of MMPs contributes significantly to age-related fibrosis, which can eventually lead to other health complications such as heart failure (Fedak et al., 2003; H. Kwak et al., 2011; Sivasubramanian et al., 2001; C. V. Thomas et al., 1998). Kwak et al. (2011) found significant changes in MMP protein expression with a 12 week endurance exercise training program commencing in 31 month old F344BNF1 rats (near-senescent). In their study, active MMP1, MMP2, MMP3, and MMP14 in the ECM was shown to decrease significantly with age, but training alleviated this age-related down regulation to near baseline levels seen at youth. In addition, Kwak's group also found a significant increase in TIMP1 protein expression in aged sedentary rats. This is in agreement with the increase in gene expression of TIMP1 with aging observed in the current study. However, converse to our current observations where endurance exercise training did not significantly reduce TIMP1 gene expression in the aged group, Kwak's group found that endurance exercise training markedly reduced TIMP1 protein expression in aged exercise trained rats, to the point of near reversal back to levels seen at youth (H. Kwak et al., 2011). The study by Kwak's group provides support to the hypothesis of the current study. In addition, Kwak et al. (2011) provides further insight as to what changes occur in protein expression of the MMP genes investigated. Although in the current study there was no significant difference between the aged groups regarding gene expression of MMP1 ($p=0.09$) or MMP3 ($p>0.05$), Kwak et al. found otherwise in regards to protein

expression of these same genes. In his study, Kwak et al. found that both MMP1 and MMP3 protein expression were increased with endurance exercise training in aged rats. This lends support to the idea that endurance exercise training in older animals results in decreased inhibition of MMPs. Decreased inhibition of the MMPs could result in a superior capacity to degrade collagen and contribute to protein turnover.

MMP1 and TIMP1 bind in a 1:1 stoichiometric ratio and form a functional unit regulating collagen turnover (Sheen et al., 2009). Comparing the ratio of gene expression of MMP1 to TIMP1 demonstrates that the 7C group has significantly less MMP1 inhibition via TIMP1 than either of the aged groups. Our observations did not show a significant difference between aged trained and sedentary groups with respect to MMP1 expression, however, there was a tendency for the 35T group to have less MMP1 inhibition than the 35C group ($p=0.13$). This is reflected by a higher MMP1:TIMP1 ratio in the 35T group than the 35C group. Although the difference between the aged groups was not significant, over time the difference seen between the two aged groups could have a substantial impact, with endurance exercise training attenuating the decrease in collagen degradation with age as seen in the 35C group. Kwak et al. (2011) found that out of the four TIMPs investigated for protein expression with a 12 week endurance exercise training program in 31 month old F344BNF1 rats, only TIMP1 demonstrated a significant training effect. Kwak et al. reasoned that this data in combination with their MMP results discussed above, indicates that the MMP1-TIMP1 pathway is a feasible pathway by which endurance exercise training could attenuate fibrosis (H. Kwak et al., 2011). Although the results of the current study regarding MMP1 and TIMP1 expression

lack statistical significance, the trends observed are in agreement with the conclusions drawn by Kwak's group, and thus provide additional support to the proposed MMP1-TIMP1 pathway by which endurance exercise training could attenuate fibrosis. A possible step preceding the MMP1-TIMP1 pathway involves TGFβ1 (W. Chen & Frangogiannis, 2010; de Souza, 2002; Petrov et al., 2002). TIMP1 expression is largely mediated by TGFβ1, a multifunctional peptide regulating proliferation and differentiation in many different types of cells including fibroblasts (H. J. Kwak et al., 2006). The higher the level of TGFβ1, the more fibroblasts are stimulated to proliferate and differentiate into myofibroblasts and synthesize collagen (H. J. Kwak et al., 2006; Petrov et al., 2002). In the study by Kwak et al. (2011), the age-related increase in protein levels of TGFβ1 was attenuated with endurance exercise training. Although the genetic expression of TGFβ1 was investigated in the current study, no changes with age or exercise training were found at the gene level. This does not preclude the changes that occurred at the protein level as a result of exercise training as found by Kwak et al., but that protein levels should also be probed for in addition to gene expression.

