DEVELOPMENT OF A METHOD FOR MECHANICAL PROPERTIES TESTING OF RAT AORTAE

by

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ABSTRACT

EMILY WIGHT ZACHERLE. The development of a method for mechanical properties testing of rat aortae. (Under the direction of DR. REUBEN HOWDEN)

INTRODUCTION: Arterial stiffness (AS) is a prevalent condition associated with increased risk of cardiovascular (CV) complications. Research has shown structural changes associated with AS, for which the precise mechanisms are unknown. Mechanical properties testing (MPT) has the potential to provide insight into the pathophysiology of AS, but studies to date have not represented physiological conditions well, limiting their contribution. PURPOSE: To develop a MPT method that overcomes these limitations. METHODS: Four groups of Sprague Dawley rats were studied: Control (C), acute diabetes (DA), chronic diabetes (DC) and insulin treated chronic diabetes (DI). Following sacrifice, each aorta was excised and cut into 4 rings. Rings were tested using cyclic and failure testing protocols. RESULTS: DI low ($p<0.05$) and high pressure ($p<0.05$) groups had significantly greater wall thickness than all other groups. DI low pressure first peak strain was lower than DA and DC low pressure groups ($p<0.05$) but was not different between high pressure groups. Modulus was not different between low pressure groups, but DI high pressure group modulus was significantly lower than all other groups ($p<0.05$). During cyclic testing, stress difference was significantly lower in DI low pressure than DC and C low pressure groups ($p<0.05$) and significantly lower in DI high pressure compared to all other high pressure groups ($p<0.05$). Strain difference was not different between groups. DISCUSSION: Our findings suggest that DI group aortas were weaker compared to the other groups. Previous research suggests that insulin therapy can induce structural alterations in the vascular wall which poorly affect cardiovascular

health. The new method of MPT used in this study was successful in measuring aortic stiffness differences between groups and may be a useful tool for future studies investigating responsible mechanisms of AS development in other disease states.

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LIST OF ABBREVIATIONS

AGE	advanced glycation end products
AS	arterial stiffness
BP	blood pressure
C	control group
CV	cardiovascular
DA	diabetic acute group
DC	diabetic chronic group
DI	diabetic insulin group
ECM	extracellular matrix
MPT	mechanical properties testing
T1D	type 1 diabetes

CHAPTER I: INTRODUCTION

Arterial Stiffness

Arterial elasticity is an essential component of CV function, allowing the arterial system to sustain minimal changes in stress despite large blood pressure (BP) changes (Kelleher et al, 2004) Although AS is a common characteristic of aging, many chronic conditions accelerate its development increasing the risk of CV complications (Zeiman et al, 2005), including hypertension, chronic kidney disease, hyperlipidemia, chronic obstructive pulmonary disease, and type I diabetes (T1D; Eren et al, 2003; Moro et al, 2008; ; Schiffrin et al, 2007; Zieman et al, 2005; Zoungas et al, 2007). Moreover, structural and cellular changes underlying AS increase susceptibility to atherosclerosis development, myocardial infarction, heart failure, stroke, dementia and mortality (Zeiman et al, 2005; Zoungas et al, 2007). Although it is known that certain diseases exacerbate vascular changes associated with AS, they may do so through a multitude of pathological mechanisms. Therefore, development of preventative measures may need to be mechanistically driven and ultimately differ by disease condition (Zeiman et al, 2005).

Mechanisms responsible for AS development all result in microstructural alterations to collagen and elastin (Zieman et al, 2005). Collagen and elastin are the primary structural proteins in the vascular wall, accounting for the strength and elasticity of the vessel, respectively (Johnson et al, 2001). Dysregulation of these components may be caused by inflammation, and changes in endothelial cell signaling or vascular smooth

muscle tone (Johnson et al, 2001). These factors lead to enhanced collagen production, abnormal elastin formation, disorganized and dysfunctional fiber distribution and eventually fiber degradation (Catell et al, 1996; Spina et al, 1976; Watanabe et al, 1996)

Clinical Measures

Common clinical measurements of AS include evaluation of larger vessels, such as the aorta, due to ease of detection (large size) and common occurrence of AS in the aortic wall versus peripheral vessels. These methods include aortic pulse wave velocity, augmentation index, ultrasonography, computed tomography and magnetic resonance imaging (Saito et al, 2012; Shahmirzadi et al, 2012). Clinical techniques are implemented to determine aortic stiffness progression, endothelial dysfunction, and the development of atherosclerosis (Wang et al, 2008). Although useful in clinical settings, noninvasive methods have not been effective at detecting specific mechanisms responsible for aortic stiffness development, which may delay or prevent effective treatment (Bolster et al, 1998; Wang et al, 2008). Consequently, use of alternative models for assessing aortic stiffness and contributing mechanistic factors are essential to understanding and delaying disease progression.

Experimental Measures

Numerous studies have examined mechanical properties of square aortic sections utilizing uniaxial and biaxial stretching (Azadani et al, 2012; Collins et al, 2011; Stephen et al, 2013; Zou & Zhang, 2012). These methods determine aortic stiffness by measuring force during deformation and corresponding stress-strain response vs. tissue thickness. Specimens have been mounted to stretching devices using hooks or bonded to linear arms while stretched in longitudinal or circumferential directions. Stiffness is assessed under

constant loading in which the tissue is stretched at a constant rate until failure, or cyclic loading, wherein tissues are stretched under predetermined amplitudes to mimic arterial BP forces during cardiac cycles (Angouras et al, 2000; Stephen et al, 2013). While, this method of MPT is useful for investigating relationships between tissue thickness and elasticity, it does not account for anisotropic properties of ECM fibers and vascular smooth muscle cell arrangement in arterial vessel walls. Collagen fibers are arranged longitudinally and vascular smooth muscle cells radially, while elastin is arranged in a three dimensional manner (Assoul et al, 2008; Lillie et al, 2007). Therefore, in order to determine stresses and strains exerted under physiological conditions, MPT of intact aortic rings provides an opportunity for more comprehensive assessment of these forces.

Few studies have tested the mechanical properties of aortic rings, in which a small cylindrical section of the aorta is tested (Arner & Hellstrand, 1981; Assoul et al, 2008; Lillie & Gosline, 2006; Lillie & Gosline, 2007; Silver et al, 2003; Tsatsaris et al, 2004). Unlike uni-axial or bi-axial testing, forces are applied to the lumen surface, similar to BP forces, by two rods or pins. Samples are mounted around two small posts and placed under constant or cyclic loading, as described previously. This method is more accurate than uni-axial and bi-axial testing in replicating actual stresses placed on collagen and elastin fibers within the vasculature induced by normal BP fluctuations (Assoul et al, 2008). Because rat aortas are relatively small in diameter, this method of testing requires custom built equipment. To date, four studies have tested aortic stiffness in a rat model, but the protocols did not replicate physiological BP forces. Moreover, the posts used to apply force to aortic samples covered a small cross sectional surface area of the whole tissue sample, focusing stresses on a small area and possibly stretching fibers and

vascular smooth muscle cells in a manner that was not physiological (Assoul et al, 2008; Arner & Hellstrand, 1981). However, the advantages of rat models in MPT are substantial because of a wide range of disease models available that may influence aortic stiffness. Therefore, developing a system by which forces closer to physiological conditions can be applied to rat aortic rings is essential for investigating mechanisms responsible for aortic tissue stiffness progression with aging and/or disease.

Combining histological analysis and MPT would be beneficial in determining mechanisms responsible for alterations in mechanical behavior of aortic tissue in aging and/or disease (Silver et al, 2003; Tsatsaris et al, 2004). Together, with improvements in testing methodology, these techniques may provide opportunities to assess aortic stiffness in more detail in diabetes, hypertension and other conditions which result in AS. Therefore, the purpose of this study is to develop a new model for MPT of rat aortas under forces representative of physiological BP fluctuations.

CHAPTER II: LITERATURE SEARCH

Although aortic stiffness is a consequence of natural aging, multiple disease states often accelerate this process, potentially developing into more serious conditions (Moro et al, 2008; Schiffrin et al, 2007; Vlassara & Palace, 2002; Zieman et al, 2005). ECM protein (collagen and elastin) turnover is modulated by a variety of factors, such as neuroendocrine changes, including the renin-angiotensin and sympathetic nervous systems, elastin and collagen gene expression, growth factors, amino acid induced cross-linking, and a host of ECM enzymes (Baxter et al, 1992; Najjar et al, 2005; Tsamis et al, 2013). Furthermore, it is not certain whether specific diseases, such as diabetes, chronic kidney disease, hypertension, and hyperlipidemia, lead to specific combinations of these mechanisms, resulting in aortic stiffness (Moro et al, 2008; Schriffin et al, 2007; Tsamis et al, 2013; Vlassara &Palace, 2002; Zeiman et al, 2005). As a result of the complexity of potential mechanisms responsible for these changes, diagnosis and treatment can provide a great challenge to healthcare providers (Zieman et al, 2005; Brands et al, 1998). Previous studies have tested mechanical properties of rat aortae, but are not representative of physiological BP conditions (Arner & Hellstrand, 1981; Assoul et al, 2008). In order to understand and determine factors responsible for aortic tissue stiffness in aging and disease, a more physiological method for MPT is essential.

Influence of Collagen and Elastin on Arterial Compliance

Aging and multiple disease states can lead to structural and compositional changes in arterial collagen and elastin. Mechanisms by which these changes occur are currently unknown, but it is well understood that changes in collagen and elastin serve as primary factors responsible for AS. In order to improve treatment in clinical settings, it is essential to understand mechanisms by which these alterations take place throughout the vasculature (Holzapfel & Gasser, 2000).

