ABSTRACT

DEVELOPMENT OF A METHOD TO EXAMINE SHEAR STRESS ON CARDIOMYOCYTES

by

Dhawal Desai

Cardiac hypertrophy is the remodeling or enlargement of the heart exhibited by individuals suffering from heart disease. Cardiac hypertrophy involves two mechanisms, mechanical stresses and humoral changes in the cardiac tissue. One such humoral change involves the Renin Angiotensin System (RAS). The primary function of RAS is maintenance of hemodynamics of the body mediated through an effector protein, Angiotensin II. However, RAS has been shown to play a significant role in the pathophysiology of the heart; contributing to hypertension, cardiac hypertrophy and myocardial infarction. RAS acts through binding of Angiotensin II at its receptors. The two forms of Angiotensin II receptors, designated type one (AT1) and type two (AT2), have been shown to have different functions and distribution in various body tissues. Receptors for both AT1 and AT2 are expressed on cardiomyocytes. The level of expression of each receptor type has been shown to have important implication. It has been suggested that the receptors may have cooperative yet opposing effects when bound by Angiotensin II. Thus, the level of expression may drive the system to or away from hypertrophy.

The current research developed techniques to allow examination of the hypothesis that in cardiomyocytes, exposure to increasing shear stress causes upregulation of the AT1 and AT2 receptors. The isolated rat cardiomyocytes were exposed to shear stress

and methods were developed to measure the change in AT1 and AT2 cellular expression resulting from the application of shear stress. The techniques required to accomplish this task including isolation of rat cardiomyocytes, flow chamber (shear stress) apparatus and use, handling of cardiomyocytes and molecular science techniques (RT-PCR) are described here in detail. The ability to track gene regulation of AT1 and AT2 receptors though RT-PCR techniques is demonstrated. The ability to attach cardiomyocytes to a culture dish surface in order to expose them to shear stress is also shown. However, to take full advantage of the flow chamber, stronger cell attachment to culture dish is required. Future research will be directed to achieving stronger adhesion between cardiomyocytes and the cell culture surface. Recent reports have suggested use of various culture surfaces and adhesion molecules including laminin, elastin, cell culture plastic, and fibronectin. Use of serum and serum free medium has also been suggested.

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To my family.

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CHAPTER 1

INTRODUCTION

1.1 Cardiac Hypertrophy

Individuals with cardiac complications such as hypertension and myocardial infarction exhibit cardiac hypertrophy. Hypertrophy or enlargement of the cardiac tissue is an adaptive response to mechanical stresses placed on the heart by existing cardiac disease.[1] The mechanical overload causes terminal cardiomyocytes to enlarge. Cell enlargement is followed by remodeling events that include loss of cardiomyocytes, production of fibroblasts and deposition of collagen.[1] All of these processes lead to loss of compliance and further deteriorate the cardiac system increasing the chances of heart failure. The mechanisms for cardiac hypertrophy are complex and not completely understood. However, research has shown that mechanical overload and humoral factors are two pathways having great influence over the development of cardiac hypertrophy. The Renin Angiotensin System (RAS) is one of the humoral systems implicated in the pathogenesis of cardiac hypertrophy.

1.2 Renin Angiotensin System

The primary function of the Renin Angiotensin System (RAS) is maintenance of hemodynamics in the body. It accomplishes this via salt retention in the kidney as well as vasoconstriction. However, this thesis the focus will be on the effects of RAS as they relate to the development of cardiac hypertrophy. It is well established that RAS through Angiotensin II (Ang II) plays a significant role in the pathophysiology of the cardiac

system, contributing to hypertension, cardiac hypertrophy and myocardial infarction.[1-3] Angiotensin II is produced in various tissues and through many different pathways that seem to be species specific.[2] The dominant pathway in Angiotensin II production is through Angiotensin Converting Enzyme (ACE). Angiotensin Converting Enzyme converts Ang I into Ang II, the molecule credited with RAS activity. However, an alternative pathway through the chymase group of enzymes has demonstrated production of Angiotensin II in the presence of ACE inhibition.[2] In vivo studies, however, credit the majority of Angiotensin II production to the ACE dependent pathway. The activity of Angiotensin II has been well documented. A recent report has shown that increased local expression of the octapeptide Ang II leads to cardiac hypertrophy.[3] The technique involved transfection of Human Angiotensin Converting Enzyme (ACE) into cardiac myocytes. The initial release of Ang II leads to a self-stimulation event, where the release of Ang II causes vasoconstriction leading to increase in pressure. Increase in pressure leads to further production of Ang II and further deterioration of cardiac tissue. The action of Ang II occurs through its binding to Ang II specific receptors. A short discussion on the therapeutic interventions is presented here before beginning an in-depth look at Ang II receptor mediated effects on the cardiac system.