4.4.3 Collagen Turnover

The quantity, and perhaps quality, of collagen in the ECM is dependent on the balance between collagen synthesis and degradation. In general, the rate of collagen turnover is slow in normal healthy myocardium (Rodriguez-Feo et al., 2005). With aging, there appears to be a decrease in the rate of collagen degradation in the myocardium (Bailey et al., 1998; P. K. Mays, Mcanulty, Campa, & Laurent, 1991; Rodriguez-Feo et

al., 2005). This results in the accumulation of collagen, because it is still being synthesized but not removed. If this decrease in collagen degradation is stopped or prevented, then the amount of fibrosis in the myocardium would be expected to be reduced. Endurance exercise training could establish its beneficial effects by reducing the decrease in collagen turnover. Although no concrete evidence regarding collagen synthesis was obtained in the current study, this hypothesis could account for the decrease in collagen cross-links seen in the current study.

4.4.4 Type of Collagen

There appears to be changes in the ratio of the two principal types of collagen, types I and III, in the myocardium with age (P. K. Mays et al., 1988). In the current study, the ratio between the two types of collagen were not statistically different, but trended to be highest in the 7C group and lowest in the 35T group ($p=0.07$). Because type I collagen has a much higher tensile strength than type III collagen (Burton, 1954), a higher type I:III collagen ratio could be a structural change sufficient enough to alter cardiac functioning (Burgess et al., 1996). A higher type I:III ratio could result in an ECM, and thus myocardium, that is stiffer than an ECM with a lower ratio of type I to type III collagen. Indeed, it has been proposed that a reduced expression and accumulation of type III collagen relative to type I is likely a result of decreased collagen synthesis rates and the maturation of collagen cross-linking (Reiser, McCormick, & Rucker, 1992; D. P. Thomas et al., 1992). It has been shown that pathological remodeling due to spontaneous hypertension of rat hearts induced in several different ways, all

increased the proportion of type I collagen (Motz & Strauer, 1989; D. P. Thomas et al., 1992). The study by Kwak et al. (2011) lends support to our observations of endurance exercise training attenuating the age-related increase in fibrosis in the heart. Kwak's group found that 12 weeks of endurance exercise training in 31 month old F344BNF1 rats resulted in a decrease in the amount of type I collagen in the ECM of the LV myocardium. They interpreted this to be a result of the decrease in collagen content in the myocardium (H. Kwak et al., 2011). Although disease states such as hypertension and dilated cardiomyopathy (but not diabetes) tend to increase the type I:III collagen ratio, it has been demonstrated that hypertension in young primates (Weber et al., 1988) and middle aged humans with type 2 diabetes (Shimizu et al., 1993) have shown variability in the relationship between the collagen types and myocardial stiffness, where a decrease in the type I:III collagen ratio was associated with an increase in myocardial stiffness (Brower et al., 2006). However, these studies were also associated with a significant increase in the quantity of collagen in the myocardium, which could also explain the increase in myocardial stiffness. Recent evidence has demonstrated that collagen quantity and not relative ratios of type I:III collagen mRNAs differ amongst various cardiomyopathies in human males (Soufen et al., 2008). Linehan et al. (2001) demonstrated that in a rat model of pressure-overload induced hypertrophy, a significant increase in type I:III collagen ratio was not associated with an increase in diastolic stiffness when overall collagen content was unchanged (Linehan et al., 2001). Thus, given the conflicting observations regarding changes in the ratio between type I and III collagens and myocardial stiffness, no definite conclusion regarding the functional consequences of a higher type I:III collagen ratio can be made (Brower et al., 2006).

4.4.5 Geometry of Myocardium

Qualitatively, in the current study the adverse remodeling in the myocardium seen in the aged sedentary animals, including the apparent disorganization of the myocytes and increased space between them (see Figure 5), and the web-like appearance of the ECM comprised of thick collagen "veins", was also observed by Kwak et al. (2006). This appearance bears a remarkable resemblance to hearts that have undergone end-stage failure in ischemic cardiomyopathy (Beltrami et al., 1994). These changes implicate sliding displacement (side-to-side slippage) of myocytes with aging, which is also seen in conditions of elevated LV diastolic pressure. Sliding displacement has been shown to contribute to an increase in LV chamber volume and a less efficient myocardial contraction due to inefficient force production and transfer in the myocardium (Beltrami et al., 1994; Olivetti, Capasso, Sonnenblick, & Anversa, 1990). In the current study, it was observed that exercise training appeared to ameliorate these adverse changes, having an observably more organized and less web-like myocardium. Although 3-D imaging was not done, these observations are in agreement with Kwak et al. (2006) who demonstrated that these adverse changes in the myocardium were associated with LV remodeling which was attenuated with endurance exercise training (H. B. Kwak et al., 2006).

