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Fluid Shear-Induced Hypertrophy in Neonatal Rat Ventricular Myocytes

A thesis submitted in partial satisfaction of the requirements for the degree of

Master of Science

in

Biology

by

Atsushi Fujimura

Committee in charge:

Professor Jeffrey H. Omens, Chair
Professor Immo Scheffler, Co-Chair
Professor Chris Armour

2009

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This thesis of Atsushi Fujimura is approved and it is acceptable in quality and form for publication on microfilm and electronically:

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Co-Chair

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Chair

University of California, San Diego

2009

To my parents, for their endless support and confidence in me.

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ABSTRACT OF THE THESIS

Fluid Shear-Induced Hypertrophy in Neonatal Rat Ventricular Myocytes

by

Atsushi Fujimura

Master of Science in Biology

University of California, San Diego, 2009

Professor Jeffrey H. Omens, Chair

Professor Immo Scheffler, Co-Chair

The cardiovascular system modifies its function and structure in response to various stimuli, affecting hemodynamic variables such as arterial blood pressure and blood flow rate. Mechanical stimuli are one such factor, in many cases eliciting a

cellular response. The responses are mediated by mechanotransduction, the mechanism by which cells convert mechanical stimuli into chemical activity.

There have been extensive studies on the effects of mechanical forces and subsequent mechanotransduction in various cell types. For example, in neonatal rat ventricular myocytes, fluid shear has been shown to affect their intrinsic beating rate. Also, in cardiac myocytes, stretching has been implicated in cellular hypertrophy. However, whether fluid shear stress imposed on the outer membrane of myocytes elicits a hypertrophic response remains unknown.

In vivo, cardiac myocytes experience fluid shear stress from the interstitial fluid between sliding layers of myocytes, and possibly due to blood flow in the heart chambers. Using a parallel plate flow chamber, fluid shear of similar magnitude experienced by myocytes in vivo can be applied. To examine the possible role of fluid shear on cardiac hypertrophy, a fluid flow chamber was used to apply shear stress on myocytes. Next, genes commonly upregulated in a hypertrophic response, such as that of atrial natriuretic factor (ANP) and brain natriuretic factor (BNP), was measured and compared to a baseline control.

It was found that neonatal rat ventricular myocytes do respond to fluid shear by increasing BNP expression, indicating a novel mechanotransduction mechanism in load induced hypertrophy.

I. Introduction

A. Background

Both the structural and functional components of the cardiovascular system regulate hemodynamic variables such as arterial blood pressure and cardiac output. Sensing mechanisms continuously monitor these variables. Significant deviations from physiologically nominal values trigger cascades of cellular events to alter cellular and organ function, which attempt to compensate for the offset. For example, an increase in end-diastolic ventricular stress (pre-load) is sensed and triggers volume-overload hypertrophy. [1] This allows increased stroke volume, offsetting the increase in end diastolic volume. In addition, pressure overload (increased afterload) has been demonstrated to cause geometric remodeling of the left ventricle, characterized by decreased compliance of the myocardium, decreasing over-stretching of the ventricle. [2] Although these responses initially are compensatory, maladaptive hypertrophy may develop, which is a common precursor to heart failure.

B. Mechanotransduction

The mechanism by which mechanical forces are sensed and translated into a chemical signal, that then elicits a cellular response, is mechanotransduction. Extensive research has been conducted on mechanotransduction in various cell types. Mechanotransduction is involved in various aspects of physiological responses such as cellular development. [3] Endothelial cells lining the walls of arteries continuously experience fluid shear due to blood pumped out at high velocity from the heart. Studies have shown decreasing blood flow to the carotid artery during development

results in a significant delay in growth of the carotid in rats. [4] Mechanotransduction may also elicit short term effects. Blood cells in circulation, also subject to fluid shear stress, use mechanotransduction to sense and respond to external loading conditions. Particularly, leukocytes respond to shear stress by retracting their pseudopods and decreasing adhesion to the endothelium. [5] The shear response of leukocytes is important in preventing them from becoming trapped in the capillaries in the absence of inflammatory mediators such as PAF. In addition, fluid shear plays a role in bone remodeling. Bone undergoing longitudinal compression redistributes the stress radially through its interstitial fluid. The resulting movement of fluid causes shear stress. [6] This causes osteoblasts and osteocytes to produce signaling factors such as prostaglandins and NO, leading to bone remodeling.

Extensive studies have been conducted on various cells that experience significant fluid shear under normal physiological conditions. These cells include vascular endothelial cells, cells in blood circulation, and cells in the bone matrix.

C. Vascular Mechanotransduction

The arterial walls are subject to two types of mechanical forces: fluid shear and tensile stress due to strain. Blood pressure generated by contraction of the heart causes mechanical strain and stresses that are tensile in the circumferential and longitudinal direction, and compressive in the radial direction. During systole the arteries distend, and relax during diastole. When arteries are subjected to increased cyclic stretch, such as with pulmonary arteries in pulmonary hypertension, mechanosensors in the surrounding smooth muscle detect the strain, transducing the signal into chemical

activity that accommodates the new condition and to restore basal levels of tensile stress in the vessel walls. [7] For example, studies indicate smooth muscles synthesize matrix macromolecules in response to stretch. [8] The increased in collagen in the arterial wall decreases arterial wall compliance, which counteracts the effects of blood pressure and decreases strain in the arterial walls.

Responses to mechanical stimuli may also be acute, rather than involving remodeling of arterial structure. One such study includes the mechanism of reactive hyperemia, where increased blood flow to an arteriole occurs by dilation following a brief occlusion. It was found that the increased pressure following a brief occlusion activates stretch receptors, which lead to an increased peak reactive dilation. [9] This allows more blood and oxygen to be delivered to oxygen deprived tissue from the occlusion, as well as carry away byproducts from anaerobic metabolism, restoring homeostasis.

As blood flows through arteries, a frictional force is exerted parallel to the vessel surface, at the blood-endothelial cell interface. As a result, the endothelial cell membranes are displaced in the direction of the flow, relative to the basement membrane where they are anchored. The magnitude of fluid shear is a function of blood viscosity, flow rate, and the vessel radius. It is expressed as: $\tau=4\mu Q/\pi r^3$. [4] The magnitude of shear stresses in large arteries is in the range of 11 to 30 dynes/cm^2. [10] Fluid shear is sensed by mechanosensors in endothelial cells, which then feedback to elicit a cellular response. Cultured endothelial cells alter their morphology in response to fluid shear. Studies have shown them to align their long axis in the direction of the

fluid shear. [11] In vivo, fluid shear affects vascular growth and remodeling. Fluid shear has been shown to enhance the ability of endothelial cells to migrate and grow. [12] The exact mechanism by which endothelial cells sense fluid shear remains unknown. However, studies have elucidated several candidate proteins. The current understanding is that externally applied forces are transmitted from the membrane embedded proteins, integrins, which are attached to the extracellular matrix, causing activation of many signaling molecules downstream. [13]

Understanding the mechanotransduction in the vasculature may shed light on the development of pathological conditions, such as the development of atherosclerosis. Atherosclerosis, a condition where regions of the artery harden due to plaque build up, occurs in regions where there is high endothelial cell permeability. The permeability between endothelial cells in turn depends on the density of connective proteins, such as VE-cadherin. It was found that different shear patterns have different effects on the expression of VE-cadherin. With a reciprocating flow, the density of VE-cadherin was decreased and endothelial cell permeability increased, as opposed to a laminar flow, the endothelial cell permeability was similar to the control, providing a basis for why atherosclerosis often occurs near vascular bifurcations, where the blood flow is turbulent. [14] These findings indicate that fluid shear is involved in the mechanism of atherosclerosis development. Moreover, the mechanism is dependent on the flow pattern.

Investigating the fluid shear stress activated mechanisms leading to pathological conditions may lead to novel approaches to prevent their development.

For example, restenosis after coronary angioplasty has been linked to changes in the local wall shear stress and mural tensile stress due to the mechanical deformation resulting from the procedure. [7] Evidence shows that arteries remodel to re-establish baseline flow and tensile stress. When fluid shear decreases, the intima remodels to narrow the lumen, increases flow and shear stress. [15] The plaque build up may prevent baseline shear levels to be established leading to closure of the lumen cross section, before the remodeling process stabilizes. Therefore, the initial dilation by the balloon which may have an effect on the long term outcome of restenosis can be modified to minimize disruption in the local flow properties.

D. Mechanotransduction of Cells in Circulation

When blood is pumped through an artery, there is a velocity profile. Velocity of blood at the blood-endothelial cell boundary is 0, and the velocity at the center of the tube is at its maximum. Because the velocity is not constant, different areas of blood cells in circulation are deformed unevenly, exerting shear stress. Leukocytes have fluid shear activated mechanisms. [5] Leukocytes must migrate from the hemopoietic pool across the marrow endothelium. Since there is only negligible fluid flow, migration is accomplished by projection of pseudopods. However, once in blood circulation, leukocyte morphology changes to a spherical geometry by pseudopod retraction. When leukocytes were subjected to fluid shear in vitro, leukocytes retracted their pseudopods, allowing them to roll and travel through blood vessels more efficiently. In addition, it facilitates movement of leukocytes through the microvasculature. When fluid shear was decreased, pseudopod projection resulted.

The response to fluid shear was absent when K+ and Ca++ channel blockers were used, suggesting the mechanism involves K+ and Ca++ channels.

E. Bone mechanotransduction

The macroscopic structure of compact bone is similar to a stiff sponge, with interstitial fluid within these pores known as lacunae and canaliculi. When a compressive force is applied to one end of the bone, a pressure gradient is generated, causing interstitial fluid to flow. The resulting fluid flow exerts a fluid shear on osteocytes. [16] The magnitude of shear stress experienced has been shown to be in the order of 8-30 dynes/cm^2. [17]

Studies have shown that the fluid shear experienced by osteocytes plays a role in bone remodeling. Osteocytes mechano-transduce this fluid shear signal into osteoblast and osteoclast recruiting signals. Osteoblasts are anabolic bone cells responsible for osteogenesis, while osteoclasts break down bone tissue. For example, pulsatile fluid flow causes osteocytes to release signal molecules nitric oxide (NO) and PGE2 in vitro. [18] NO inhibits osteoclast activity in vitro [19] while PGE2 recruits osteoblasts, [20] resulting in adaptive bone remodeling.

F. Cardiac Mechanotransduction

The heart regulates hemodynamic variables in order to maintain adequate perfusion to peripheral cells and tissues. This is accomplished by responding to feedback to variables such as blood pressure, vascular resistance, and neurohormonal stimuli, which alter the function of the heart. Although studies have shown that this

feedback includes many mechanotransduction mechanisms, the variables which trigger the response, as well as the exact pathways remain unknown.

G. Stress and Strain in the Heart

The walls of the heart experiences large magnitudes of stress and shear during each cardiac cycle.

1. Strain

During systole, the average human left ventricle experiences an increase in pressure from about 10 mmHg to 120 mmHg. In addition, the ventricular volume decreases from 140mL to 65mL. [21] As a result, the ventricular walls experience significant deformation and wall strain. Wall strain has been measured using a number of different marker techniques such as sonomicrometry and biplane cineradiography.