Arterial walls comprise three separate layers, intima, media and adventitia, each of which has its own biomechanical and structural properties (Han et al, 2009; Tsamis et al, 2013). The intima is predominately composed of endothelial cells, a basal membrane, and sub-endothelial collagen fibers. The media is composed of vascular smooth muscle cells, elastic laminae and a network of collagen and elastin fibers. The separation of these fibers by elastic laminae gives this layer its transversely isotropic structure, in which the interconnection between collagen, elastin, laminae and smooth muscle cells are arranged circumferentially in a continuous fibrous helix (Holzapfel & Gasser, 2000). The adventitia contains a thick helical layer of collagen fibers, surrounded by connective tissue (Tsamis et al, 2013). In healthy aortae, connective ECM fibers, elastin and collagen, provide elasticity and strength to vessels that are responsible for systemic blood flow supply (See figure 1; Han et al, 2009; Najjar et al, 2005; Tsamis et al, 2013). Conversely, as a result of aging and pathological mechanisms, changes in quantity and structure of these fibers are often responsible for mechanical dysfunction and hence a reduction in arterial compliance (Alford et al, 2008; Tsamis & Stergiopulos, 2007; Tsamis et al, 2009, 2011, 2013).

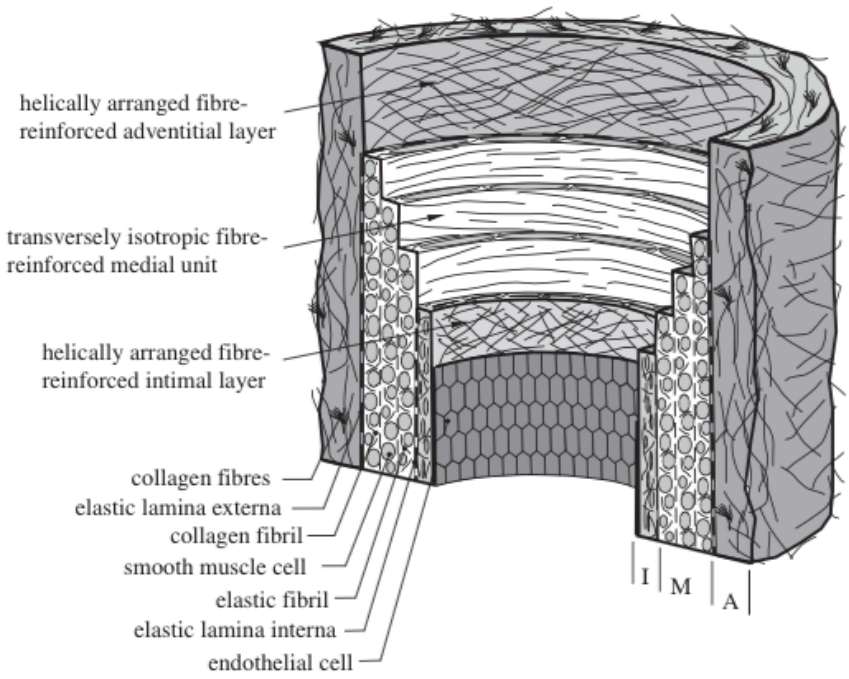

Figure 1: Architecture of a healthy human artery. Adapted from Gasser et al. 2006.

Histological studies have shown the concentration and arrangement of elastin and collagen fibers to be altered in aging and disease (Huang and Kang, 2007; Marque et al, 1999; Tsamis et al, 2013). Early reports speculated that decreases in total elastin content account for reduced arterial compliance (Faber & Oller-Hou, 1952; Hass, 1942). However, more recent studies suggest that increased collagen is primarily responsible for arterial inelasticity, while total elastin content minimally changed with little effect on arterial compliance (Schlatmann & Becker, 1977; Lansing et al, 1950). Elastin fragmentation has also been associated with aging and hypertension as a consequence of wall strain experienced by blood vessels over a lifetime of cardiac cycles (Avolio 1998; Greenwald, 2007; Sans & Moragas, 1993; Tsamis et al, 2013). Advanced age and diseases, such as diabetes and atherosclerosis, have been associated with an increase in

collagen cross-linking, irregularly arranged collagen and elastin fibers, reductions in elastin cross-linking and uncontrolled extracellular matrix (ECM) remodeling (Bobbink et al, 1997; Vlassara & Palace, 2002). An increase in cross-linked, or chemically bound, collagen fibers results in markedly increased stiffness and reduced elasticity. Extensive research has shown a variety of genetic, molecular and neuro-hormonal factors as responsible for ECM changes associated with aging, but it remains unclear if aging and disease result in aortic stiffness via different pathological mechanisms (Tsamis et al, 2013).

MPT and histological analysis are often used in combination to provide a detailed overview of aortic stiffness consequences on CV function and injury (Greenwald & Berry, 1978; Kassab, 2006). This mixed-methods approach allows investigators to determine the relationship between alterations in elastin and collagen content (i.e. crosslinking, degradation, concentration) and mechanical properties of the aortic wall.

Mechanical Properties Testing

Numerous studies have examined alterations in structural and mechanical properties of the aortic wall to determine the effects of aging and disease on collagen and elastin content and CV function. Most studies have utilized uniaxial and biaxial testing instruments to characterize properties of small, aortic sections (Azadani et al, 2010; Collins et al, 2011; Lally et al, 2004; Kassab, 2006; Li et al, 2008; Okamoto et al, 2002; Vaishnav et al, 1987; Vorp et al, 2013; Zou & Zhang, 2012). Although these testing methods are widely used, the method for testing the mechanical properties of arteries is not physiologically representative of BP conditions. Conversely, few studies have tested tensile properties of intact aortic rings, in which samples are mounted on small posts and

stretched under forces similar to physiological conditions (Ameer et al, 2014; Arner & Hellstrand, 1981; Assoul et al, 2008; Fitch et al, 2006; Lillie & Gosline, 2006, 2007; Papadopoloulos & Delp, 2002; Silver et al, 2003; Tsatsaris et al, 2004). In order to gain a better understanding of mechanisms responsible for aortic stiffness in multiple disease states, an improved model for rat aortic MPT is needed (Greenwald & Berry, 1978).

Mechanical Properties Testing: Measurements of Aortic Stiffness

MPT of arterial tissue involves placing a specimen under a pre-determined load and measuring the degree of deformation. The direction of the applied load should be representative of BP forces exerted under physiological conditions (Greenwald & Berry, 1978). Prior to testing, sample dimensions, such as wall thickness and diameter, are used for stress-strain analysis. A stress vs. strain curve is used to calculate tissue stiffness and elasticity (Figure 2; Azadani et al, 2012).

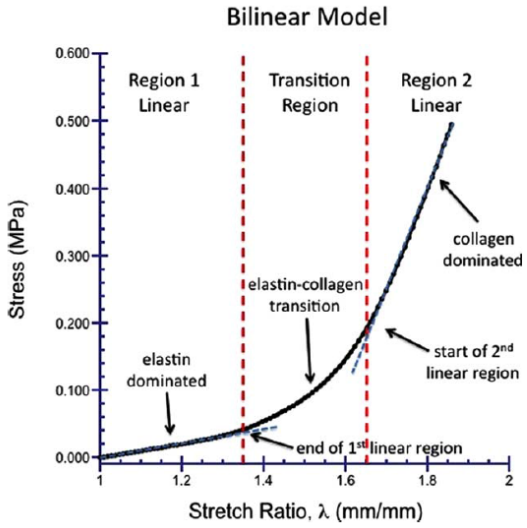

Figure 2: Stress vs. Strain curve. Adapted from Stephen et al. 2013.

The linear region of the curve defines modulus (material stiffness), marked by reversibility, a property of elastic materials. The point at which the slope of the linear region decreases defines the plastic region, in which deformation is irreversible. Irreversibility is a consequence of damage to the material's structural components or irreparable disruption of these components. Beyond the plastic region, stress returns to zero, at which point the material has slipped or torn at a failure point. The area under the curve in a failure test is the energy required to break a tissue sample. In a cyclic test, differences between the area under the loading curve and unloading curve is the energy lost during each cycle (Guinea et al, 2010; Humphrey et al, 2002).

From stress-strain outputs, tensile modulus, the ability of a tissue to resist deformation, can be used to calculate tissue stiffness. Stress, force per unit area, and strain, ratio of deformation over initial length, are measured as a tissue is stretched over a period of time (Azadani et al, 2012). Multiple equations exist to estimate tissue tensile modulus, including Young's modulus, which is defined as the ratio of stress to strain along a given axis. For materials experiencing small deformations, Cauchy stress is used to calculate stress inside a tissue, assumed to be homogeneous, that has been deformed (Azadani et al, 2012). Tensile stress for larger deformations is calculated by Kirchhof's expression. Multiple deformation measures exist to allow for dissimilar tissue structure (anisotropic vs. isotropic), each of which is useful in describing different types of structural changes (Zou & Zhang, 2012).