4.4.6 Detection of Advanced Glycation Endproducts in Myocardium

The detection of individual AGEs directly is difficult (Wolff & Dean, 1987). Fortunately, age-associated collagen cross-linking is closely correlated with AGE

formation (Kochakian et al., 1996). In the current study, the 35T group had significantly less AGE fluorescence than the 35C group. The degree of reduction in the 35T group was to the extent that the AGE fluorescence decreased to near levels observed at young adulthood in the 7C group. This attenuation in AGE fluorescence caused by endurance exercise training is in agreement with previous studies investigating changes in collagen cross-linking in older, but not senescent, endurance exercise trained animals (Choi et al., 2009; D. P. Thomas et al., 2001). In a study by Thomas et al. (2001), 10 weeks of endurance exercise training in 26 month old F344 rats completely reversed age-related increases in collagen cross-linking, as determined by pyridoxamine versus hydroxyproline content via liquid chromatography (D. P. Thomas et al., 2001). In a study by Choi et al. (2009), an indirect measurement of collagen cross-linking via myocardial solubility by pepsin digestion showed that a 12-week endurance exercise training program in 25 month old F344 rats resulted in an attenuation of collagen cross-linking in trained rats to levels observed in young rats (Choi et al., 2009). In this same study, the attenuation of collagen cross-linking was observed with a concomitant increase in myocardial contractility. Interestingly, in very young (~1 mo) rats, exercise training had no effect on collagen cross-linking (Woodiwiss et al., 1998). A possible explanation for this observation is that the prevalent form of cross-linking at this age is the enzymatic cross-links formed via lysyl oxidase during development and maturation. In the current study, gene expression of the three essential enzymes associated with enzymatic cross-linking (Lox, Loxl3, and Irf1) showed no change with age or training (see Figure 11). The enzymatic type of cross-linking has been shown to have the greatest influence on the formation of cross-links during development and maturation, while non-enzymatic cross-

linking through AGEs formation has been shown to have the greatest effect during the aging process after physiological maturity has been reached (Avery & Bailey, 2005). It therefore stands to reason that endurance exercise training has its greatest effect on non-enzymatic cross-links formed via AGEs that occur in aged tissue. The results of the current study are in agreement with the group of studies which indicate that endurance exercise training can attenuate the age-related increase in collagen cross-links formed via AGEs in the myocardium of the free wall of the LV of the heart (Choi et al., 2009; D. P. Thomas et al., 2000). The novelty of the current study lies in the age of the animals studied, and the methodology used. AGE fluorescence and collagen cross-linking have never before been tested with an endurance exercise training program initiated in late middle age where detrimental age-associated changes in the heart had already begun to accumulate. At the age where the rats commenced training, significant fibrosis and cross-linking had already accumulated in the heart (Choi et al., 2009; D. P. Thomas et al., 2001). That exercise can still have a significant positive impact on the heart at advanced ages through into senescence is relevant and critical knowledge to aged individuals. To the best of our knowledge, no one has ever measured AGEs using immunohistochemistry in-situ. The benefits of this methodology include the removal of risks associated with chemicals used in former methodologies of hydroxyproline analysis following pepsin-digestion (Stegemann & Stalder, 1967; Wossner, 1961). The methodology used in the current study also enables measurement in the original non-homogenized tissue, and thus is easily able to look at location-specific areas of the tissue, such as the ECM and subendocardium.

The attenuation of the decrease in degradation of collagen in the ECM could account for the attenuation of the formation of collagen cross-links represented by the attenuation of AGE formation seen via immunohistochemical staining, which is in agreement with the literature (Choi et al., 2009; D. P. Thomas et al., 1992). Thomas et al. (1992) found that a 10 week endurance exercise training program with aged female F344 rats reduced collagen cross-linking by 50%, which although still higher than the level observed in young adult rats was markedly closer to those levels at youth (D. P. Thomas et al., 1992). Interestingly, training had no effect on young adult rats, demonstrating that endurance exercise can have a measurable effect on attenuating age-related changes.