2. Fluid Shear Stress

The ventricular wall is composed of synctium laminar sheets of myocytes that are organized transmurally. During systole, myofiber shortening and ventricular wall thickening occur. Consequently, these laminar sheets move relative to each other, causing fluid shear to occur between them. In addition, fluid shear may occur in the interstitium between individual myocytes within laminar sheets as they move relative to each other. [22] Lastly, fluid shear occurs at the blood-myocyte interface in the ventricles as blood is pumped out during each cardiac cycle.

Using the equation: $\tau = \frac{6\mu Q}{ab^2}$ [23], where μ is dynamic viscosity, volume flow rate Q, the width of the chamber *a*, and the height of the flow region ***b***, the magnitude fluid shear can be calculated. Given the following parameters, the fluid shear during

systole in vivo was approximated to be 50 mdyn/cm^2: distance between fiber sheets – 10 micrometers, relative velocity 50 micro meter/sec. [24]

H. Myocyte Response to Fluid Shear Stress – Increased beating rate

Cardiac myocytes, which also experience shear in vivo exhibit fluid shear stress activated mechanisms. Cultured neonatal rat ventricular myocytes beat spontaneously and when subjected to low shear rates, increase their beating rate instantaneously. Although the initial beating rate in non-sheared myocytes may be variable between cultures, they all exhibit a relative increase in beating rate of 80 ± 14%. Similar results were obtained when shearing media with higher viscosity were used, thus suggesting that the myocytes are more sensitive to shear rate rather than shear stress. The increase in beating frequency isn't dependent on oxygen concentration, which was demonstrated by measuring the oxygen concentration in the chamber during shear. A transport phenomenon appears unlikely to be involved in the response. Using gadolinium, a potent stretch activated channel blocker, it was shown that stretch activated channels do not appear to be involved. However, a study reported that fluid jet pulses can trigger action potentials in NRVMs, and that this is mediated through stretch activated channels. [38] The reported mechano-electric feedback may be related to the shear response as well and warrants further study. Interestingly, when cells are sheared with serum free media, the response was significantly attenuated. It appears that a component in the media is necessary to elicit the fluid shear response. Moreover, the response appears to share a similar pathway with the beta-adrenergic stimulation. However, the beta adrenergic receptors are not required for the response,

and instead the overlap between the pathways occurs further downstream. Lastly, the response is mediated by adhesion molecules such as integrins interacting with the extracellular matrix.

I. Cardiac Hypertrophy

Cardiac hypertrophy is the response by cardiomyocytes to mechanical (i.e. pressure and volume overload in the heart) and neurohormonal stimuli (i.e. growth factors, angiotensin II, aldsoterone).

This leads to the enlargement of the heart and the thickening of the ventricular walls due to increased cardiomyocyte size with increased protein synthesis and changes in the organization of the sarcomeric structure. The phenotypic pattern of organization of sarcomeres is dependent on the type of mechanical stimuli, and can be broadly categorized as either eccentric or concentric. Concentric hypertrophy, where sarcomeres are assembled in parallel and resulting in a relative increase in width of the cardiomyocytes, occurs in response to pressure overload. This pattern of hypertrophy may result from chronic hypertension and aortic valve stenosis is accompanied by increased thickness of the ventricular wall and septum, decreasing the ventricular chamber volume. In eccentric hypertrophy, which occurs in response to volume overload, sarcomeres are assembled in series and results in a relative increase in length of the cardiomyocytes. This pattern of hypertrophy may result from myocardial infarction and is associated with cardiac dilation and dilated cardiomyopathy.

At the molecular level, there is commonly the reinduction of the fetal gene program; so called for it similarity of expression pattern during embryonic

development. Some commonly activated genes include the immediate early genes (c-jun, c-fos, c-myc) and the fetal genes (Arterial Natriuretic Peptide ANF, Brain Natriuretic Peptide BNP), which are commonly used as hypertrophic markers.

J. Sensing Mechanisms involved in Mechanical Load Induced Hypertrophy

1. Stretch Activated Ion Channels

Mechanosensitive ion channels respond to mechanical stimuli by regulating the current of ions into the cell. For example, mechanosensitive ion channels are responsible for senses such as touch, hearing, and balance. Mechanosensitive ion channels have also been implicated as the transduction mechanism from load to protein synthesis in cardiac hypertrophy. The study utilized a patch clamp technique to show that stretch applied to cardiomyocytes increase the influx of Na^+, which may then increase protein synthesis. [25]

In another study a calcium-dependent phosphatase calcineurin has been shown to be involved in cardiac hypertrophy. Transgenic mice that express the activated form of calcineurin developed hypertrophy and heart failure. [32] Using a calcium binding fluorescent dye, it was determined mechanical stress induces calcium influx in cardiac myocytes. [27] These studies show that mechanosensitive ion channels are good candidates as initial responders in mechanically induced hypertrophy. The subsequent change in ionic fluxes may affect the electrical activity and beating rate of cardiac myocytes. It is plausible that fluid shear, which affects the beating rate of cardiac myocytes, may also induce an ion channel mediated hypertrophic response.

2. Cell cytoskeleton-Extracellular Matrix Interaction

Interactions between the cell cytoskeleton and the extracellular matrix have been implicated in transducing mechanical stress. Integrins are an example of such transmembrane proteins that bind the intracellular cytoskeleton to ECM proteins. Stretch-induced activation of the hypertrophic pathway involving p38 MAPK was significantly reduced using PTEN, a novel inhibitor of the outside-in signaling of integrins. [28] Morever, studies indicate that the interaction between B1 Integrins and extracellular matrix proteins play a role in the hypertrophic response of rat ventricular myocytes. Agonists such as phenylephrine (PE) are used to create a model for hypertrophied neonatal ventricular cardiomyocytes. Ventricular myocytes that are plated on an anti-B1 integrin antibody and stimulated with PE induces expression of hypertrophic markers. However, ventricular myocytes plated on an integrin independent substrate gelatin, shows no response to PE. [29] These results collectively suggest that integrins and their interaction with the ECM play a significant role in sensing mechanical strain and transducing it to induce a hypertrophic response in cardiac myocytes. Interestingly, beta 1 integrins have also been implicated in the chronotropic response of cardiac myocytes to fluid shear. [24] Based on this involvement of integrins in the shear response to increase the beating rate, it is plausible that the shear response may also include a hypertrophic response.

K. Hypertrophic Signaling Cascades

Studies have shown that many signaling cascades are involved in the activation of gene expression that's associated in cardiac hypertrophy. Some of these include the calcineurin-NFAT circuit, p13K-AKT-GSK-3 Dependent Signaling, MEF2/HDAC pathway, G Protein Coupled Receptors, and MAPK pathways. [30] It is increasingly becoming evident that there is considerable overlap between these different pathways and the second messengers. The interactions of these signaling cascades that lead to cardiac hypertrophy is complex and remains poorly understood.

1. MAPK Signaling Pathway

Mitogen Activated Protein Kinases (MAPK) signaling pathways provide a link between external stimuli (such as stretch) and the reprogramming of cardiac gene expression. MAPKs consists of three major subfamilies: extracellularly responsive kinases (ERKs), c-Jun –terminal kinases (JNKs), and p38 MAPKs. Activated MAPKs phosphorylate intracellular targets, including transcription factors that regulate gene expression. For example, overexpression of MAPK phosphatase, an enzyme which inhibits MAPKs, blocked pressure-overload associated hypertrophy in vivo. [31] This provides evidence that the MAPKs play a significant role in mechanical stress induced hypertrophy.

2. NFAT/Calcineurin pathway

Calcineurin is a Ca2+ dependent serine/threonine protein phosphatase. Transgenic mice expressing activated calcineurin induce heart enlargement and eventual heart failure, indicating its significant role in cardiac hypertrophy. [32] When

intracellular Ca2+ levels increase, calcineurin becomes activated, which then bind to NFAT transcription factors. Calcineurin dephosphorylates NFAT, which then move into the nucleus leading to the activation of genes characteristic of cardiac hypertrophic response. [33]

L. Gene Expression in Hypertrophic Myocytes

Cardiomyocytes respond to hypertrophic stimuli by undergoing successive changes in their gene expression. Mechanical stress has been shown to activate the early response genes, which include c-Fos, c-Jun, c-Myc, and BNP, as early as 30 minutes from the initial stimulus, and peaks within the first hour. [34, 35] The magnitude of stretch experienced by the ventricular wall appears to correlate with the amount of c-Fos expression in extracorporeal perfusion experiments. [36] Studies show the upregulation of these genes, with the exception of BNP, are transient, and return to baseline levels after a few hours, even when the hypertrophic stimulus persists. [35]

Within 6 to 12 hours of the hypertrophic stimulus, the fetal program (so-called because it results in the re-induction of structural proteins from the adult form to the fetal isoforms) becomes upregulated. [37] The upregulated gene products, which include ANP, ß-myosin heavy chain, skeletal α-actin (sACT) amongst others, regulate contractility and calcium handling. These changes affect cardiac function, and underlie the progression of cardiac hypertrophy to heart failure.

1. Beating rate and ANP Expression

The contractile activity in neonatal rat ventricular myocytes has been shown to modulate the expression of atrial natriuretic factor. For example, when the spontaneous contractile activity of NRVM was arrested, the level of ANP expression was markedly decreased. Different calcium blockers were used to delineate the affects of contractile activity and intracellular calcium, and their affect on ANP expression. These studies showed that the inhibition of contractile activity significantly decreased ANP expression independently of intracellular calcium. [39]

When the contractile rate of cardiac myocytes was increased using electrical stimulation to 3 times its intrinsic rate (from 1 Hz to 3 Hz) over 3 days, the ANF expression increased nearly 8 fold. [40]

2. Substrate Stiffness and Gene Expression

Substrate stiffness has been shown to affect the functional maturation in isolated NRVMs. [41] Cells plated on a substrate similar to the native myocardium generated the greatest mechanical force, had the greatest calcium handling proteins, compared to those plated on softer or stiffer substrates. Additionally, surface texture has been shown to affect the expression of ANF. [42] ANF and β myosin heavy chain were shown to be expressed higher when cells were plated on plasma etched films. These results demonstrate that in addition to the interaction of specific cell membrane-extracellular matrix proteins, substrate stiffness and surface texture can affect gene expression in cardiomyocytes.

M. Significance

The traditional notion was that cardiac hypertrophy is a compensatory response that transiently normalizes the biomechanical stress and optimizes cardiac pump function. However, prolonged hypertrophy has been implicated in arrhythmogenicity and the development of heart failure.

The complex molecular processes that lead to cardiomyocyte growth involve membrane receptors, second messengers, and transcription factors. The specific mechanism by which myocytes sense mechanical stress and transduce them to chemical signals which induce cardiac hypertrophy remain poorly understood. Thus, it is crucial to investigate possible stimuli and their signaling pathways that are involved with hypertrophy.