Mechanical Property Testing: Uniaxial and Biaxial Methods

Uniaxial MPT was one of the first methods used to quantify biomechanical analysis of aortic function and characterize the effects of structural alterations on aortic

tissue stiffness (Adham et al, 1996; Angouras et al, 2000; Lally et al, 2004; Li et al, 2008; Stephen et al, 2013; Vaishnav et al, 1987). This is the simplest form of MPT, in which aortae are cut into rectangular sections, sutured to two linear arms of a testing unit and tested under cyclic or failure protocols, in which each arm of the unit stretches the sample in two linear, opposite directions (See Figure 3; Adham et al, 1996; Mohan and Melvin, 1982; Sherelorn et al, 1989). Uniaxial testing units are capable of testing aortae from smaller species, such as mice or rats, allowing for the study of aortic stiffness in knockout strains predisposed to diabetes, hyperlipidemia, hypertension and other conditions which constitute a high risk of CV disease (Guo et al, 2002; Huang et al, 2006; Kassab, 2006). Furthermore, many studies have tested porcine aortic mechanical properties, which are especially advantageous because of the similarities to humans in size distribution of arteries and hemodynamic properties, such as BP and heart rate (Kassab, 2006). Therefore, uniaxial testing in both species allows for biomechanical analysis of aortic function essential for advancing knowledge of aging and disease in human CV physiology.

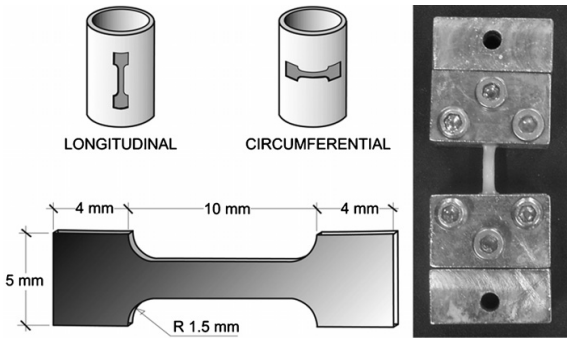

Figure 3: Longitudinally and circumferentially excised aortic sections mounted on a uniaxial testing unit. Adapted from Guinea et al. 2010.

Current literature on uniaxial testing is characterized by multiple limitations regarding the nature of testing protocols, which do not represent physiological conditions and inconsistency in protocols utilized between investigators (Guinea et al, 2010; Holzapfel et al, 2000). Holapfel et al (2000) noted that mechanical behavior of tissues is dependent on physical and chemical environmental factors, such as temperature, pH, pO2, and pCO2, of test media. Therefore, it is essential that comparative studies test samples in a uniform test media, otherwise inconsistent quantification of aortic biomechanical properties may result. Additionally, composition of the aortic wall is not uniform throughout; hence the stress-strain curve shape depends on the anatomic location from which aorta samples are harvested (Kassab, 2006; Holzapfel et al, 2000). A review by Guinea et al (2010) reported dissimilar values in aortic strength between studies, highlighting the need for uniform testing protocols to quantify aortic compliance with and without pathology. Furthermore, the authors noted an inability of uniaxial testing to reproduce complex loading consistent with aortic physiological conditions, providing an incomplete analysis of anisotropic structure of aortae. Consequentially, several studies have emphasized the need for uniform MPT, which better describes the anisotropic behavior of arterial walls (Guinea et al, 2010; Holzapfel et al, 2000).

Biaxial MPT units were developed in an attempt to more accurately describe the complex, anisotropic structure of aortae under physiological conditions. This method of testing involves square aortic sections sutured to wire hooks or glued to four tensile unit arms and stretched under cyclic loading or until failure in four linear directions (See figure 4). This method of testing can be used to determine tissue responses under both circumferential and axial loads, unlike uniaxial testing, which only tests one or the other

(Okamoto et al, 2002). Consequently, multiple studies have compared aortic stress-strain variation between uniaxial and biaxial testing, but results have been inconsistent (Lally et al, 2004; Andel et al, 2003; Shulze Bauer et al, 2003). Lally et al (2004) found that mean slope of the uniaxial stress-strain curve was 10±7 MPa, whereas the mean slope of the biaxial stress strain curve was 26±9.5 MPa, which implies that there is a difference in aortic stiffness results between the two testing modalities. Although, the authors noted a great deal of variation in both uniaxial and biaxial tension, and no significant difference was found between testing modalities (Lally et al, 2004).

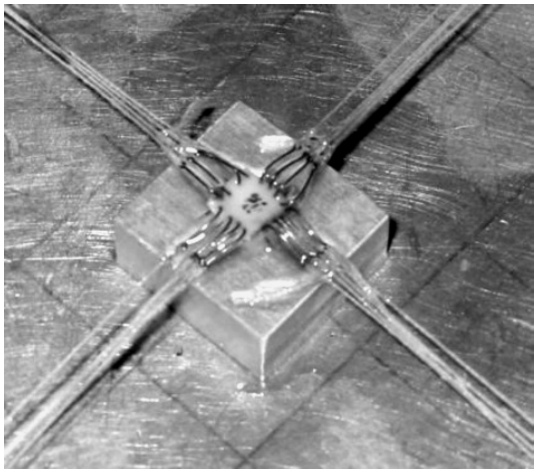

Figure 4: A square aortic section, mounted on four linear arms of a biaxial testing unit. Adapted from Lally et al. 2004.

Although biaxial testing applies loads to aortic samples in four linear directions further investigation is needed to determine if this method of testing is more representative of physiological conditions in comparison to uniaxial testing. Authors note that uniaxial and biaxial studies are often difficult to compare because aortic stiffness

varies between subjects and along the length of the aorta within a subject (Lally et al, 2004; Andel et al, 2003). Further, wire hooks commonly used to attach to samples causes tearing in stiffer specimens and are subject to slippage or failure at attachment points (Okamoto et al, 2002). Atherosclerosis, diabetes and hypertension are often characterized by higher arterial BPs, therefore testing models need to assess arterial mechanical properties when exposed to more extreme physiological loads (Greenwald & Berry, 1978). Biaxial testing of specimens excised from aortic arches removes tissue curvature, altering the natural position of ECM components, limiting reproducibility of physiological conditions (Guinea et al 2010; Okamoto, et al, 2002). As a consequence of uniaxial and biaxial limitations, an alternative model for MPT of intact aortic rings was developed to investigate mechanisms responsible for aortic stiffness by testing samples under forces more physiologically representative conditions, accounting for the anisotropic structure of arterial walls (Ameer et al, 2014; Arner and Hellstrand, 1981; Assoul et al, 2008; Fitch et al, 2006; Lillie and Gosline, 2006, 2007; Silver et al, 2003; Tsatsaris et al, 2014).

Mechanical Properties Testing: Intact Aortic Rings

Arner and Hellstrand's (1981) research on aortic compliance in spontaneously hypertensive rats was one of the first studies to investigate mechanical properties of intact, cylindrical aortas. Aortas were mounted around two parallel pins (total diameter, 0.6mm, ~30% aorta cross sectional surface area), one of which measured load with a force transducer. Results confirmed elevated stiffness in spontaneously hypertensive rat aorta, theorized to be a result of alterations in arterial wall composition. Conversely, histological analysis revealed no change in total amount of collagen or elastin and no

change in collagen and elastin concentration. The authors stated further investigation was needed to determine causal factors of aortic stiffness in spontaneously hypertensive rats (Arner and Hellstrand, 1981). Later studies investigated mechanical, structural and biological properties of porcine thoracic aorta for artificial tissue engineering and porcine models of stenosis (Lillie and Gosline, 2006, 2007; Tsatsaris et al, 2004). It was found that elasticity was reduced in distal arteries, elastic fiber integrity determined long term durability and aortic stiffening was induced by higher concentrations of collagen fibers (Lillie and Gosline, 2006, 2007; Tsatsaris et al, 2004).

Similar testing of aortic rings investigated variation in mechanical properties between regions of the aorta, the vena cava and carotid artery of porcine and rat models. (Ameer et al, 2014; Assoul et al, 2008; Silver et al, 2003). It was concluded that regional variation in collagen and elastin content was evident along the length of aortae and between the studied vessels, but causal factors responsible for these regional differences remains unknown (Assoul et al, 2008; Silver et al, 2003). Furthermore, Ameer et al (2014) studied regional differences in aortic stiffness and calcification in hypertensive Lewis polycystic kidney rats, spontaneous hypertensive rats, normotensive Lewis rats induced by vitamin D3 and Nicotine. MPT and histological analysis demonstrated regional variation between abdominal and thoracic aorta, but these differences were not uniform across all models. The mechanisms by which vascular calcification and aortic stiffness occur in chronic kidney disease remain unknown, but Ameer et al (2014) and other studies have shown stiffness and calcification to occur as a result of elastin degradation and loss of elastin integrity, respectively (Aikawa et al, 2009; Smith et al,

2012). The authors stated that alternative mechanisms responsible for aortic stiffness, such as AGE accumulation, require further investigation (Ameer et al, 2014).

As mentioned previously, rodent knockout strains are useful in MPT to study the differences in aortic stiffness among conditions predisposed to CV complications (Kassab, 2006). Limited studies have assessed aortic ring mechanical properties of knockout models, therefore comparative analysis is not plausible between current literature (Ameer et al, 2014; Fitch et al, 2006; Assoul et al, 2008; Papadopoulos & Delp, 2002). Among these studies, testing of rodent models of calcification, hypertension, nitric oxide deficiency and angiotensin II have been performed (Fitch et al, 2006; Ameer et al, 2014). Nitric oxide maintains vessel elasticity, while angiotensin II is a potent vasoconstrictor, which in higher concentrations leads to increases in BP (Ramchandra et al, 2005; Safar et al, 2010). Fitch et al (2006) were the first group to investigate important CV disease risk factors, including nitric oxide deficiency, angiotensin II, and vascular stiffening. Chronic angiotensin II treatment and nitric oxide deficiency were evaluated alone and in combination to determine variation in aortic stiffness. It was found that chronic treatment of angiotensin II and L-NAME (nitric oxide inhibitor) resulted in a significant increase in aortic stiffness, in contrast to angiotensin II or L-NAME alone. Further, it was concluded that combined treatment increased collagen/elastin ratio, adventitial collagen deposition and enlargement contributed to aortic stiffness. These findings suggest that angiotensin II and nitric oxide regulation may form the basis for targeted treatment to reduce aortic stiffness.