The longer a synthesized protein exists, the more exposure it has to stress and detrimental factors such as sugar (glycation), ROS, etc. With increased degradation of long-lived proteins, older proteins would be safely disposed of, and thus reduce the likelihood of forming cross-links. Consequently, the chance of a protein reacting with a detrimental factor increases with the length of its existence. If the rate of protein turnover decreases with age (Masson et al., 2005), then the number of old proteins increases, and therefore the number of damaged proteins would also accumulate, including AGE formation and also cross-linked proteins. However, if the decline in protein turnover is prevented or attenuated, for example by endurance exercise training (Takala et al., 1991), then the number of old and damaged proteins would not increase or accumulate to the same degree with advancing age. Thus less AGEs and cross-links would form, a point consistent with the observations in the current study. Specifically, the aged trained group

had significantly less AGE formation than their sedentary counterparts, and thus would have significantly less collagen cross-links and a higher quality of collagen.

4.5 Function of Heart

No measurements of the functioning of the animals' hearts were taken, nonetheless, some reasonable conclusions can be made based on the structural data that was collected. Having previously viewed experimentally induced transmural infarcts (Yoshiyama et al., 2005), given the extensive thinning of the myocardium and composition of connective tissue in the infarct area, no evidence of myocardial infarction was found in the histological tissue sections of the current study.

The literature states that with endurance exercise, the pressure-volume (P-V) loop of an individual shifts to the right compared to that of a normal healthy heart (Choi et al., 2009). This means that the heart, and specifically the LV, has increased the volume of blood that the LV can receive during diastole and consequently eject during systole (i.e., SV). In addition, it accomplishes this without increasing the pressure needed to overcome afterload. Under disease conditions such as pathological hypertrophy and hypertension, the P-V loop extends upwards compared to that of a normal healthy heart. This means that the heart must generate enough force to overcome an increased afterload to eject blood during systole, and accomplish this without increasing the SV. The shift in the increase in SV of the heart with endurance exercise training could be due to physiological remodeling. This remodeling could include a reduction in the quantity of collagen in the

heart or an improvement in the quality of collagen in the heart due to a decrease in the amount of cross-links formed between collagen molecules. Both of these changes could result in a myocardium that is less stiff. To increase the SV during physiological remodeling, the myocardium has to be allowed to stretch more to receive a larger quantity of blood. Therefore, changes in collagen quantity or quality in the ECM must occur.

Recent evidence by Willemsen et al. (2011) has shown an association in late middle aged humans between tissue AGEs and both early diastolic functioning and exercise capacity (VO_{2peak}). They found that an increase in tissue AGEs measured by skin autofluorescence was independently associated with a decrease in early diastolic function. They hypothesized that this could account for the reduced exercise capacity in those with higher levels of AGEs in diabetic and HF patients (Willemsen et al., 2011). This could help to explain the exercise effects of the animals in the current study. The 35T group had a higher exercise capacity than their sedentary age matched controls. According to the association described by Willemsen et al. (2011), this would mean that the 35T group also had lower levels of AGEs, which was found to be true in the current study. Lower levels of AGEs are associated with increased diastolic functioning as described by Willemsen et al., and thus could help to explain the increased health and survival of the 35T animals.

4.6 Conclusion

In the current study, "snapshot" measurements were taken at two time points being 7 months and 35 months of age in F344BNF1 rats. With regard to the aging process, detecting the small changes over time which yield significant changes with age, are far less likely to be measurable with "snapshot" measurements, as this kind of measurement may not decipher what occurs during a process over time. Therefore, measurements that were taken that were approaching statistical significance could possibly have a meaningful impact on the heart during the aging process. Being cognizant of this limitation in methodology, in the current study the 35T group underwent endurance exercise training and significant age and training effects were observed. It was found that the 35T group had both a decrease in the quantity of collagen in the ECM via attenuation in the accumulation of collagen in the subendocardium of the free wall of the LV, with a concomitant increase in the quality of the collagen assessed by an attenuation of the age-associated accumulation in the quantity of AGEs in the ECM compared to the 35C group. A decreased quantity of AGEs correlates to a decreased quantity of collagen cross-links. A decreased quantity of cross-links means that the collagen is less rigid and thus more compliant.