Studies have shown that external mechanical stress increases protein synthesis rate and induces hypertrophy in neonatal myocytes. [49] In all previous studies, these stresses have been applied via cell stretch – applying normal stress to cells in either the axial or traverse directions with respect to the cell axis or with more arbitrary stresses applied to "stellate" neonatal cells. These external mechanical strains promote hypertrophy by increasing the rate of expression of proteins N-cadherin, Connexin 42, Actin, BNP and ANP. [50] BNP and ANP are acute hypertrophic markers, whose expression is upregulated in response to such external mechanical stress [30].

In the heart, cardiomyocytes are subjected to both fluid shear and shear between adjacent layers of cells. As of yet however, the role of shear stress applied to myocytes cell membrane remains unknown. Moreover, under pathological conditions such as volume overload, the shearing pattern of myocytes may be affected. This

shearing of myocytes may be a contributing factor in volume or pressure overload induced hypertrophy as well.

Ultimately, identifying the similarities and differences between the signaling systems responsible for promoting the generation of a pathological, versus a compensatory hypertrophic response may enable us to modulate hypertrophic growth, achieving a clinical benefit without provoking hemodynamic compromise.

N. Hypothesis

Both mechanically induced cardiac hypertrophy and the chronotropic response of cardiac myocytes response to fluid shear share common mediators such as integrins and Ca2+ influx. We will test the hypothesis that neonatal rat ventricular myocytes can sense externally applied *fluid-shear stress*, and this type of stress can regulate the hypertrophic response of the cells. Fluid-shear stress will be applied to a confluent layer of neonatal rat ventricular myocytes using a fluid flow chamber. The expression of hypertrophic markers will then be assessed using RT-PCR to assess whether they are upregulated. If fluid shear stress can regulate the hypertrophic response, we expect to see a higher expression level.

II. Materials and Methods

A. Preparation of Substrates to Plate the Cardiomyocytes

Three glass microscope slides (75 x 38 mm) were placed in each square cell culture dish. The dishes were then sterilized by exposing them to UV light in the fume hood for 7 minutes on each side. The slides were then coated with 7mL of Sure Coat, Collagen type II, or Fibronectin solution and placed in the incubator at 37° C for 2 hours to allow protein adsorption. The solution was removed and the slides were set to dry in the fume hood for 30 minutes.

Three transparent silicone elastomeric membranes (75 x 38 mm, 0.25 mm thick gloss finish; Specialty Manufacturing, Saginaw, MI) were prepared using the same methods described for the glass microscope slides.

Silicone membranes were installed in the equibiaxial cell stretch device. The elastic membrane was clamped into the square groove using a rubber O-ring. The membranes were UV sterilized for 15 minutes, coated with 7 mL of Sure Coat solution and placed in the incubator at 37° C for 2 hours. The solution was removed and set to dry in the fume hood for 30 minutes.

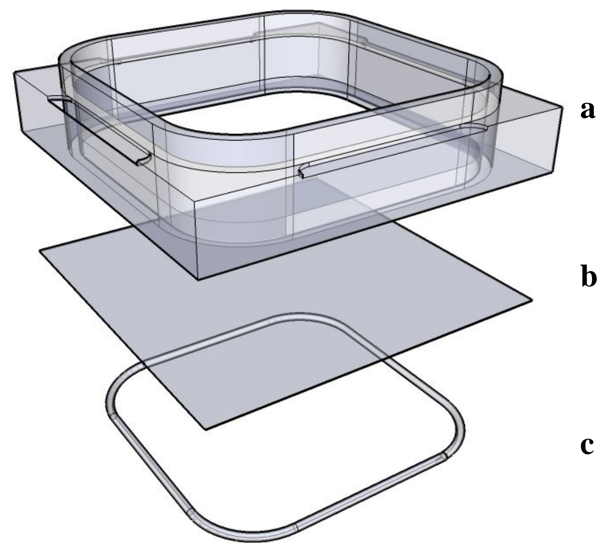


Figure 1: 3 Dimensional Schematic of Silicone Membrane for culturing cardiomyocytes. (a) Membrane holder (b) Silicone Membrane (c) O-ring

B. Isolating Neonatal Rat Ventricular Myocytes

Primary cultured myocytes were prepared from ventricles of neonatal (1-2 day old) Sprague-Dawley rats using a standard isolation kit (Cellutron Life Technology, Highland Park, NJ). Rats were washed by briefly submerging them in a 70% ethanol solution. They were decapitated and their hearts excised quickly. Any connective tissue and atria were carefully trimmed away from the heart. Ventricles were then stirred for 20 minute periods in a digestion buffer (collagenase) at 37º C. The cell suspension was centrifuged for 2 minutes at 1200 rpm and the supernatant was removed. This cycle was repeated 5 additional times. The cells were counted and diluted to 1 million cells/ 1mL of solution. The cells were then pre-plated in uncoated plates for 1-2 hours at 37ºC in order to reduce the contamination of cardiac fibroblasts. The cells were plated onto slides (7 million/dish) and membranes previously prepared

(7 million/membrane).(Figure 1) 15 mL of plating medium, containing 15% horse and fetal bovine serum was added to promote myocyte differentiation and placed in the incubator at 37º C. Plating medium was exchange for standard maintenance medium, containing 6% serum, after 24 hours. The maintenance medium was replaced every 24 hours thereafter. The culture was incubated for 4 to 5 days to allow them to spread to a monolayer with observed spontaneous beating.

C. Laminar Flow Assay

A mechanical syringe pump and flow chamber were used to impose shear stress on cultured myocytes. (Figure 2)

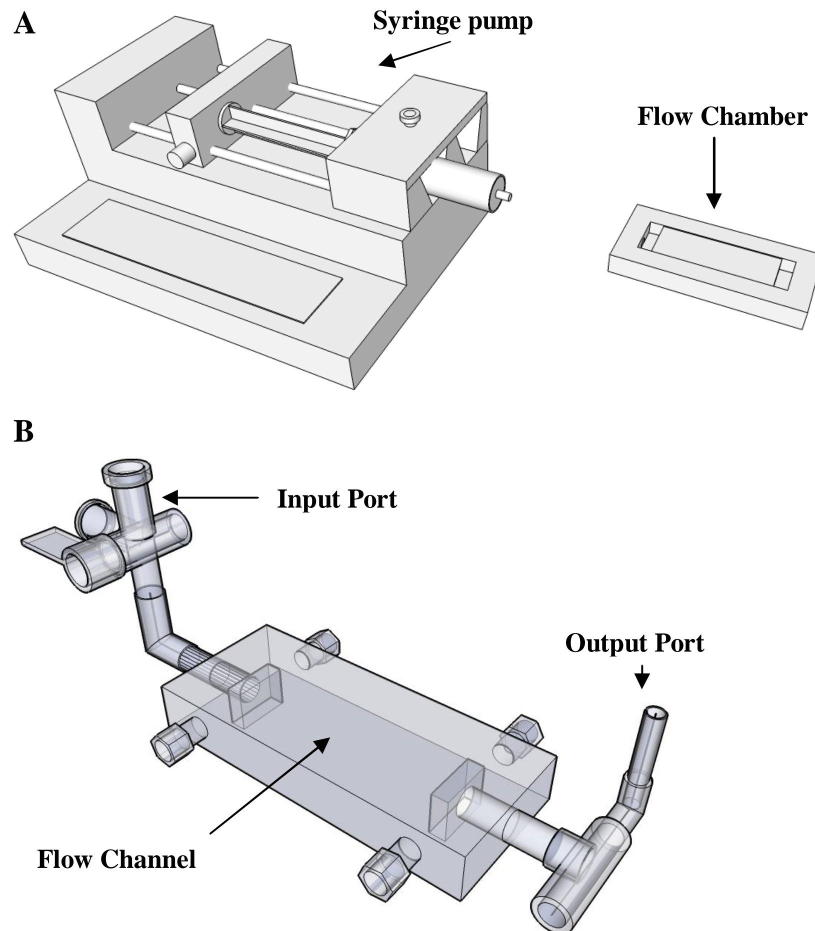


Figure 2: A) Schematic of parallel plate flow chamber apparatus. B) Parallel plate flow chamber

75 x 38 mm glass slides (0.025 cm in height, 2.5 cm in width, and 5.0cm in length), (Figure 3)and silicone membranes (Figure 4) with a confluent myocyte monolayer was mounted in a rectangular flow channel created by sandwiching a silicone gasket between the flow chamber and the myocyte monolayer.

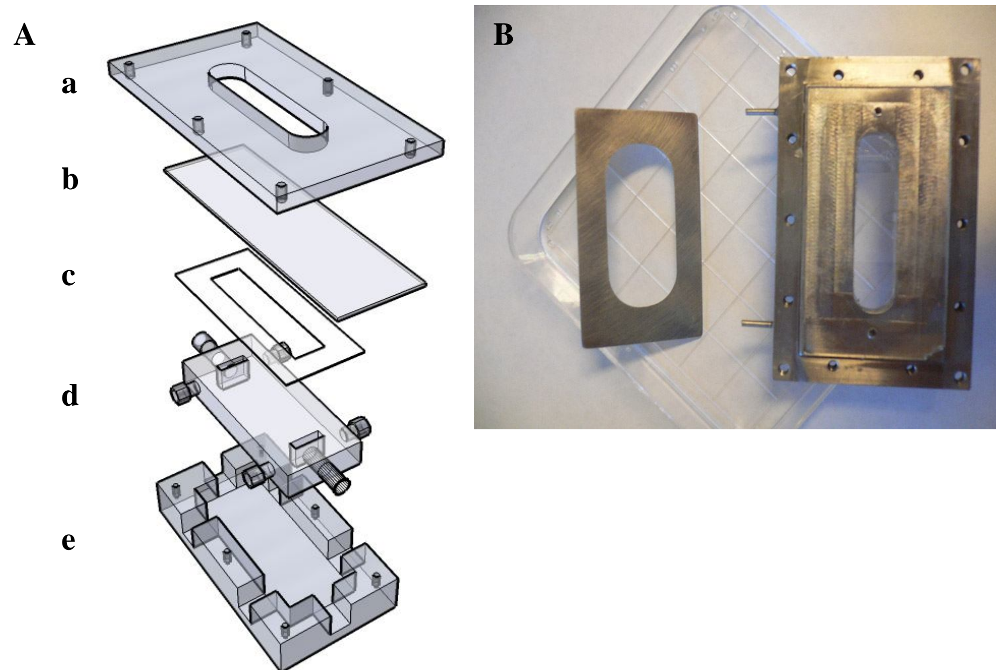
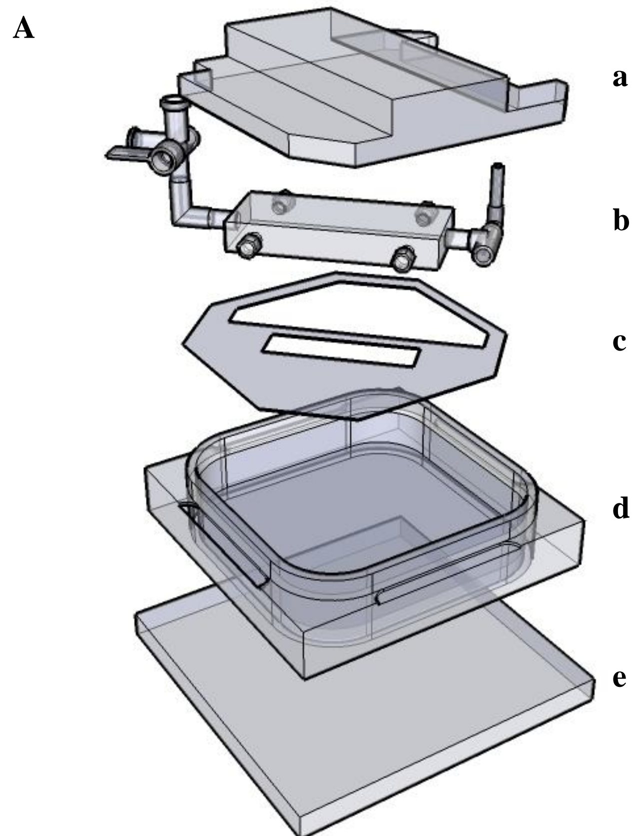


Figure 3: (A) 3 dimensional schematic of flow chamber apparatus used for cardiomyocytes plated on glass microscope slides: (a) Top plate (b) Glass Microscope Slide (c) Silicone Gasket (d) Flow Chamber (e) Bottom Plate (B) Picture of Flow Chamber Apparatus. Parameters for calculating Shear Rate and Shear Stress: Gasket thickness: 127 µm. Flow channel width: 2.5 cm.