Although MPT of intact aortic rings is more representative of physiological conditions in aging and disease, there are significant limitations that can only be

overcome with further development and investigation of this technique. The most prominent limitation has been surface area used to apply loads to aortic rings. Rat and mouse aortae are small in diameter; therefore cross sectional surface area covered by pins must be similar to the inner circumference of aortae. The studies mentioned previously, utilized pins ranging from 0.4mm-0.8mm in total diameter (20-40% aorta cross sectional surface area), applying force across a small surface area, therefore poorly representing physiological vascular wall stresses induced by BP (Ameer et al, 2014; Assoul et al, 2008; Arner and Hellstrand, 1981). In order to overcome this limitation, it is essential that a MPT unit is developed in which pins apply forces across a larger surface area, better representing BP loads placed on the aortic wall and structural fibers. Furthermore, Ameer et al (2014) cycled aortic rings between equivalent BP forces of 80mmHg and 120mmHg, but hypertensive cyclic loading was not tested. Individuals affected by aortic stiffening commonly develop hypertension, therefore it is essential to compare mechanical properties under elevated and normal BP forces ex vivo (Dart et al, 1993; Eren et al, 2004). MPT of aorta only under normotensive conditions inaccurately assesses mechanical responses of vessels, which normally experience higher BP forces. Improvements in these aspects of testing will allow researchers to more accurately investigate differences in mechanical properties of healthy, diseased and aged aortae by simulating physiological BP forces and accounting for histological properties of arterial vessels. Without testing samples similar to physiological conditions, assessment of mechanical properties may inaccurately measure aortic responses, providing an incomplete understanding of AS in aging or pathological conditions.

Summary

Results of previous studies highlight the need for further development of a MPT instrument capable of testing aortic tissue representative of physiological conditions. Evidence suggesting multi-factorial mechanisms for aortic stiffness in aging and disease raise attention to the need for custom-built MPT equipment to determine whether these differences exist between conditions. The discovery of responsible mechanistic factors would allow for improved CV outcome in individuals at a high risk of CV complications and mortality.

CHAPTER III: METHODS

Experimental Model

All experiments were performed by Dr. Chad Carroll's laboratory at Midwestern University in Phoenix, AZ. Methods and procedures were approved by the Midwestern University Institutional Animal Care and Use Committee. Aortae were donated to the Laboratory of Systems Physiology at UNC Charlotte by Dr. Carroll.

8 week old, male Sprague Dawley rats (n=40) from Charles River Laboratories in Wilmington, MA were housed in pairs on a 12 hour light-dark cycle and given access to food and water. After one week of housing, rats were randomly assigned into one of four groups (10 per group): healthy non-diabetic control (control; C), 10 weeks of diabetes (chronic; DC), 10 weeks of diabetes with insulin therapy (insulin; DI) or 1 week of diabetes (acute; DA). All animals were maintained in pairs for the duration of the study. Rats assigned to diabetic groups were induced by 60 mg/kg of streptozotocin (in citrate buffer; pH 4.5) via injection of the penile vein under isoflourane anesthesia. C animals were injected with citrate buffer to avoid effects induced by the citrate buffer alone in treatments groups. The DA group was injected during week 9 of the experiment. The DI group was administered subcutaneous Humulin-N (Human rDNA produced insulin; 0.6-0.8 units/100 grams; Moore Medical LLC, Famington, CT), equivalent to the intermediate acting insulin in humans.

All groups were sacrificed after 10 weeks (19 weeks of age; 12.68 human years)

by decapitation. Body weight and plasma glucose levels were measured at the time of termination by urine glucose (Diastixs, www.walgreens.com, product #666850). Intact aortae from 32 rats (10 C, 8 DC, 7 DA, 7 DI) were excised post mortem and stored in a -80°C freezer.

Mechanical Properties Testing

Each aorta was sectioned into 4x 2 mm cylindrical rings, with 18 lost due to ring failure or inability to obtain 4 complete rings per aorta, for a total of 110 rings. Rings were cut individually, immediately tested and the remainder of the aortae was returned to -80°C to avoid thawing. Rings were placed in 1xPBS media for 5 minutes prior to testing, which allowed the specimen to thaw entirely and hydrate. Rings were mounted around two 0.5mm parallel aluminum posts (1mm total diameter) and immersed in room temperature 1xPBS solution.

Morphology

Deben MPT unit (Figure 5) (200N Tensile Stage; Deben UK Ltd. 1999-2012) with custom built mounting posts and software (Deben MicroTest V6.1.83 ©Deben UK Ltd 1999-2012) were used to calculate specimen stress/strain responses to pre-determined loads. All specimen images were captured under a Nikon SMZ 2100 microscope and measured using ImageJ software (Image Processing and Analysis in Java 1.47v, Wayne Rusband, NIH, USA). Wall thickness at no load and circumference at no load, were measured (Figure 6). Initial diastolic and systolic force were estimated from measurements taken of the tissue at no load. The rings were positioned at initial diastolic force and measured again for baseline wall thickness and circumference, which determined baseline diastolic and systolic force. Two rings from each aorta were exposed

to loads equivalent to normotensive BP (120mmHg/80mmHg) in Sprague Dawley rats. The remaining 2 rings were to be exposed to loads equivalent to hypertensive BP (160mmHg/100mmHg). During the cyclic test, each ring was cycled between two peak loads, normotensive diastolic and systolic load or hypertensive diastolic and systolic load. Normotensive and hypertensive loads were converted from mmHg to N (1 mmHg = 0.000133322368 N/mm^2).

Pictures at no load and initial diastolic force were captured using StCamSWare software (StCamSWare 2005-2011 v3.05, Sensor Technologies America, Inc.) directly above a given sample.

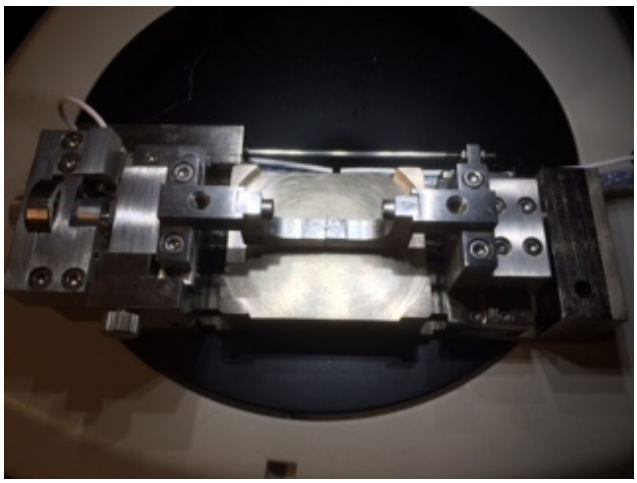

Figure 5: Deben rig

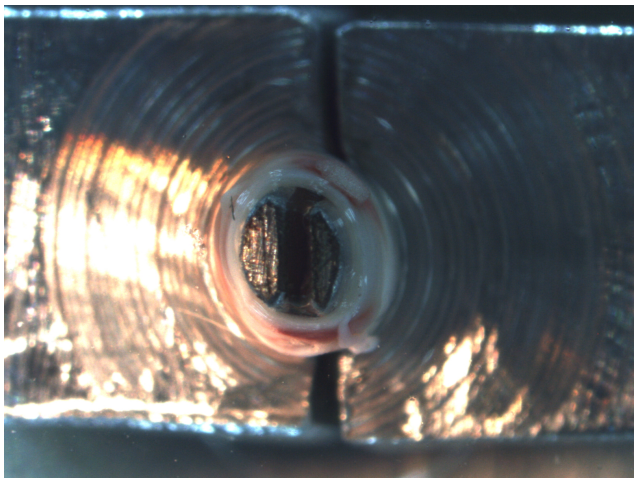

Figure 6: Aorta mounted on pins

Cyclic and Failure Testing

Following calculation of baseline diastolic and systolic force, rings were set to a sampling time of 100ms and a gain of 50x. Gain referred to the vertical scale of the acquisition window, meaning the 200N load cell with a 1x gain ranged from 0N-200N (full scale). For a 50x gain, the scale ranged from 0N-4N, until the force exceeded 4N, in which the gain automatically lowered to 20x while the motor was running. The speed in which the pins moved to stretch the sample was set at 6mm/min. Pilot studies were performed to determine that specimens stabilize after approximately 15 cycles, therefore 25 cycles were performed, with the last 5 cycles used for calculations. Minimum and maximum deformation were set to baseline diastolic and systolic force, respectively. Upon completion of the cyclic test, rings were returned to initial diastolic force before the failure test began (see below). A video was taken of cyclic testing using StCamWare software directly above the testing unit.

Prior to starting the failure test, wall thickness and circumference were measured at initial diastolic force. Percent change between pre- and post-cycling was calculated to determine

the effects of cyclic loading on aortic dimensions**.** Failure testing parameters were set to 100ms sampling time, 50x gain and 6mm/min motor speed. Rings were stretched until failure, the point at which the tissue tore or broke. A video was taken of the failure test using StCamSWare software and the specimen breaking point (location of the tear or break) was noted.