Physiologically, the 35T group completely reversed the increase in body mass and %BF seen with aging in the 35C group. The 35T group also had a significantly lower absolute heart mass, and thus a reduced degree of cardiac hypertrophy than the 35C group. These physiological changes in the 35T group are indicative of a healthier group

of animals as compared to their sedentary age-matched controls. This was also reflected in the percentage of 35T group that survived to more advanced ages as compared to the 35C group. A decrease in quantity (percent connective tissue) and increase in quality (AGE levels; type I:III collagen ratio) of collagen in the LV of the 35T group compared to the 35C group was observed in the current study. The proposed explanation of these changes in the myocardium is enhanced collagen protein turnover due in part to an attenuation in the age-related decrease in collagen degradation. Further evidence in support of the proposed mechanism includes trends found towards a decrease in TIMP1 gene expression coupled with an increase in MMP1 gene expression when expressed relative to the young controls. In addition, MMP1:TIMP1 gene expression trended to increase in the 35T group. This could be interpreted in that there was enhanced collagen degradation and perhaps collagen turnover in the 35T animals compared to the 35C group. Cumulatively, the changes in the myocardium would promote improved early diastolic functioning in the 35T group. Functionally, the hearts of the trained animals should be better able to fill during diastole, particularly during the early passive phase of ventricular filling, and eject the blood during systole, thereby improving their SV, and also be better able to respond to stress. Being better able to respond to stress would be an advantage to survive, as it is well known that with aging, one experiences a reduction in the ability or capacity to respond and adapt to stressors in the body (Arking, 2006).

As mentioned above, one novel aspect of this study is the advanced age of the animals. To the best of our knowledge, this study is the first time AGE fluorescence and collagen cross-linking have been tested with an endurance exercise training program

initiated in late middle aged and carried through into senescence. At the age of training initiation, detrimental age-associated changes in the heart had already begun to accumulate, such as significant fibrosis and collagen cross-linking (Choi et al., 2009; D. P. Thomas et al., 2001). This is of importance as it demonstrates that exercise training can still have a significant positive impact on the heart at advanced ages through into senescence. This is especially relevant and critical in regards to potential new health care options and prescriptions for aged individuals. Potentially, exercise could offer an effective and exciting low-cost and low-risk intervention to treat diastolic dysfunction leading to heart failure. This is an especially attractive notion given the huge burden on the health care system as the baby boomers continue to age and experience detrimental changes to their hearts due to aging. In addition, the knowledge gained from the current study and others to discover the mechanisms by which endurance exercise training ameliorates these detrimental age-related changes in the heart is critical to pave the way for the creation of pharmaceutical drugs for those individuals who are not able to exercise safely.

Another novel aspect of the current study is the immunohistochemical staining of AGEs in-situ. To the best of our knowledge, no one has ever measured AGEs using this technique. Although it remains to be validated against traditional methods of measuring collagen cross-linking, the benefits of the methodology used in this study are considerable. Benefits include a safer, easier, and possibly quicker procedure compared to former methodologies of hydroxyproline analysis following pepsin-digestion (Stegemann & Stalder, 1967; Wossner, 1961). The methodology used in the current study

also enables measurement in the original non-homogenized tissue, and thus facilitates study of location-specific areas of the tissue, such as the ECM and subendocardium. This enables further insight into where AGEs form that would not be possible using whole-tissue homogenates.

4.7 Limitations

There are several limitations to the current study. The first and foremost is that no functional physiological measurements were taken of the rats. Therefore there is no indication if the structural findings have functional consequences or if measured significant changes would cause a significant positive change physiologically in the endurance exercise trained group. Another limitation to the study is that there were only two time points measured (7 mo and 35 mo). Thus, there is no information as to what happened to the areas of measurement. In addition, it is unknown if any differences occurred between the groups in regard to the pattern of fibrosis and collagen cross-linking (i.e., what happened to the groups between 7 mo and 35 mo).

The method used to measure Collagen cross-linking detection via AGE measurement would have benefited from also using the current standard of hydroxyproline measurement following pepsin digestion (Choi et al., 2009). This would have allowed comparison with and a degree of validation to the current technique. Furthermore, it would be pertinent to also validate specificity of staining for the Van Geison's stain. The differences between the red-brown of the cardiomyocytes and vibrant

pink of the collagen can be unobvious. By using a different staining with colours of greater contrast such as Masson's trichrome stain, these differences would be more apparent and allow for comparison with the findings of the current study.

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