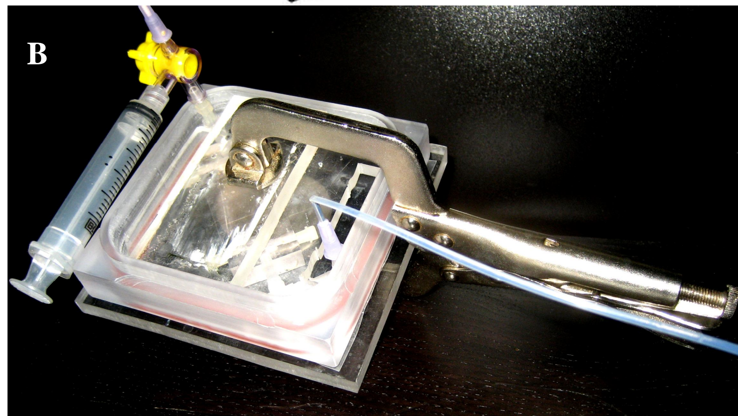


Figure 4: (A) 3 Dimensional Schematic for Flow Chamber Apparatus for shearing Cardiomyocytes plated on Silicone membranes. (a) Top Spacer Plate (b) Flow Chamber (c) Gasket (d) Silicone Membrane Holder (e) Bottom Plate. (B) Picture of Flow Chamber Apparatus for Shearing Cardiomyocytes plated on silicone membranes. Parameters for Calculating Shear Stress and Shear Rate: Gasket thickness: 118 μm. Flow channel width: 1.1 cm.

The channel has an inlet and outlet for perfusing the cultured cells allowing medium flow to be introduced as laminar flow over the entire width of the chamber. Myocytes were sheared by introducing medium into the flow chamber apparatus using a mechanical syringe infusion pump (Harvard Apparatus, Holliston, Ma.). (Figure 5)

Figure 5: Mechanical Syringe Infusion Pump

During the flow experiments, the system was kept at 37º C in a constant temperature hood, and the circulation medium was ventilated at 95% humidified air and 5% CO_2.

D. Beating Rate Measurement

The beating rate of the myocytes before and after shear, were determined using a pixel value threshold algorithm. Using a frame grabber software, a rectangular area of interest was selected on the microscopic image. Images were sampled at 10 frames

per second for a duration of 1 minute and compiled into a stack of digital TIFF images. The algorithm was used to crate an M-mode image by selecting a linear cross section of each of the TIFF image frames, then plotting them over time.

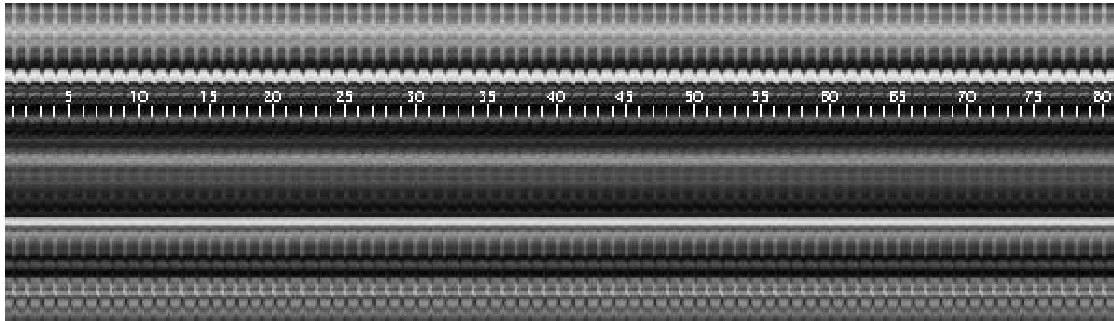


Figure 6: Myocyte Beating Frequency M-Mode Image

The maximum and minimum pixel value from the M-mode Image was then used to determine the beating rate. This was done by taking the halfway point between the maximum and minimum values, and using this as the threshold. Whenever the pixel value is not changing over time, the myocyte is contracting. The number of contractions was counted and the average value was determined.

E. Isolating RNA

Total RNA was extracted from the myocytes in the control and shear groups by using 500 microliters of Trizol reagent (Invitrogen) to lyse the cells. The samples were collected into 1 mL centrifuge tubes and incubated at room temperature for 5 minutes. 20% of chloroform by volume was added to solution, and then shaken for 15 seconds. The solution was incubated for 2 minutes at room temperature and centrifuged. This separated the solution into an aqueous phase and an organic phase. RNA remained exclusively in the aqueous phase, while DNA and proteins separated into the interphase and organic phase respectively. The organic phase was removed, and 50%

isopropanol by volume was added. The solution was incubated for 10 minutes at room temperature and centrifuged at 2-8 C for 10 minutes at 12,000g. Addition of isopropyl alcohol precipitated RNA pellets, which was washed with 75% ethanol and dissolved in RNAase free water. The purity of the extract was determined measuring the optical density at 260 and 280 nm.

F. Reverse Transcribing RNA to cDNA

The isolated RNA was reverse transcribed into cDNA using the iScript™ cDNA Synthesis Kit. The kit contains a RNA-dependent DNA polymerase (i.e. reverse transcriptase), buffers, and oligonucleotides used to construct the complementary DNA strand. By reverse transcribing RNA to cDNA makes it possible to run semi-quantitative PCR in the subsequent experiments to quantify gene expression levels. The success of reverse transcription was verified by measuring the optical density at 260 and 280 nm.

G. Primer Design

Exon sequences for rat ANP and BNP were obtained from the NCBI Blast Database (http://130.14.29.110/BLAST/). Primers were designed to amplify exon sequences for ANP and BNP using primers complementary to sequences flanking the amplicom. (Operon technology)

	Sequence 5' to 3'
ANP Forward	CTC TTG AAA AGC AAA CTG AGG GC
ANP Reverse	TGG GCT CCA ATC CTG TCA ATC
BNP Forward	TAG CCA GTC TCC AGA ACA ATC CAC
BNP Forward	TGT CTC TGA GCC ATT TCC TCT GAC
GAPDH Forward	AGA CAG CCG CAT CTT CTT GT
GAPDH Reverse	CTT GCC GTG GGT AGA GTC AT

Figure 7: Primer Sequence

H. SYBR Green RT-PCR

RT-PCR was performed using the iQ SYBR Green Super Mix (BioRad). 45 amplification cycles were performed with PCR denaturation set at 95° C, PCR annealing at 58° C, and PCR extension at 72° C. cDNA was quantified by measuring fluorescence from the SYBR-green dye and plotting it against the cycle number, which binds to ds-DNA products. The Ct value, which indicates the fractional cycle number where the fluorescence reaches an arbitrary value, was obtained. The differences in the Ct values between the target genes (ANP and BNP) and the reference gene (GAPDH) were used to calculate the relative amount of ANP and BNP expression using the $2^{-\Delta \Delta Ct}$ method.

I. Primer Fidelity Assay

Primer fidelity was assessed performing western blots on the PCR products.

J. Statistical Analysis

All data are expressed as means ± SEM. Paired student's t-tests were performed to compare the relative expression of BNP and ANP normalized to GAPDH in control and shear groups. Differences at $p<0.05$ were considered statistically significant.

III. Results

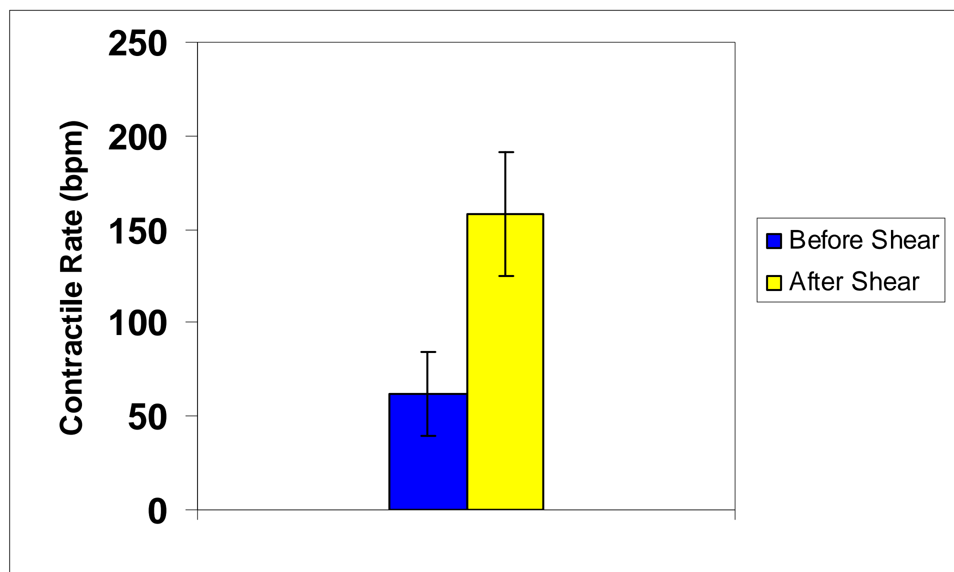


Figure 8: Beating rate of cardiomyocytes before and after shear. Shear rate: 58 sec^{-1}

Figure 8 shows the increase in spontaneous beating rate of myocytes due to shear. Beating rates increase nearly 3 fold, which is similar to previous studies conducted. ($n=6$, 22 ± 22 bpm vs. 158 ± 33 bpm, $p<0.01$) [24] This shows that the shearing apparatus used applied fluid shear successfully to the confluent layer of cultured myocytes.

Table 1: OD Values of RNA Extract and cDNA

	OD 260	OD 280	OD 260/280
RNA extract	0.051	0.027	1.89
cDNA	0.007	0.003	2.33

The purity of RNA extract was determined using the ratio of spectrophotometric absorbance at 260 nm:280nm. This ratio was above 1.8 for the RNA extract, indicating there were no contaminants such as proteins or phenol and

thus successful. The ratio for the cDNA sample was above 2.0 at 2.33, indicating the RNA was successfully reverse transcribed into double stranded DNA.