1.3 Therapies against cardiac hypertrophy

Two therapeutic interventions aimed at prevention of cardiac hypertrophy have been proposed. One of the methods looks at the production of Ang II. Since ACE is the dominant pathway leading to Ang II production in the body, this method aims to inhibit ACE activity. ACE blockers have shown to be effective in reducing patient mortality and

morbidity by reducing Ang II production.[4, 5] However, complete suppression through ACE inhibition has not been achieved nor is it considered possible. As shown earlier, multiple sources and multiple pathways are responsible for production of Ang II in the body. In fact, it has been shown that in cases where ACE inhibitors have been utilized alone the level of systemic Ang II returns to normal levels through other production pathways.

The second mode of intervention aims at prevention of Ang II binding to its receptors. Initial studies with Losartan, an Ang II receptor antagonist, have shown to be cardio protective. However, researchers have suggested that combining these approaches, including ACE and chymase inhibition and Ang II receptor antagonism, may prove even more beneficial.

1.4 Angiotensin Receptors and Actions

The Angiotensin II receptors have been credited with various responses upon binding of Ang II. Based on their affinity for receptor antagonists such as Losartan and PD123177, as many as five Ang II receptors have been identified.[6] Angiotensin II type 1 (AT1) and Angiotensin II type 2 (AT2) receptors are credited with most of the functions resulting from Ang II binding. A good overview of functions attributed to these two Angiotensin receptors is presented by Busch et al 2000.[7] AT1 mediates all hemodynamic functions, including vasoconstriction, renal salt retention and release of aldosterone and vasopresin. The hypertrophic response to Ang II is also thought to be though AT1 receptor via growth factor β1 and endothelin-1. A great debate is taking place over the role of AT2. One group claims a counter-balancing role between the two receptors with AT1 causing

the pathological effects observed in cardiac hypertrophy and AT2 demonstrating a cardio protective role.[8] Through the use of type selective antagonists, they demonstrated that growth or antigrowth response is a consequence of receptor density rather than level of Ang II present. The second group of researchers believes that there is little evidence to substantiate the claim that AT2 restores cardiac health by counteracting the effects of Ang II binding to AT1 receptors. Meanwhile, other researchers believe further investigation is required to bring a conclusion to the debate.[9, 10]

The receptor story, however, is much more complex. Along with the functions of each receptor the issues of tissue expression and receptor density must also be considered. The expression of these receptors is both tissue and species specific.[11-15] It has been reported that adult rat cardiac cells only express AT1 binding sites whereas in human myocardium the level of AT2 receptors dominates with a two to one ratio.[16] Furthermore, AT1 and AT2 expression is regulated developmentally and therefore tends to fluctuate. The number of AT2 receptors tends to decline after birth to a lower number.[13, 17] Finally, in a failing heart or infarcted hearts the AT1 and AT2 receptors are selectively upregulated.[18] Specifically, in an infarcted animal the number of AT1 receptors decreases while the number of AT2 receptors shows an increase one-day post infarction. Other studies involving failing human hearts have shown that AT1 expression decreases while AT2 remains unchanged, causing a relative increase in the level of AT2 receptors.

The reason for placing emphasis on receptor expression here is because this may be the driving force behind Ang II mediated remodeling of the heart. If Kestern and group are correct about a cooperative relationship between Ang II type 1 and 2 receptors

and their claim that receptor density is more important than levels of Ang II, it will have a profound effect on the future direction of research and treatment. Studies are continuing and no consensus has been reached.