Tissue Stiffness

Peak modulus, the maximum slope of the stress/strain curve, was calculated to determine elastic modulus. Strain was defined as the relative specimen elongation or percent change in length from baseline, expressed as:

(L-Lo)/Lo

Where L was the length at a given time during stretching and Lo was the length at a force equivalent to diastolic pressure (resting length).

The stress was defined as the load (F) per unit cross sectional surface area (CSA) carrying the load, expressed as:

F/CSA (N/mm^2)

Where F was force applied to the specimen at d_{bl}. CSA was the longitudinal cross sectional area throughout the aorta.

Data Analysis

A 2-way ANOVA [group x cyclic force (diastolic vs. systolic)] was used to assess differences in peak modulus, stress vs. strain and distance traveled between two different loads between groups (C, DI, DA and DC). The Student Newman-Keuls post-hoc test was used for data that produced significant results and to identify the significance of specific differences.

Assumptions

All samples within groups and testing conditions were expected to behave similarly. The testing unit accurately measured stress and strain produced by each specimen. The investigator performed each test in a synonymous manner, exposing each aortic ring to the same testing conditions and testing duration.

CHAPTER IV: RESULTS

A total of 110 aortic rings were tested under cyclic and failure testing. Mean BP ranges during cyclic testing were higher than anticipated (normotensive 139.10mmHg/69mmHg; hypertensive 186.02mmHg/84.29mmHg), therefore the two loads will be referred to as low pressure and high pressure loads. Fifty-two rings were exposed to low-pressure loads (DC=10, C=19, DI=12, DA=11) and 58 were rings exposed to high-pressure loads (DC=13, C=20, DI=12, DA=13). During failure testing, specimens tore at multiple points during the test. The first tear, or first peak, was used in calculations of first peak stress, strain and modulus, as this represents the maximum stress and strain achieved before initial failure.

Sample Dimensions

At baseline diastolic force prior to cyclic testing, DI-low pressure group mean wall thickness was greater than all other C-low pressure groups (DC 0.104±0.011mm, C 0.102±0.007mm, DI 0.133±0.006mm, DA 0.109±0.008mm; DI vs. C $p=0.010$; DI vs. DC $p=0.028$; DI vs. DA $p=0.039$). DI-high pressure group mean wall thickness at diastolic load was greater than all other high pressure groups (DC 0.111±0.010mm, C 0.101±0.006mm, DI 0.150±0.006mm, DA 0.115±0.004mm; DI vs. C $p<0.001$; DI vs. DC $p=0.007$; DI vs. DA $p=0.012$: See Figure 7).

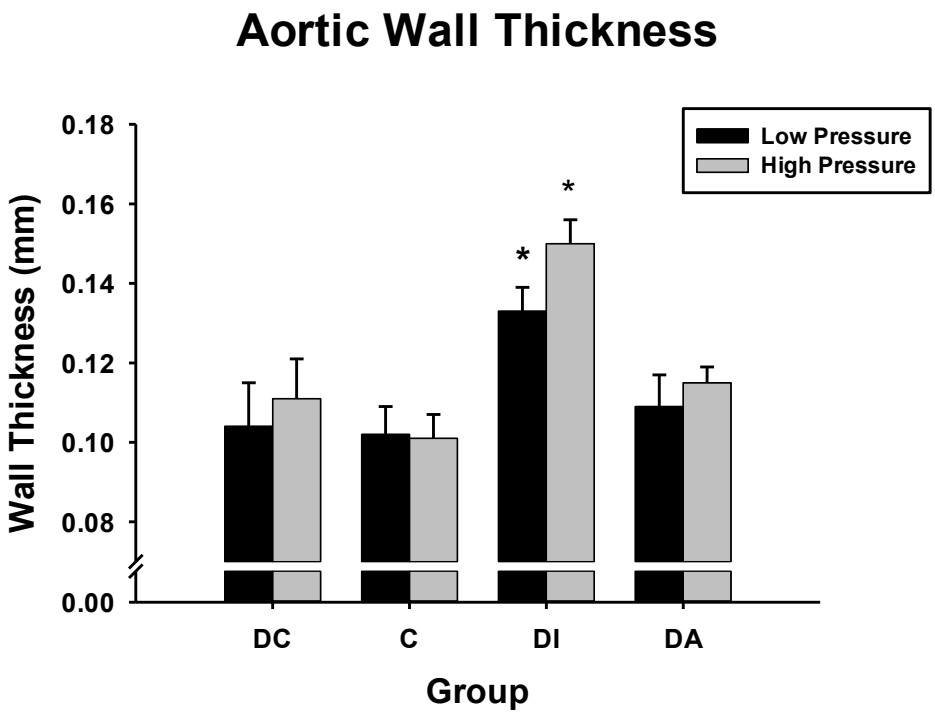

Figure 7: The thickness of the aortic wall, from the inner circumference to the outer circumference of the aorta, was measured. DI low and high pressure wall thickness was significantly greater than all other groups. (values are group means ± SEM ; * = statistical significance)

C-low pressure group mean circumference at diastolic load was greater than all other low pressure groups (DC 6.39±0.13mm C 6.98±0.15mm, DI 6.59±0.10mm, DA 6.41±0.17mm; C vs. DC p=0.013; C vs. DA p= 0.007; C vs. DI p=0.31). C-high pressure group mean circumference was significantly greater than DC (DC 6.64mm±0.12mm, C 7.15±0.12mm, DI 7.05±0.12mm, DA 6.98±0.16mm; C vs. DC p=0.021) but not different from DA or DI high pressure groups (p= 0.58 and p=0.57, respectively; See Figure 8).

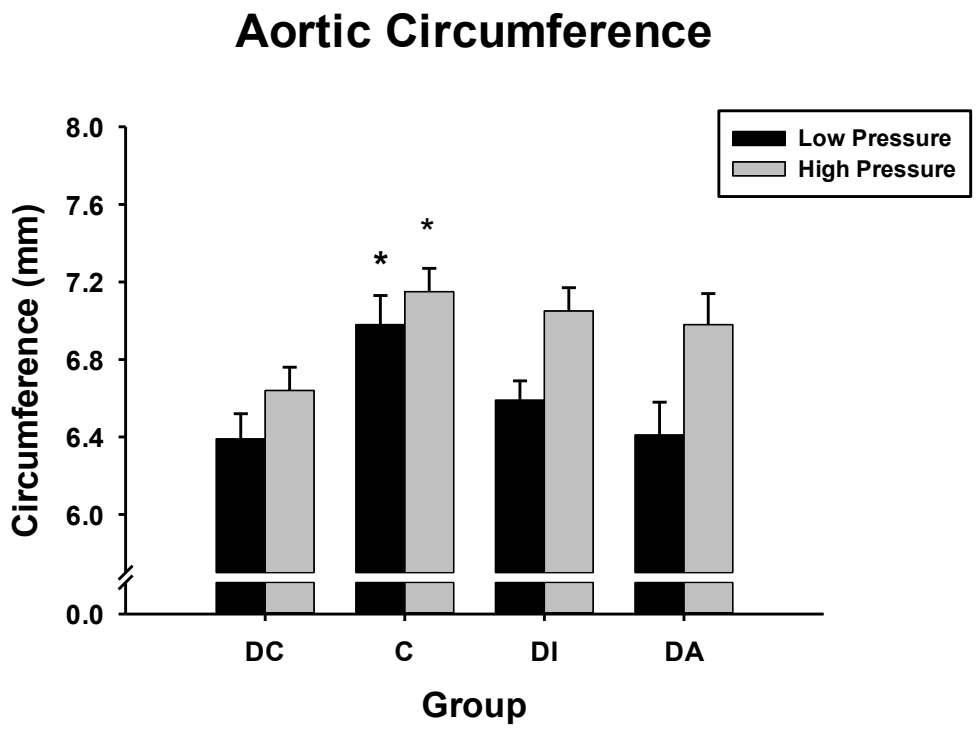

Figure 8: Circumference was calculated my measuring the inner circumference, or lumen, of each aortic ring. C low pressure circumference was significantly lower than all other groups. C high pressure circumference was significantly greater than DC high pressure. (values are group means ± SEM ; * = statistical significance)

Following cyclic testing, average circumference was greater in low pressure groups (DC +15.18%, C +12.75%, DI +11.99%, DA +14.51%) and high pressure groups (DC +16.42%, C +11.75%, DI +12.34%, DA +15.19%) in comparison to circumferences at baseline diastolic load.

Failure Testing

Stress at first peak, the stress of the sample just before initial failure, was significantly lower in the DI low pressure group compared to DC and DA low pressure groups (DC 7.14±1.22MPa, C 5.16±0.79MPa, DI 4.06±0.53MPa, DA 6.86±0.79MPa; DA vs. DI $p=0.025$; DC vs. DI $p=0.032$) but not different from C ($p=0.345$). Stress at

first peak was not different between high pressure groups (DC 5.39±0.92MPa; C 5.39±0.92MPa, DI 3.70±0.43MPa, DA 4.69±0.46MPa; p>0.05; See Figure 9).