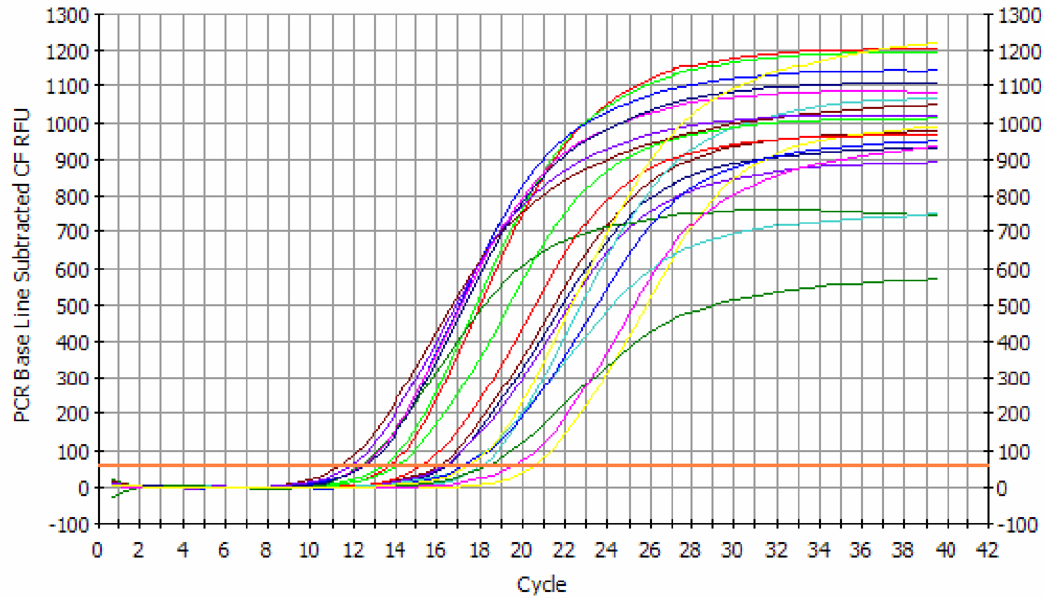


Figure 9: Changes in Fluorescence during RT-PCR

Figure 9 is a sample of the changes in fluorescence detected during RT-PCR. As the target gene is amplified, the detected fluorescence increased. The cycle number at which the fluorescence reached a critical value was used in subsequent steps to calculate relative gene expression levels.

Table 2: Ct Values Obtained from the Changes in Fluorescence during RT-PCR

	Ct values
GAPDH No Shear	19.69
GAPDH Shear	15.53
BNP No Shear	20.05
BNP Shear	17.80

The Ct values were used to calculate the gene expression of BNP and ANP relative to GAPDH using the using $2^{-\Delta \Delta Ct}$ method. Sample calculations are shown below.

<u>BNP No Shear normalized to GAPDH No shear</u>
½ (BNP No Shear – GAPDH No shear)
=1/2 (20.05-19.69)
=1.2

<u>BNP Shear normalized to GAPDH Shear</u>
=1/2(BNP Shear – GAPDH Shear)
=1/2(17.80 – 15.53)
=2.27

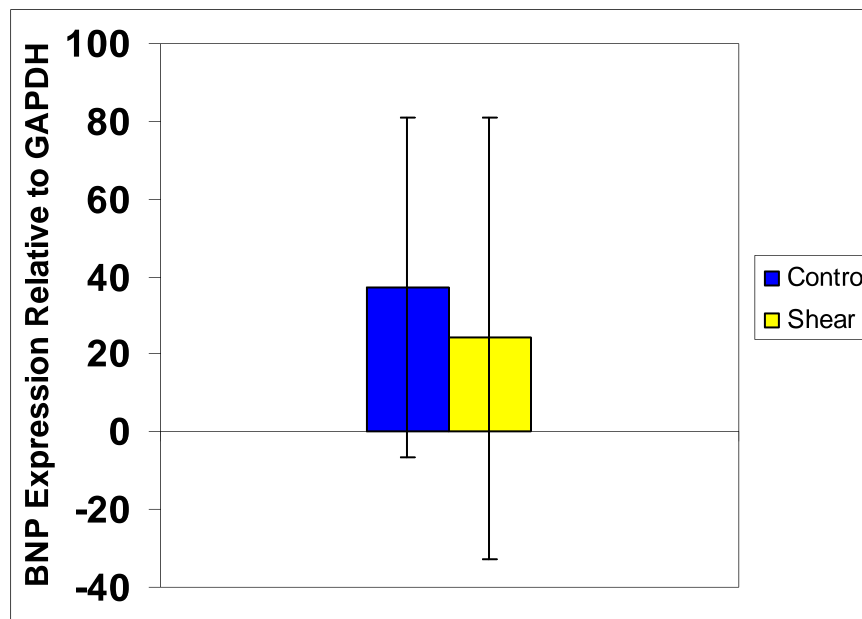


Figure 10: BNP Expression relative to GAPDH for Cardiomyocytes on Glass Slides + Type II Collagen. Shear rate: 58 sec^{-1}

Figure 10 shows the expression level of BNP normalized to internal control GAPDH for cardiomyocytes plated on glass slides coated with Type II Collagen. The cells were sheared with maintenance media, a Newtonian fluid, at a shear rate of 58 sec^{-1}. It was inconclusive whether there was a significant difference observed in the BNP level relative to GAPDH, between the NS and the sheared group, due to high variance in the data sets ($n=17$, 37.42 ± 43.73 vs. 24.17 ± 56.83, $p=0.472658$)

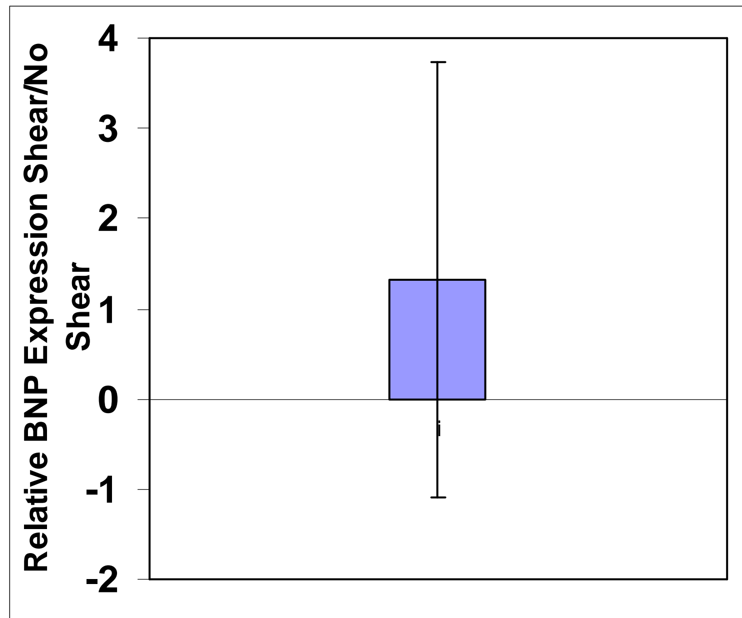


Figure 11: Shear/No Shear Ratio for BNP for Cardiomyocytes on Glass Slides + Type II collagen. Shear rate: 58 sec^{-1}

In order to see if myocytes responded to shear despite variability in the controls, the ratio of BNP expression of shear to no shear was taken for individual data sets.(Figure 11) There was no conclusive difference found before and after shear in BNP expression levels (n=17, 1.32 ± 2.42, p=0.600)

Figure 12: Primer Fidelity Assay

Gels were run using the PCR products to see if the primers were responsible for the variance in gene expression levels, as determined from Ct values using RT-PCR. Figure 12 shows 1 band in each lane, indicating 1 amplicon. Therefore, it is unlikely that primer fidelity is responsible for the variance in gene expression of GAPDH and BNP.

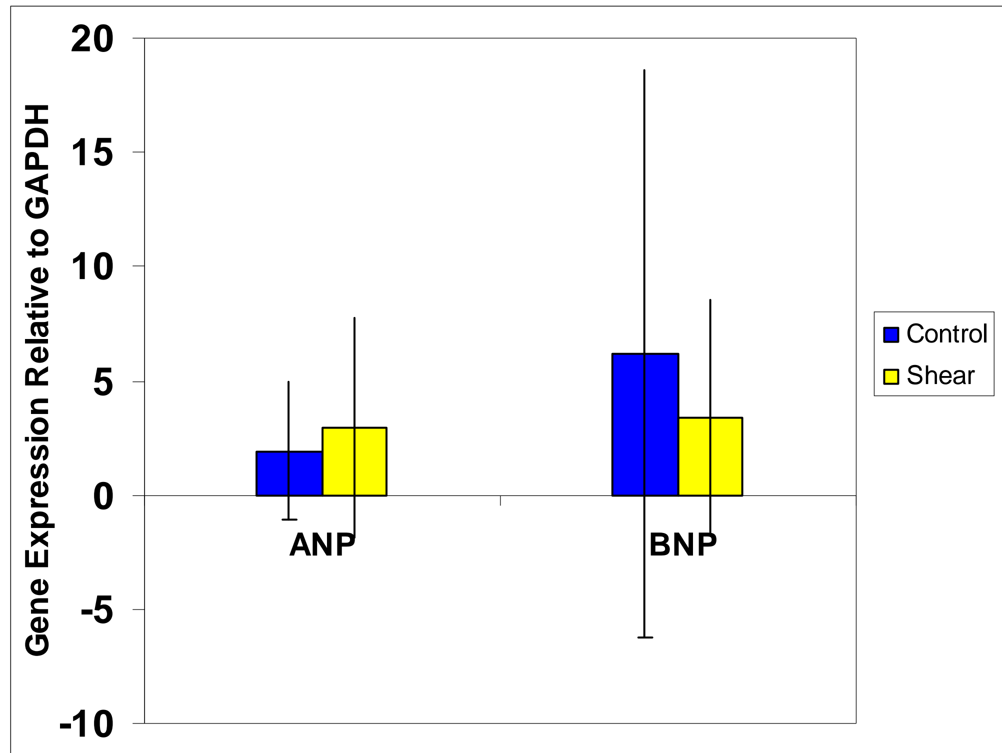


Figure 13: BNP and ANP Expression relative to GAPDH for Cardiomyocytes on Glass Slides + Surecoat. Shear rate: 58 sec^{-1}

An experiment looking at different plating substrates was performed to obtain a control that shows a consistent expression level of BNP/GAPDH and ANP/GAPDH. Figure 13 shows the expression level of BNP normalized to internal control GAPDH for cardiomyocytes plated on glass microscope slides coated with Surecoat. And a shear rate of 58 sec^{-1}. The large variance in BNP and ANP expression levels, even within the Non-sheared (NS) group, led to an inconclusive difference observed between NS and the sheared group. (BNP; n=18, 1.91 ± 3.01 vs. 2.93 ± 4.83, p=0.459) (ANP; n=18, 6.21 ± 12.41 vs. 3.38 ± 5.19, p=0.378)