1.5 Signal Transduction Pathways

This section looks at the down stream effects of Ang II binding to its receptors. It is believed that Ang II mediated hypertropic response may involve more than one signal transduction pathway. It has been shown that binding of Ang II to its receptors invokes second messengers such as Inositol Triphosphate IP3, Ca^{2+} and activation of G protein coupled STAT/JAK Kinase and Protein Kinase C (PKC). McWhinney and group have shown that in neonatal rat cardiomyocytes binding of Ang II to AT1 leads to formation of sis-inducing factor (SIF) protein.[19] SIF occurs in three varieties SIF-A, SIF-B, SIF-C. SIF-B consists of a heterodimer of activated Stat1 and Stat3 proteins and SIF-A and SIF-C are homodimers of Stat3 and Stat1 respectively. The activation of Stat proteins involves phosphorylation of a tyrosine residue, which takes place through JAK tyrosine Kinase activity. Inhibition of JAK or tyrosine kinase activity prevents SIF complex formation. SIF complex translocates to the nucleus, binds SIE element and promotes transcription of downstream genes. The same group also reported a JAK dependent Prolactin Inducing Factor (PIF).[20] PIF results from a complex formed between Stat5a and Stat5b proteins that subsequently translocate to the nucleus and influence transcription. All of these proteins also exhibit ability to bind the AT1 receptor. It is thought that binding of Ang II to the AT1 receptor leads to activation of the receptor bound JAK kinase, followed by activation and translocation of Stat proteins. Ang II

binding to AT2 does not result in JAK/STAT transduction event. However, pressure overloaded rat heart remodeling events involve the JAK/STAT transduction pathway. In overloaded hearts, an early and late response is observed. The early response is mediated by pressure directly and the late response results from pressure induced release of Ang II.[21]

Research also exists to show that hypertrophic response to Ang II is directed through a Ca^{2+} related signaling pathway. In adult rat cardiomyocytes, the increase in Ca^{2+} is mediated equally through both AT1 and AT2 receptors.[22] Others have reported that cardiac hypertrophy is mediated by Ca^{2+} activation of PKC that leads to induction of early gene such as c-fos.[23] They also showed that Ca^{2+}, AT1, and PKC inhibition leads to suppression of mRNA and protein synthesis in cardiomyocytes. The details of AT2 activation and signal transduction have not yet been reported. Significant evidence however is present to conclude that binding of Ang II to AT1 results in downstream signal transduction and protein synthesis responsible for the hypertrophic response in cardiomyocytes. Further investigation is required to determine the AT2 transduction pathways and the role of these various pathways leading to a hypertropic response via AT1 receptor.

1.6 Current Research

The aim of the current research was to develop techniques that would allow testing of the hypothesis that in cardiomyocytes, an increased exposure to shear stress leads to upregulation of AT1 and AT2 receptors. As shown in section 1.4, the receptors play a significant role in development of cardiac hypertrophy. For the experiments performed

for this thesis, a flow chamber was used to generate varying velocities of a fluid through the flow chamber. A detailed discussion of the flow chamber is presented in the materials and methods section. In the same section, the rat cardiomyocytes isolation procedure, shear stresses within the flow chamber and cell culture procedures for handling cardiomyocytes are discussed. Finally, the preliminary findings are presented and the future direction of research is suggested.

CHAPTER 2

MATERIALS AND METHODS

The experiment is complex and requires many steps to complete. For convenience, the methods section is divided into four parts. These include: isolation of cardiomyocytes, messenger RNA (mRNA) extraction, RT-PCR and cell culture procedure as well as steps involved in exposing the cardiomyocytes to shear stress.

2.1 Isolation of Cardiomyocytes

For the experiment, Sprage-Dawley rats weighing between 175-200 grams were used. Cells were isolated using an enzymatic method described previously by Mitra and Morad.[24] The following materials and solutions are required for the isolation technique.