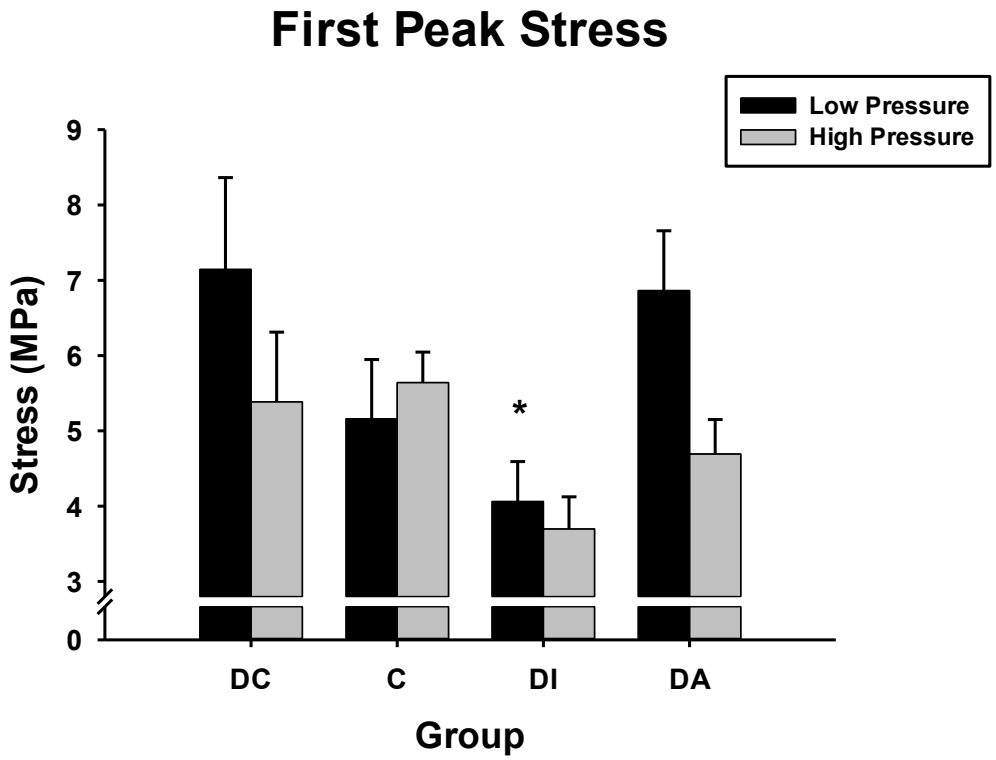

Figure 9: First peak stress is calculated by averaging the stress of each sample within a group just before sample failure (i.e. tear). A stronger or stiffer material (aorta) will be able to withstand larger stresses than a weaker material (aorta). During failure testing, the DI low pressure group had a significantly lower first peak stress than all other low pressure groups. (values are group means ± SEM ; * = statistical significance)

Strain at first peak, the strain of the sample just before initial failure, was significantly greater in the DA low pressure group than all other low pressure groups (DC 29.49±2.78%, C 26.69±1.92%, DI 28.30±1.92%, DA 36.10±3.02%; DA vs. C p=0.004; DA vs. DI p=0.035; DA vs. DC p=0.042). Strain at first peak was not different between

high pressure groups (DC 22.52±1.69%, C 24.48±0.73%, DI 22.87±1.65%, DA 24.72±1.72%, p>0.05; See Figure 10).

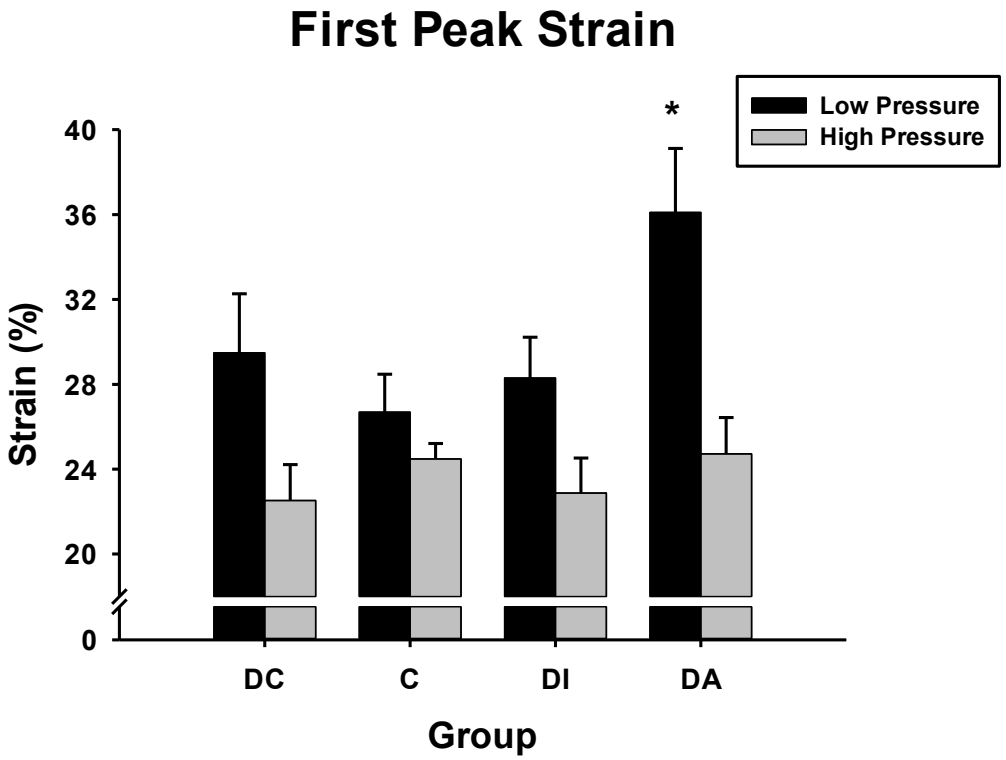

Figure 10: First peak strain is calculated by averaging the strain, percent change in length, of each sample within a group just before sample failure. A stronger or stiffer material will have a lower strain when compared to weaker materials. During failure testing, strain at first peak was significantly greater in the DA low pressure group than all other low pressure groups. (values are group means ± SEM ; * = statistical significance)

Modulus at first peak was not different between low pressure groups (DC 22.97±4.56; C 27.24±3.23; DI 18.01±1.81; DA 28.48±4.59; p>0.05). Modulus at first peak was significantly lower in DI high pressure compared to C high pressure (DC 22.76±2.78; C 32.30±2.95; DI 20.25±1.78; DA 22.84±1.95; C vs. DI p=0.047), but not

different from DA and DC high pressure groups (p=0.809 and p=0.735, respectively; See Figure 11).

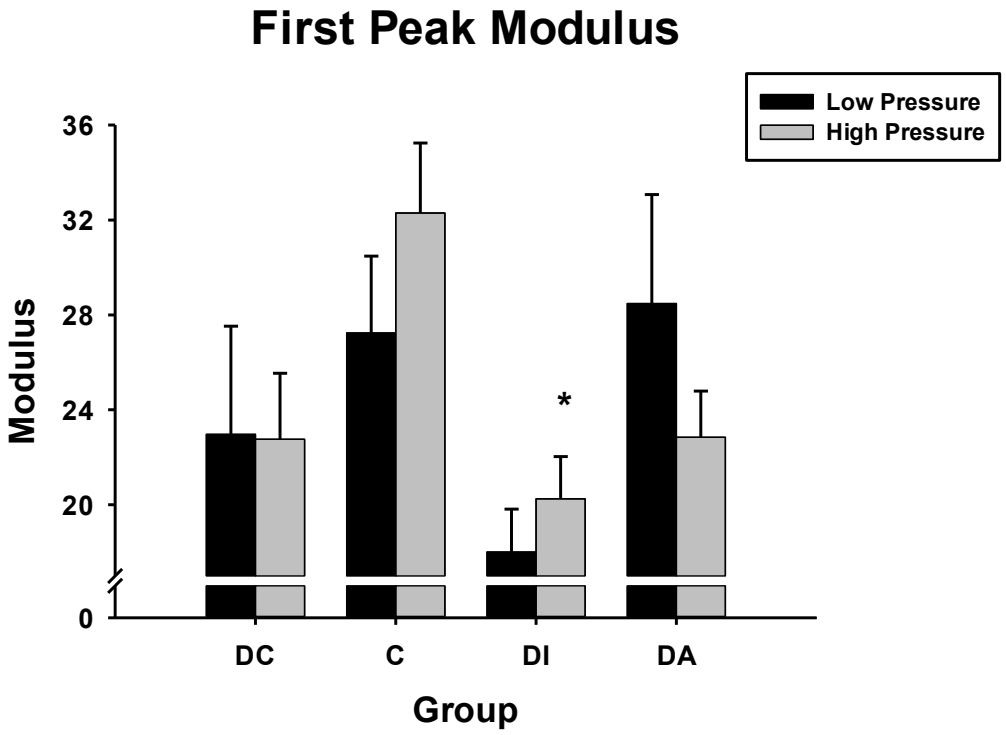

Figure 11: Modulus is defined as the stiffness of a material and is a function of both stress and strain (modulus = stress/strain). A stiffer or stronger material will have a greater modulus than weaker materials (aortas). During failure testing, the DI high pressure group modulus at first peak was significantly lower than first peak modulus of the C high pressure group. (values are group means ± SEM ; * = statistical significance)

Cyclic Testing

Stress difference was significantly lower in DI low pressure than DC and C low pressure groups (DC 0.35±0.04MPa; C 0.33±0.02MPa; DI 0.23±0.01MPa; DA 0.29±0.02MPa; C vs. DI p=0.024; DC vs. DI p=0.035) but not different from DA (p=0.175). Stress difference was significantly lower in DI high pressure compared to all

other high pressure groups (DC 0.44±0.038Mpa; C 0.49±0.03MPa; DI 0.34±0.017MPa; DA 0.41±0.015MPa; C vs. DI $p<0.001$; DC vs. DI $p=0.031$; DA vs. DI $p=0.042$; See Figure 12).