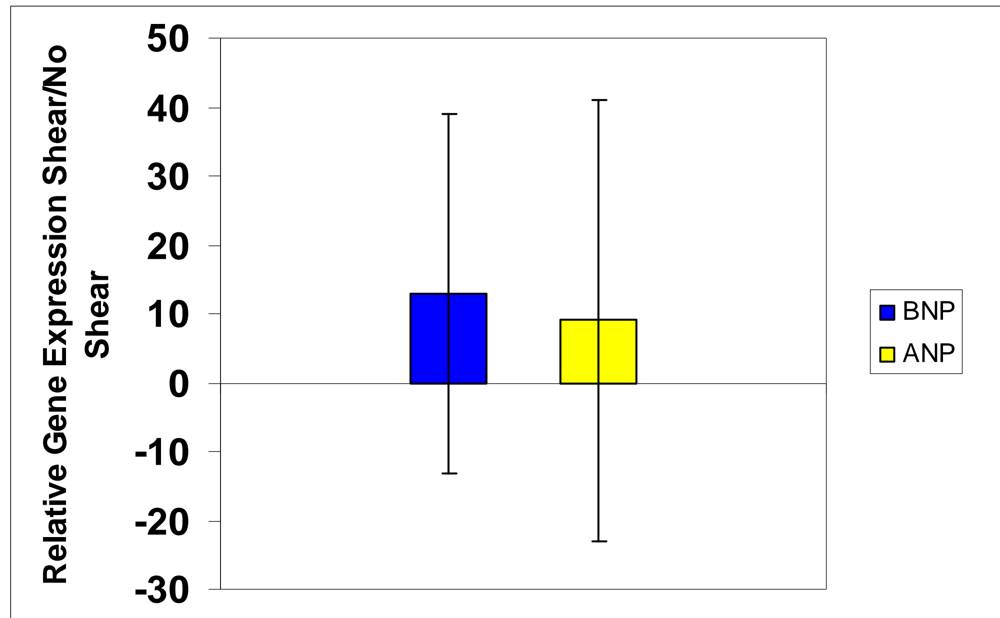


Figure 14: Shear/No Shear Ratio for BNP & ANP for Cardiomyocytes on Glass Slides + Surecoat. Shear rate: 58 sec^{-1}

In order to see if myocytes responded to shear despite variability in the controls, the ratio of BNP expression of shear to no shear was taken for individual data sets. (Figure 14) There was no conclusive difference found before and after shear in BNP expression levels (n=18, 1 ± 0.05 vs. 13.00 ± 26.73 p=0.07) and ANP expression levels. (n=18, 1 ± 0.05 vs. 9.02 ± 32.07, p=0.28).

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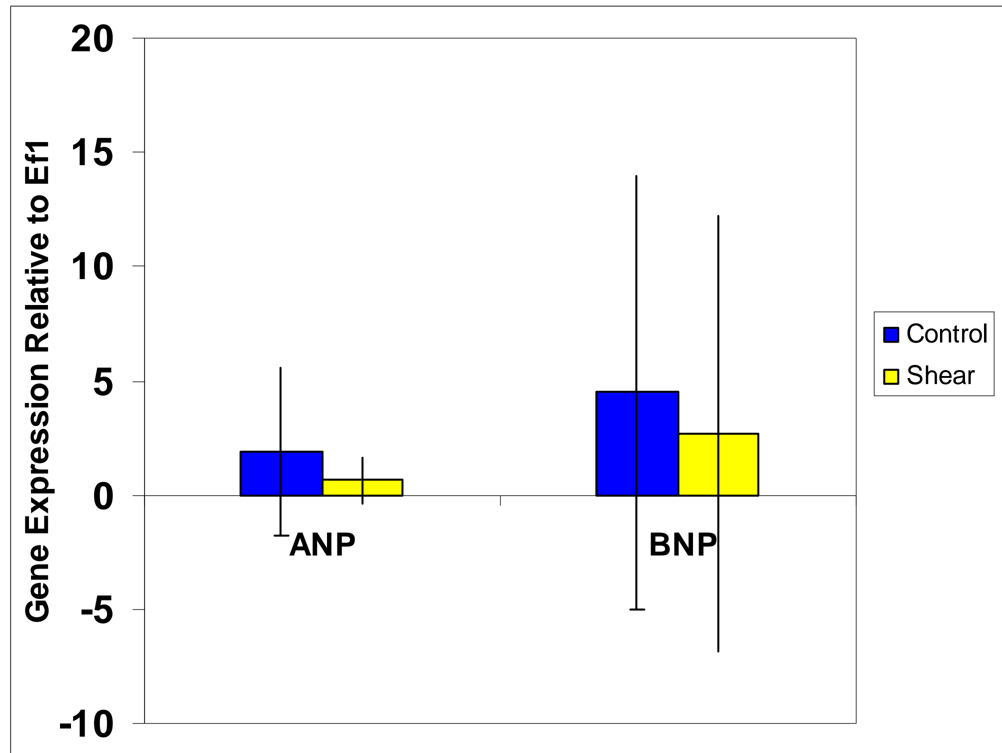

Figure 15: BNP and ANP Expression relative to Ef1 for Cardiomyocytes on Glass Slides + Surecoat. Shear rate: 58 sec^{-1}

An experiment was conducted to investigate whether a different internal control, other than GAPDH, will provide a more consistent expression. The graph shows BNP and ANP levels normalized using elongation factor 1 (EF1), for cardiomyocytes plated on glass microscope slides coated with Surecoat. (Figure 15) The cells at a shear rate of 58 sec^{-1}. A large variance in BNP and ANP expression levels led to an inconclusive difference observed between NS and the sheared group. (BNP; n=8, 4.49 ± 9.51 vs. 2.68 ± 9.51, p=0.667) (ANP; n=8, 1.91 ± 3.65 vs. 0.63 ± 1.02, p=0.377)

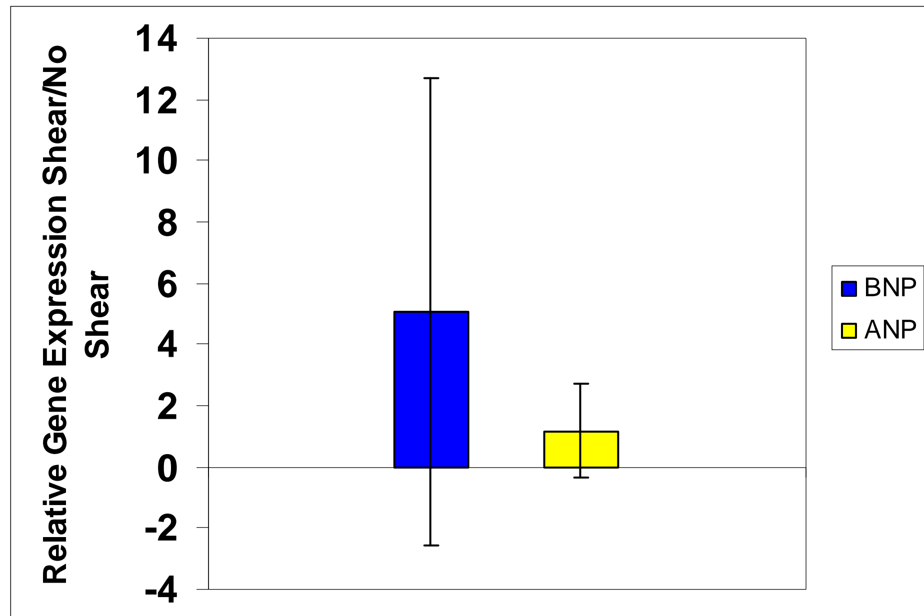

Figure 16: Shear/No Shear Ratio for BNP & ANP for Cardiomyocytes on Glass Slides + Surecoat. Shear rate: 58 sec^{-1}

In order to see if myocytes responded to shear despite variability in the controls, the ratio of BNP expression of shear to no shear was taken for individual data sets. (Figure 16) There was no conclusive difference found before and after shear in BNP expression levels (n=8, 1 ± 0.05 vs. 5.04 ± 7.63 p=0.177) and ANP expression levels. (n=8, 1 ± 0.05 vs. 1.17 ± 1.53, p=0.749).

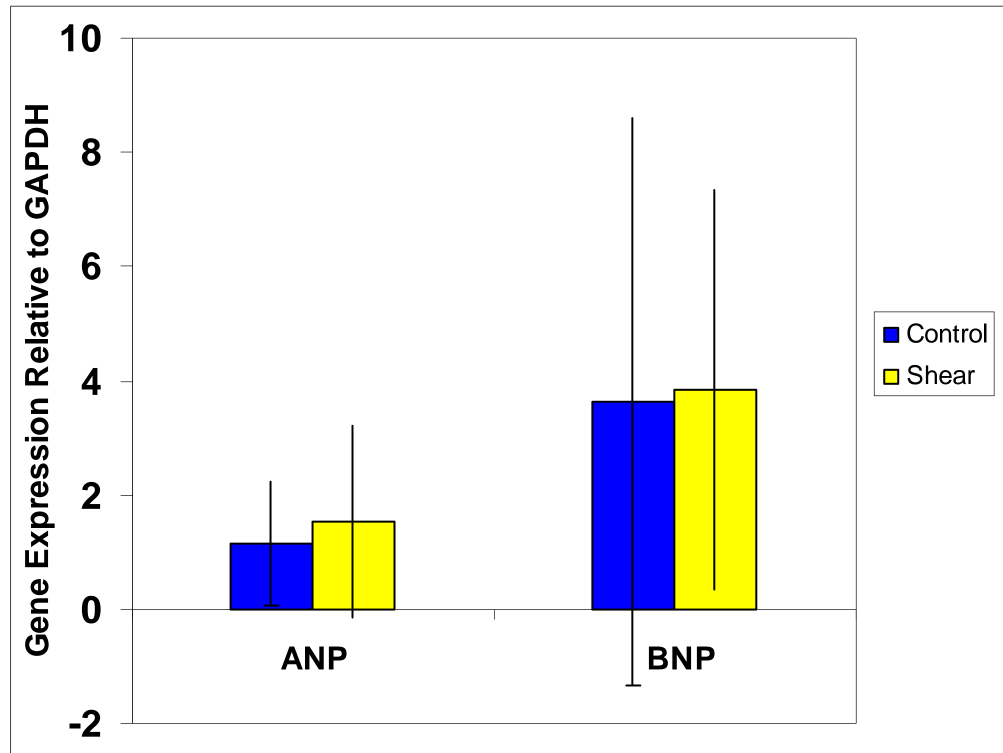

Figure 17: BNP and ANP Expression relative to GAPDH for Cardiomyocytes on Silicone + Surecoat. Shear rate: 65.2 sec^{-1}

Figure 17 shows the expression level of BNP normalized to internal control GAPDH for cardiomyocytes plated on a silicone membrane coated with Surecoat. The cells at a shear rate of 65.2 sec^{-1}. There is large variance in the average BNP and ANP expression levels and hence an inconclusive difference observed between NS and the sheared group. (BNP; n=6, 3.647 ± 4.97 vs. 3.85± 3.49, p=0.897) (ANP; n=8, 1.15 ± 1.09 vs. 1.55 ± 1.68, p=0.644)