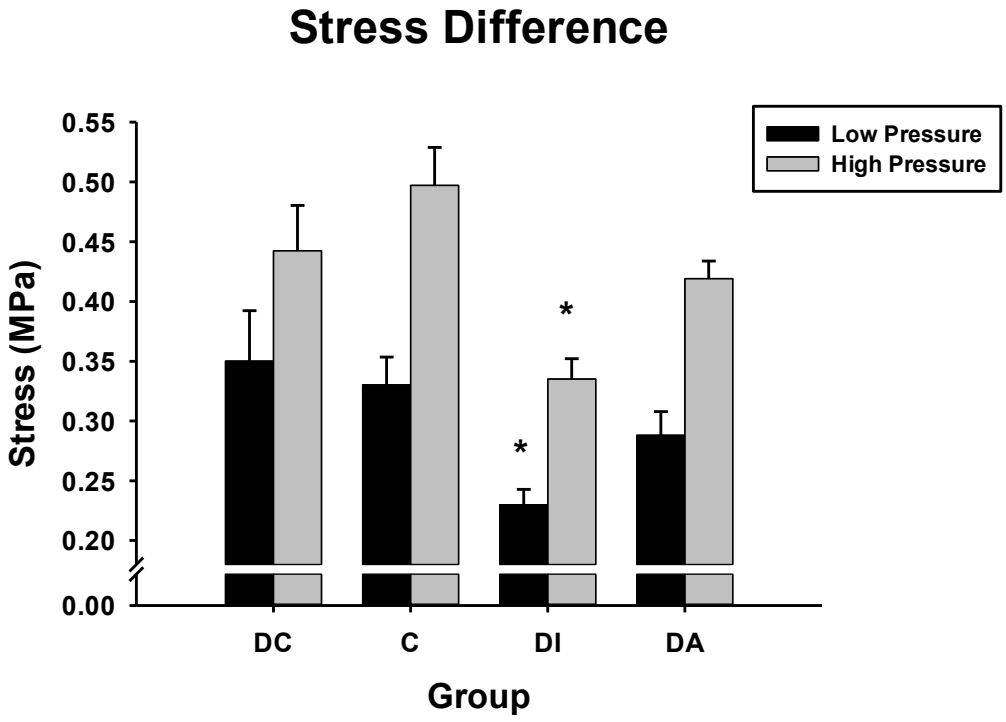

Figure 12: Stress difference is calculated by subtracting the stress at diastolic load from the stress at systolic load. The resulting value, in combination with strain responses, determines the stiffness or strength of the material (aorta) under cyclic stresses. During cyclic testing, stress difference was significantly lower in DI low pressure than DC and C low pressure groups. Stress difference was significantly lower in DI high pressure compared to all other high pressure groups. (values are group means ± SEM ; * = statistical significance)

Strain difference was not significantly different between low pressure (DC 1.84±0.22%; C 2.09±0.17%; DI 2.19±0.14%; DA 2.13±0.18%; $p>0.05$) or between high pressure groups (DC 1.82%±0.13%; C 1.86%±0.09%; DI 1.77%±0.09%; DA 1.87%±0.14%; $p>0.05$; See Figure 13).

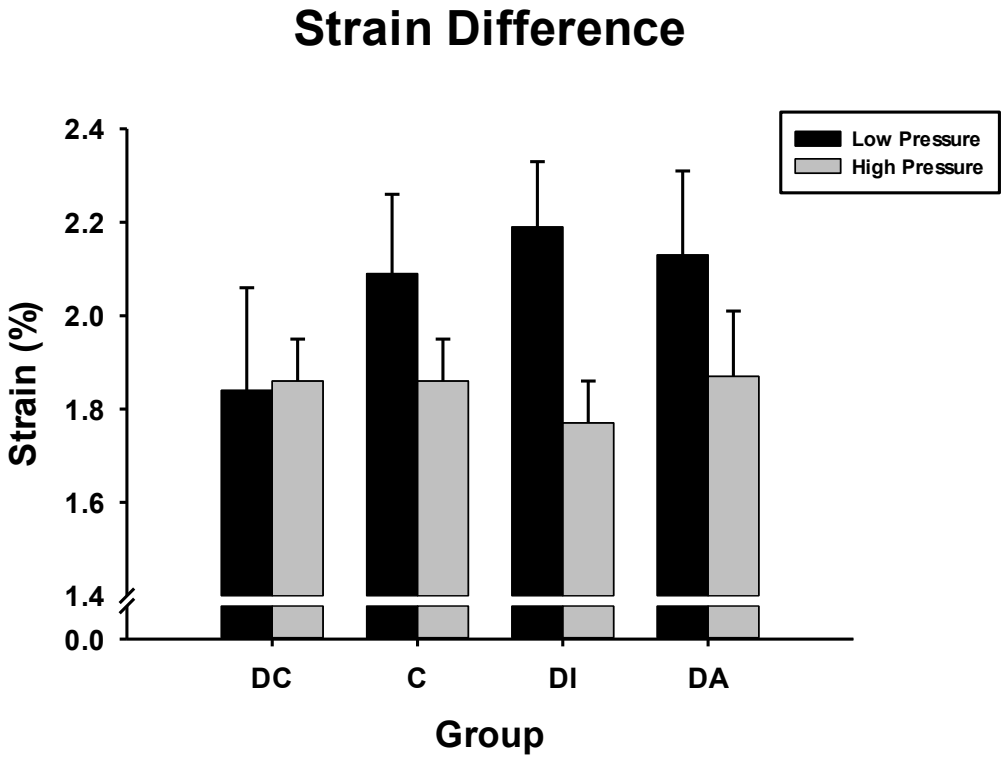

Figure 13: Strain difference is calculated by subtracting the strain at diastolic load from the strain at systolic load. The resulting value determines the ability of the material (aorta) to stretch under varying stresses. During cyclic testing, there was no statistical difference in strain difference between low pressure groups or high pressure groups. (values are group means ± SEM)

CHAPTER V: DISCUSSION

Few studies have assessed the mechanical properties of intact aortic rings in a rat model (Arner & Hellstrand, 1981; Lillie & Gosline, 2006, 2007; Papadopoulo & Delp, 2002; Silver et al, 2003; Tsatsaris, 2004). Furthermore, no study to date has assessed the mechanical properties of diabetic rat aortas. The purpose of this study was to develop a new method for MPT of intact rat aortae, using diabetes as a model for disease which results in arterial stiffening. Previous studies utilized pins, covering 20-40% of the lumen cross sectional area, which may expose samples to forces in a non-physiological manner (Arner & Hellstrand, 1981; Assoul et al, 2008; Lillie & Gosline, 2006; Lillie & Gosline, 2007; Silver et al, 2003; Tsatsaris et al, 2004). The new method for MPT used in the current study used pins which cover approximately 50% (or more) of the total lumen cross sectional area, which more accurately replicates the BP forces experienced by the aorta throughout the cardiac cycle. This model for MPT in combination with collagen/elastin staining will be a useful tool in better understanding the mechanisms responsible for AS development and whether those mechanisms differ by disease state.

Our findings suggest that insulin treated rats had a weaker vessel in comparison to all other groups, which was not an anticipated outcome of the study. Insulin therapy is commonly used to treat diabetes in an attempt to lower risks associated with poor glycaemic control (Muis et al, 2004). However, recent evidence and the results of

the current study suggest that exogenous insulin therapy may contribute to CV complications associated with diabetes (Muis et al 2004; Stout 1990).

Collagen and elastin are predominant components of the aortic wall. The tensile strength and elastic modulus of elastin are low, while that of collagen is high. The relative composition of both components determines the elastic modulus, which is a function of both stress and strain (stress/strain; Matsuda et al, 1987). Therefore, it is reasonable to assume that the increase in wall thickness, and reduction in stress and elastic modulus found in DI rats were attributable to alterations in collagen content relative to elastin as a result of insulin therapy. It is possible that insulin therapy, although beneficial in other aspects of diabetic complications, may have negative effects on the cardiovascular system that outweigh potential benefits (Muis et al, 2004).

DI rats had a thicker vessel wall than all other groups. A plausible reason for this finding was previously studied in a T1D population (Muis et al, 2005). It was found that diabetic patients treated with insulin had significantly greater intima-media wall thickness compared to individuals without diabetes, or those who had been on insulin therapy for fewer years (Muis et al, 2005). Furthermore, animal studies have shown that exogenous insulin leads to greater atherosclerosis risk, and therefore increased CVD risk (Stout, 1990). Development of atherosclerosis consists of plaque formation in the vasculature, which causes arterial lumen narrowing (stenosis), possibly accounting for the greater wall thickness seen in DI groups of the current study (Rekhter, 1999).

Other studies have shown no relationship between intensive insulin treatment and artery wall thickness (Saito et al, 2000). This may be due to differences in pharmacodynamics/kinetics between regular and intermediate acting insulin. Regular

insulin treatment results in immediate, large insulin peaks followed by a rapid clearance, while intermediate acting insulin results in lower insulin peaks with a slower rate of clearance (Woodworth et al, 1994). It has therefore been suggested that cumulative use of regular insulin 8 to 12 years is more harmful than the profile of intermediate-acting insulin, although studies have shown both to result in poor CV outcome (Muis et al, 2005). Without clear distinction between the dose of insulin used, it is difficult to determine whether regular vs. intermediate acting insulin results in different CV outcomes. DI rats in this study were administered insulin doses equivalent to intermediate acting treatment for a period of 6.67 human years. Although it must be noted that rats were given Humulin-N, a type of synthetic insulin used in treating human diabetic patients, which may have a different pharmacodynamic/kinetic profile in rats.

MPT indicated that DI rats had lower modulus compared to DC, C and DA groups, which was significant compared to C aortas. Since diabetes is associated with atherosclerosis development, it was expected that all groups would have a higher elastic modulus (i.e. a stiffer vessel). Typically, when testing mechanical properties of arterial tissue, a lower modulus suggests a more compliant vessel (Karimi et al, 2013). Vessel compliance is the ability of a vessel to increase in volume and distend with increases in BP. It was assumed that the control group would have a compliant vessel with the lowest modulus and the diabetic groups, including DI, would have higher moduli. But the lower modulus in DI groups found in this study may suggest that insulin treatment causes structural alterations resulting in a weaker vessel, with reduced tensile strength.

During cyclic testing, stress difference was significantly lower in DI groups, without a significant difference in strain difference between all groups. This

further suggests reduced resistive force, or tensile strength, of DI aortas during cyclic testing. Failure and cyclic testing results suggested that insulin therapy causes alterations in the structural components of vascular walls (ie collagen or elastin), which may negatively affect aortic strength. If insulin does in fact increase the risk of atherosclerosis development, the results of this study concordant in that the strength of DI aortas was reduced, which may be attributable to the structural adaptations that occur with disease progression.

Although insulin is known to have beneficial effects on the vasculature, such as stimulation of NO release, it has been found that hyperinsulinemia stimulates sympathetic nervous system activity (Arauz-Pacheco et al, 1996). More specifically, those with hypertension may have more sympathetic nervous system activity in response to hyperinsulinemic environments. It is well documented that sympathetic mechanisms, including catecholamine release, are a responsible factor in hypertension development (Weber, 1993). Furthermore, hypertension stimulates inflammatory responses, including macrophage and T-lymphocyte migration, which overtime forms the basis of plaque formation in atherosclerosis (Marvar et al, 2011). Animal models of atherosclerosis suggest that initial inflammatory responses leads to stenosis, characterized by intima thickening, wall stiffness and luminal narrowing. In response to this, consequential changes in shear stress occur and affect the medial layer structure (Golledge & Norman, 2010; Ward et al, 2000). Excessive medial ECM remodeling expands and weakens arteries, possibly explaining well established medial layer thinning and aneurysmal development commonly associated with atherosclerosis (Golledge & Norman, 2010; Ward et al, 2000).

Although T1D patients are commonly referred to as insulin deficient, substantial research suggests that insulin resistance is prevalent in T1D populations. The mechanisms responsible for insulin resistance in T1D remain poorly defined and research suggests a multitude of possible mechanisms for this correlation. It has been suggested that excess growth hormone concentration, infections, insulin induced weight gain, and stress may play a prominent role (Donga et al, 2013; Islam et al, 2013). Despite little understanding of these potential mechanisms, research has shown that insulin resistance in T1D may play a role in micro- and macrovascular complications. Furthermore, inflammatory responses have been associated with higher risk of these complications (Llaurado et al, 2012; Fernandez-Real, 2003). Therefore, it is plausible that the development of insulin resistance in T1D rats, and therefore hyperinsulinemia induced by exogenous therapy seen in the current study may explain the correlation between insulin therapy, atherosclerosis and reduced aortic strength.

Interstingly, a correlation between insulin therapy and CVD risk may be explained by the age of diabetic patients, duration of diabetes, number of comorbidities and degree of insulin resistance (Rensing et al, 2010). Therefore, research is needed to determine the mechanisms responsible for CV complications associated with insulin use in T1D. Although mechanisms underlying the relationship between insulin therapy, atherosclerosis, stenosis and aneurysms are not completely understood, this may be a plausible mechanism for a relationship between prolonged exogenous insulin treatment and increases in aortic wall thickness, resulting in a weaker vessel. Since DI circumference was not larger than other groups, perhaps these aortas were in preliminary stages of aneurysmal development, in which wall thickening had already taken place, and

ECM matrix degradation had just started, accounting for the reduced strength found in this study. However, without testing additional samples, plus staining for collagen and elastin content, this cannot yet be determined.

Although studies have not tested mechanical properties of insulin treated rat aortas, previous studies testing weaker vessels, such as Marfan syndrome and aneurysmal aortas, have shown reduced strength when compared to younger groups (Okamoto et al, 2002; Perejda, 1985; Raghavan et al, 1996). Marfan syndrome is characterized by low levels of fibrillin, which is a protein responsible for elastin production. Consequently, vessels such as the aorta lose their strength with age, become weak and may balloon (aneurysm) at a particularly weak section, which may rupture (Okamoto et al, 2002). Although DI aortas in the current study did not show signs of dilation (ie circumference was not larger than controls), it is possible that the tested samples were in the early stages of abnormal collagen synthesis and breakdown, which is commonly associated with atherosclerotic aneurysms (Rehkter, 1999). This may have caused the mechanical behavior of insulin treated rat aortas to behave in a similar manner.

Conversely, other studies assessing mechanical properties of atherosclerotic aortas have shown increased modulus, or stiffness, when compared with controls (Duprey et al, 2010; Karimi et al, 2013; Teng et al, 2009). It is well documented that atherosclerotic plaque either partially or totally impedes blood flow, causing a cascade of events that leads to arterial wall thickening and stenosis (Karimi et al, 2013). The diseased segments become progressively thicker and stiffer, which has been attributed to collagen accumulation and elastin degradation (Karimi et al, 2013). As previously mentioned, collagen has a high elastic modulus, which would explain the increased

modulus found in other models of atherosclerosis (Duprey et al, 2010; Karimia et al, 2013; Teng et al, 2009). If insulin does accelerate atherosclerotic processes, our results suggest that although stiffness is associated with this condition, MPT revealed a much weaker vessel, with similar properties to aneurysmal models. As atherosclerosis is known to result in aneurysms, our findings suggest that insulin treatment may accelerate the progression from one to the other.

Few studies have investigated the effects of exogenous insulin therapy on cardiovascular outcomes in animal models. In a non-diabetic animal model, it was found that chronic insulin injections increased aortic lipid content and intima thickening, while oral administration decreased lesion size (Sato et al, 1989; Shamir et al, 2003; Stout, 1970). An additional study, investigating the effects of insulin in diabetic dogs found that daily infusion resulted in increased arterial fatty acid and cholesterol content (Cruz et al, 1961). Although together these observational and animal studies do not provide concrete evidence that insulin is pro-atherogenic, they do suggest that insulin is indicated in atherosclerosis progression (Rensing et al, 2010). Due to a lack of studies providing sufficient evidence, the mechanisms responsible for pro-atherogenic effects of insulin in T1D are not well understood. As high endogenous insulin levels increase CV disease risk, it is reasonable to assume an increase in cardiovascular disease risk would be a consequence of exogenous insulin treatment (Muis et al, 2005). Studies investigating potential mechanisms for the increased cardiovascular risk associated with insulin therapy in T1D needs to be further investigated.

The original purpose of this study was to develop a model for MPT of intact aortic rings. Previous studies revealed limitations in the ability to reproduce physiological

changes in BP. Our method exposed forces to a greater lumen cross sectional area in comparison with studies previously performed. This new method was successful and produced novel findings associated with insulin treatment in T1D. Our results suggest that insulin therapy causes structural alterations to the arterial wall, reducing strength of the vessel. It was assumed that control groups would have the lowest modulus, implicating a more compliant vessel, but our results suggests that insulin treatment reduces modulus further, which may signify a reduction in vessel strength. As this method has never been tested in a diabetic model, additional MPT and histological studies are needed to investigate the relationship between insulin treatment, arterial wall structure, and mechanical behavior. Furthermore, this method for MPT will be useful in future studies investigating AS in multiple disease states. Perhaps more accurately replicating physiological BPs utilizing the method developed in the current study, in combination with histological analysis, will provide the knowledge needed to determine to whether mechanisms responsible for AS development differ by disease status or condition.

Limitations

This study contained limitations. Although all aortae were exposed to both diastolic and systolic forces, physiological BPs of rats prior to sacrifice may not be equivalent to those tested by the MPT unit. Small sample size, specimen failure and large variation within each group's results limited the number of tested samples used for analysis and may have masked significant differences between groups. Although rats within each group were exposed to synonymous treatment and housing conditions prior to sacrifice, individual vascular characteristics (i.e. stiffness) may have differed due to

exogenous factors. The testing unit and methodology of the current study has not been performed prior to this experiment; therefore complications not previously expected arose while testing specimens. BP loads were higher than anticipated, which may have affected the results. Therefore, future studies utilizing this method for MPT will involve recalculations of diastolic and systolic loads. Finally, because there is not a standard modulus range for healthy aortas in this rat model, it was difficult to determine at what point a modulus is too low, suggesting a weak vessel and adverse health effects. Future studies will allow for this calculation by refining diastolic and systolic loads to be closer to physiological BP and testing healthy Sprague Dawley aortas using the MPT method used in this study in an attempt to determine standard modulus values.

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