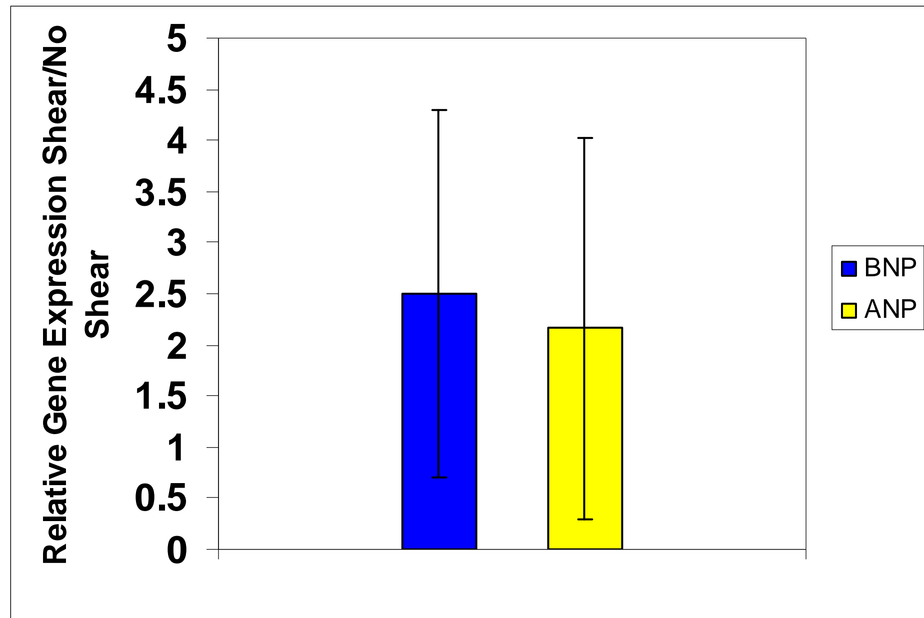

Figure 18: Shear/No Shear Ratio for BNP & ANP for Cardiomyocytes on Silicone + Surecoat. Shear rate: 65.2 sec^{-1}

In order to see if myocytes responded to shear despite variability in the controls, the ratio of BNP and ANP expression of shear to no shear was taken for individual data sets. (Figure 18)Although statistically not significant ($p>0.05$), there appears to be increased BNP expression after shear anywhere from 1.6 to 5 fold. ($n=8$, 1.0 ± 0.05 vs. 2.50 ± 1.79 $p=0.09$). There was no conclusive difference observed between NS and the sheared group for ANP expression levels. ($n=8$, 1 ± 0.05 vs. 2.16 ± 1.87, $p=0.223$).

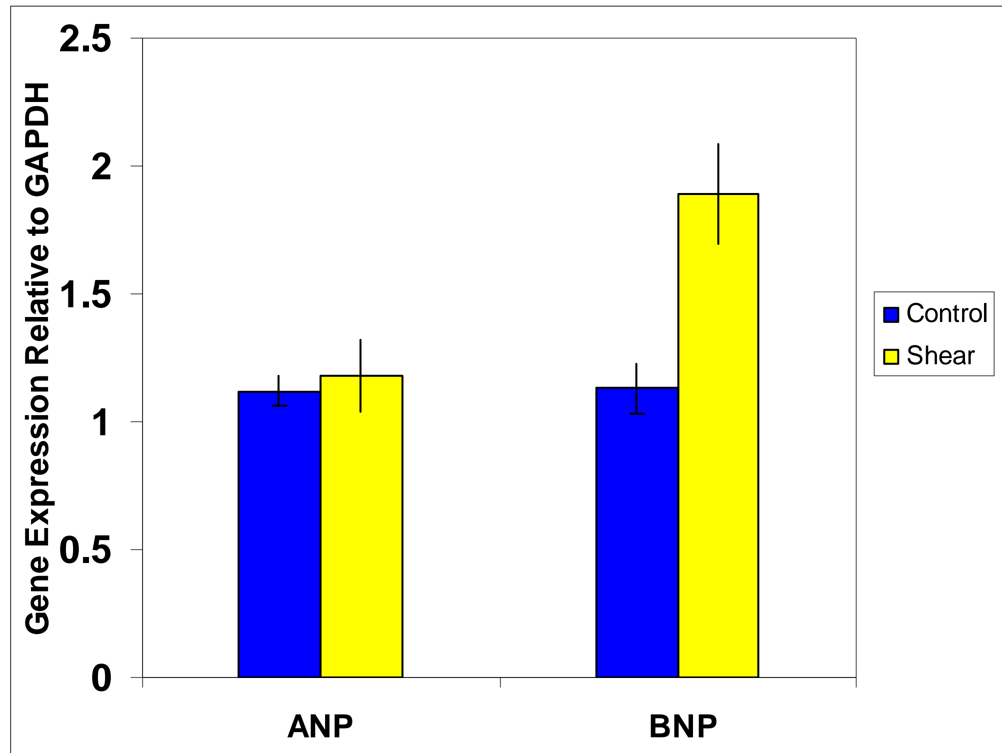

Figure 19: BNP and ANP Expression relative to GAPDH for Cardiomyocytes on Silicone + Surecoat. Shear rate: 65.2 sec^{-1}

In order to minimize variability among individual sets of cell samples, variables including substrate preparation, cell incubation time, CO2, humidity, temperature were regulated. Figure 19 shows the average BNP and ANP mRNA levels compared to the internal control gene, GAPDH, for cardiac myocytes plated on Silicone coated with Surecoat. The cells at a shear rate of 65.2 sec^{-1}. The graph shows that exposure to 1 hour fluid shear increases BNP expression in neonatal rat ventricular myocytes. (n=6, 1.13 ± 0.10 vs. 1.89 ± 0.198, p= 0.01). There is no significant different in ANP in cardiac myocytes after 1 hour exposure to fluid shear. (n=6 1.12 ± 0.06 vs. 1.18 ± 0.14, p=0.18)

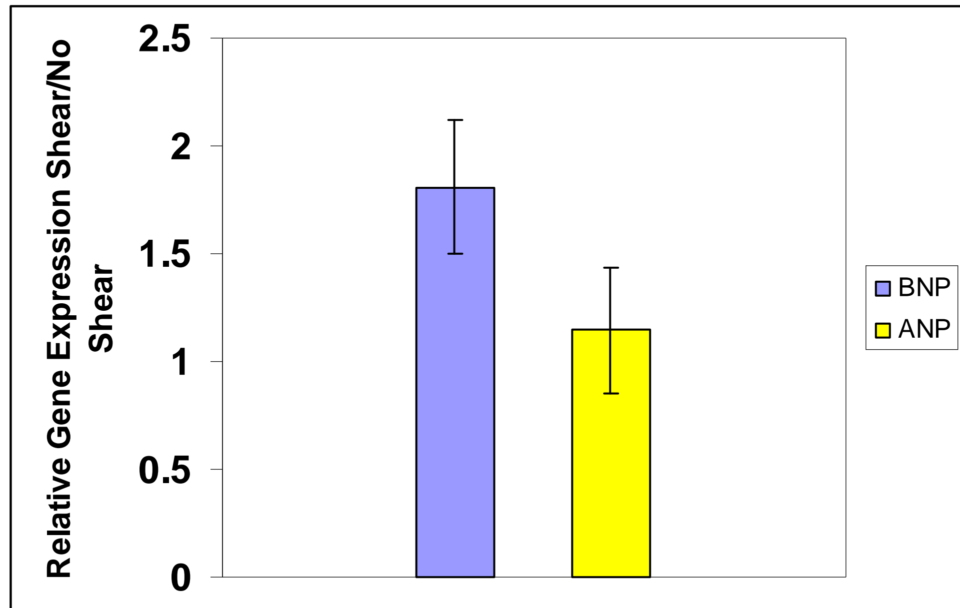

Figure 20: Shear/No Shear Ratio for BNP & ANP for Cardiomyocytes on Silicone + Surecoat. Shear rate: 65.2 sec^{-1}

Figure 20 shows the ratios of BNP and ANP before and after shear. BNP after shear is nearly 2 fold greater than before (n=6, 1.81 ± 0.31, p = 0.01), while ANP expression is the same before and after shear (n=6, 1.15 ± 0.29, p = 0.43).

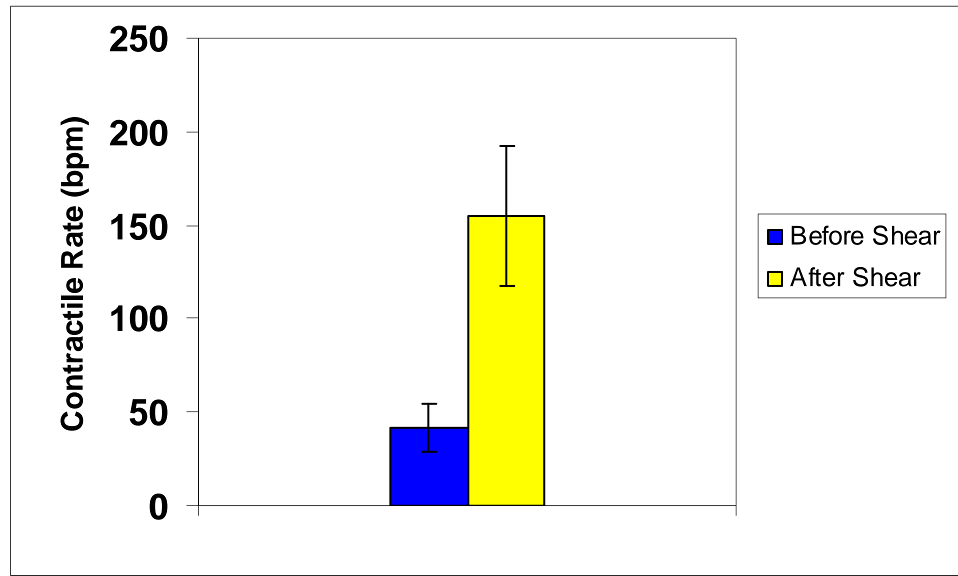

Figure 21: Beating rate of cardiomyocytes before and after shear. Shear rate: 32.6 sec^{-1}

Figure 21 shows the increase in spontaneous beating rate of myocytes due to shear, at a shear rate 32.6 sec^{-1}. Beating rates increased significantly. (n=6, 42 ± 13 bpm vs. 155 ± 37 bpm, $p<0.01$) However, there was no significant difference in the absolute increase in beating rate compared to when the cells were subjected to a higher shear rate.

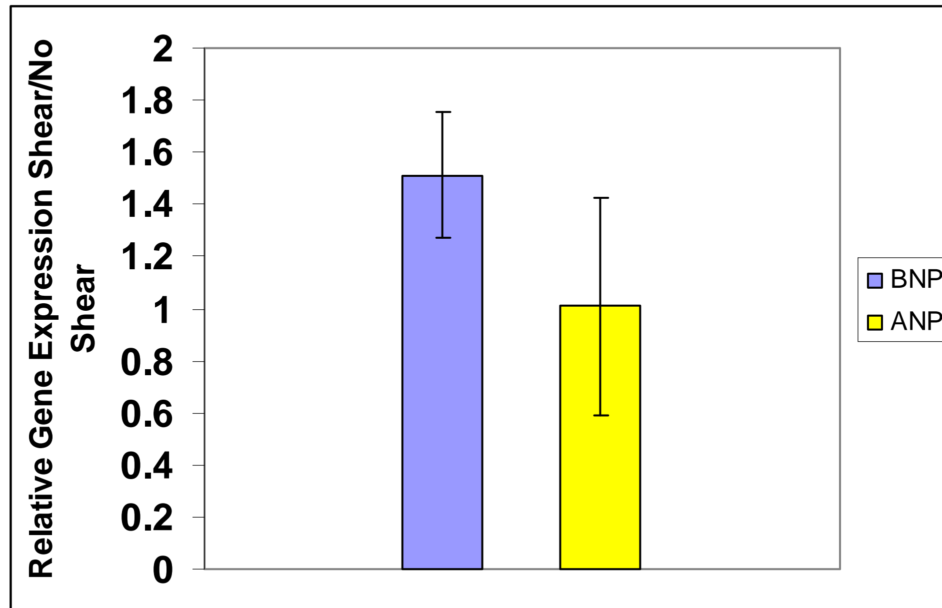

Figure 22: Shear/No Shear Ratio for BNP & ANP for Cardiomyocytes on Silicone + Surecoat. Shear rate: 32.6 sec^{-1}

The cells were sheared at a shear rate of 32.6 sec^{-1}. Figure 22 shows the ratios of BNP and ANP before and after shear. BNP after shear is nearly 2 fold greater than before (n=6, 1.51 ± 0.24, p = 0.01), while ANP expression is the same before and after shear (n=6, 1.01 ± 0.41, p = 0.96).

IV. Summary of Results

These results clearly show that a 1 hour laminar fluid shear induces a hypertrophic response in neonatal rat ventricular myocytes by increasing the expression of hypertrophic marker BNP. Initially, there was much variability in the expression of hypertrophic markers ANP and BNP among the individual sets of cardiac myocytes. Experiments were conducted to determine the conditions that would produce consistent gene expression levels. The primer fidelity assay determined that the primers were not responsible for the variability. Different internal controls, GAPDH and Ef1 were used, which attributed to the variability to some other factor. Next, different combinations of plating substrates were used to see if gene expression levels would show more consistency. Substrates Type II Collagen/Glass Slides and Surecoat/Glass Slides produced variable controls. In experiments using cells prepared on these substrates, the data showed to be inconsistent due to a high variance and limited amount of experiments available. In order to look at the effect of fluid shear in individual sets of cell samples used, the ratio of ANP and BNP before and after shear was taken. The Surecoat/Silicone Membrane substrate showed that although cells harvested on different days still varied in gene expression levels, their response to fluid shear and the subsequent increase in BNP levels was fairly consistent. Therefore, cells were prepared using the Surecoat/Silicone Membrane substrate, with the same incubation duration after isolation, CO2, temperature, and humidity, with similar morphology. These cell samples were then subjected to fluid shear for 1 hour, at shear rates of 65.2 sec^{-1}, resulting in an increase in BNP expression of 1.81 fold. When the

cells were sheared at a lower shear rate of 32.6 sec^{-1}, the BNP expression increased 1.51 fold. There was no statistical difference in the increase in BNP expression level between cells subjected to these 2 shear rates. ($p = 0.68$).

V. Discussion

A. Plating Density

Although the experimental procedure was fairly consistent, obtaining a consistent culture of viable myocytes for the experiments was challenging. A large variance was observed in the confluency of the cells. These occurred not only among cells isolated on different weeks, but even those from the same week in different dishes. This may be because the current plating procedure relies on chance to obtain a desired density of myocytes. The cell suspension containing a known concentration of myocytes is poured onto a silicone membrane coated with a ECM substrate, Surecoat. The degree to which myocytes adhere to this substrate, which determines the plating density and cell confluency, was variable even when the density of cells in the cell suspension used was consistent. Although generally, a higher count of cells used per dish results in a higher plating density, there was still variability. This may be attributed to variabilities such as the percentage of non-myocytes (such as fibroblasts), agitation of the cells during the isolation procedure, during plating, transporting them into the incubator, and when changing the media, duration of plating, and other environmental factors such as CO2 concetrations and temperature.

Because the effect of shear on the beating rate of myocytes might influence the shear mediated hypertrophic response, it's important to obtain cultures with consistent beating characteristics. Because myocytes do not divide, the number of myocytes that adhere to the substrate determines whether they spread out to form a confluent synctium through gap junctions. Generally, confluent cells beat synchronously with

large amplitude. Individual myocytes often do not beat or beat inconsistently, and show little or no response to shear. Therefore, a high plating density is more desirable.

However, it should be noted that with a high plating density, the force of the synchronously beating myocytes caused them to detach from the substrate.

B. Variability in Gene Expression

The interaction between the cell cytoskeleton and the ECM proteins are known to be involved in the hypertrophic signaling mechanisms. [43] Therefore, the high level of variability seen in the baseline gene expression levels of hypertrophic markers may be due to the inconsistency seen in the degree of attachment of the myocytes to the substrates. Additionally, the difficulty in determining and obtaining a consistent myocyte to cardiac fibroblasts ratio during cell isolation may be an underlying reason as well. Myocytes hypertrophy more by increasing their surface area when they are co-cultured with fibroblasts, compared to myocytes alone. [44] Thus, the varying ratio of fibroblasts to myocytes that are plated will affect the expression level of hypertrophic markers. While myocytes do not divide, fibroblasts continue to do so even in vitro. Waiting longer to use the cells for experimentation after being plated increases the fibroblasts and would be expected to affect the expression level of hypertrophic markers. There was variability as to when cells were used because they were left in the incubator until the myocytes had spread to a confluent monolayer. Generally a low initial plating density required a longer time for the cells to become confluent.

BNP and ANP are commonly used as hypertrophic markers. However, after 1 hr shearing, there was an increase in BNP expression, while no significant difference was observed in ANP expression levels compared to the control group. In stretch activated hypertrophy, ANP induction takes up to 12 hours, while BNP induction may occur within 30 minutes of the initial mechanical stimulus. The similarities in the pattern between stretch and shear activated induction of these hypertrophic markers may suggest they do so through a common signaling pathway.

C. Possible Mechanism of Increased BNP Expression

These results have clearly demonstrated that fluid shear elicits a hypertrophic response by upregulating hypertrophic marker, BNP, as well as contribute to an increase in its spontaneous beating rate. The fact that fluid shear contributes to both hypertrophy and increased beating rate may provide the link between arrhythmia and heart failure. The increase in BNP was relatively insensitive to the shear rate. Moreover, consistent with previous studies, the absolute increase in beating rate was insensitive to the shear rate. [24] These findings collectively highlight the possibility that the mechanism mediating the increase in spontaneous beating rate, and increased BNP expression may share a common pathway. However, it is possible that the increase in BNP was a result of the increased contractile activity due to shear.

Arginine-Glycine-Aspartate (RGD) peptides contain a motif that binds competitively to Integrins. Therefore, RGD peptides can be added to the shearing media to disturb the Integrin-ECM interaction to see if hypertrophy due to shear is blocked.

Studies have shown that the increase in beating rate may be mediated by the Beta-1 Integrin and extracellular matrix interaction. With RGD peptides, competitive agonists for Integrin binding sites, the cardiomyocytes' response to fluid shear was significantly diminished. [24] In stretch activated hypertrophy, the cell ECM interaction has also been shown to be involved. When the outside in signaling of integrins were blocked using PTEN, the p38 MAPK pathway involved in stretch activated hypertrophy was attenuated. [47] These results further support the possibility that the fluid induced hypertrophic and chronotropic response shares a common pathway as the stretch activated hypertrophic response.

In addition, studies have shown that beta 1 integrins are linked to L-type calcium channels. [45] Therefore, the increase in beating rate of myocytes may be mediated by Integrin dependent pathway that increased L-type Ca2+ current. In another study using a calcium sensitive dye, mechanical stress has been shown to increase Ca2+ influx through stretch-activated channels. [46] In either case, the resulting increase in calcium may trigger the hypertrophic response through calcium dependent hypertrophic pathway, the NFAT-calcineurin pathway. This would explain the overlap between the chronotropic and hypertrophic pathways due to fluid shear.

It is not sure whether the type of media used to shear affects the fluid shear response. There may be substances in the shearing media that may have caused the upregulation of hypertrophic marker BNP. Some candidates include horse serum and fetal bovine serum, which may contain growth factors that illicit a hypertrophic response. Therefore, the cells may have been responding to the higher availability of a

particular substance, when the shearing media was introduced into the flow chamber. Diffusion studies and consumption analysis may be performed to determine whether the concentrations are uniform.

It is not clear whether the cells responded to the shear stress or shear rate. The media that was used to shear the cells is a Newtonian fluid with a constant viscosity. Therefore, shear stress equals shear rate times viscosity. In order to determine whether the cells responded to shear stress or shear rate, a media with a higher viscosity can be used. This media can be prepared by adding a non-reactive macromolecule such as ficoll. If the cells produce a greater hypertrophic response, then it will demonstrate that it is the shear stress plays a greater role in eliciting the response.

D. Suggestions for Further Investigation

Several interesting studies can be performed as follow up studies. To determine if the increase in the contractile rate is necessary for shear to illicit the hypertrophic response, the beating activity may be first arrested prior to shearing. This can be accomplished in several ways. A voltage gated Na+ channel blocker, TTX may be added to the medium to prevent depolarization required for contraction. The extracellular Na+ may be removed, or using a cardiac arrest solution with K+ may be used to hyperpolarize the cell.

Mechanical strain in vivo is multiaxial and anisotropic. Moreover, myocytes in vivo are rod shaped and aligned. Studies have looked at the effect of anisotropic stretch on the hypertrophic response of cardiomyocytes. In it showed that stretch applied transverse to the major axis of cardiomyocytes elicits a greater hypertrophic

response than stretch applied longitudinal to the major axis. [48] Thus, the effect of anisotropic fluid shear on the hypertrophic response could be examined. This would be accomplished by the use of photolithographic and microfluidic techniques to micropattern extracellular matrix components on silicone membranes. [48] This method produces confluent, elongated, aligned myocytes which can then be used to examine the effect of fluid shear parallel and perpendicular to its major axis.

In endothelial cells, the dynamic characteristics of flow differentially modulate the cellular response. For example, steady laminar fluid shear increases NO production while turbulent flow does not. [26] Flow conditions that could be tested include pulsatile laminar and oscillating turbulent flow. In addition, different shear rates could be tested to determine whether the hypertrophic response is dependent on shear magnitude.

When cells were sheared, there may have been signaling molecules being secreted by the myocytes that affects the hypertrophic response. Looping the shearing media back into the flow chamber or to virgin cells to see if it produces a different result may highlight this possibility.

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