2.1.1 Materials

1. Zero Ca^{+2} – Langendorff solution
2. 50 mM $CaCl_2$
3. Collagenase (Worthington Biochemical Corporation, Lakewood, NJ O8701)
4. Protease (Sigma Chemical Com. Louis, MO 63178)
5. Pentobarbital (Veterinary Laboratories Inc. Lenexa, KS 66215)
6. Surgical Instruments:

Large scissor (1)	Small scissor (1)
Artery clip (1)	Hemostat (1)
Small dish (1)	Suture

Stock Solutions are prepared in advance:

Zero Ca^{+2} – Langendorff Solution pH of 7.2:

Add 135mM NaCl,

5.4mM KCl,

1mM$MgCl_2$,

0.33mM NaH_2PO_4,

10mM HEPES solution.

0.2mM Ca^{+2} – Langendorff Solution:

The 0.2 mM Ca^{+2}– Langendorff solution is prepared by adding 50mM $CaCl_2$ solution to Zero Ca^{+2}- Langendorff Solution (1:250 dilution). Both Langendorff solutions are titrated to pH 7.2.

2.1.2 Procedure

The water bath is turned on 30-45 minutes prior to animal sacrifice to warm the apparatus to 37 degrees Celsius. Four to five 50ml eppendorf tubes are marked accordingly. The Langendorff apparatus is readied by washing with 1 liter of deionized water 30 minutes prior to sacrifice. The 0.2 mM Ca – Langendorff solution (250ml) is prepared and warmed by placing in the water bath. The Zero Ca – Langendorff solution (100ml) is also prepared. Then Collagenase (25mg) and Protease (5mg) are measured out and placed in a small bottle or beaker. For these experiments the above amounts of Protease and Collagenase proved best. However, depending on animal size and other factors these

amounts are adjusted. The animal is anesthetized by administering 50-100 mg/kg dose of pentobarbital.

Upon checking for pain reflexes, the heart is excised and placed in a small dish containing Zero Ca – Langendorff solution. Then the heart is, placed on the apparatus by cannulating the ascending aorta and perfused with 0.2 mM Ca – Langendorff solution at a rate of 5-10 ml/min. The heart is secured in place with an artery clip and suture. A small incision is made at the top of the heart to enable the heart to empty of blood and Langendorff solution. Perfusion is continued until blood is completely removed from the heart, approximately two to three minutes. At this point, flow is switched to Zero Ca – Langendorff solution for 5 minutes. During this time, 25 ml of the warm Zero Ca – Langendorff is added to the premixed enzymes and dissolved well. Then, the enzyme solution is added to the chamber containing Zero Ca – Langendorff solution. Additional Zero Ca – Langendorff solution is added to the chamber to bring the final volume to 50 ml. Perfusion is continued with the enzyme solution for five to seven minutes (5:15 yielded the best results).

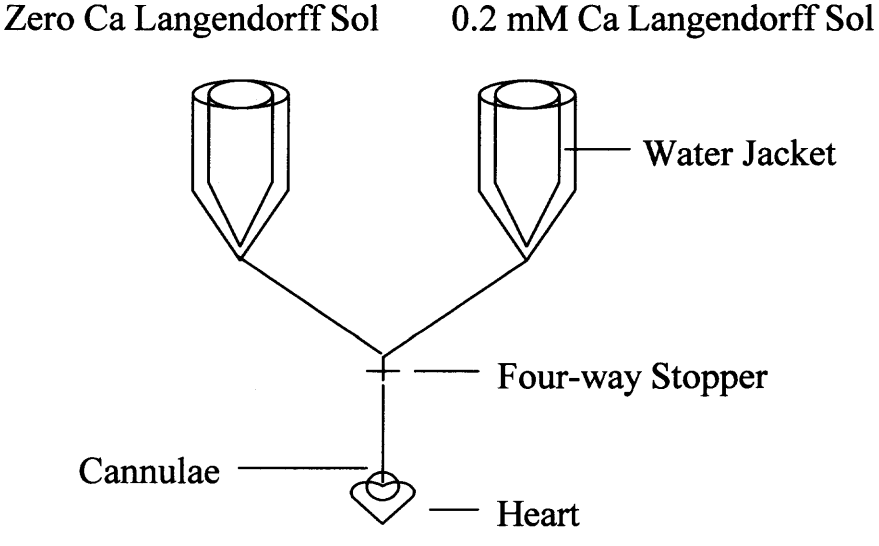

Figure 2.1 Langendorff apparatus.

2.1.3 Cell Isolation

The heart is removed from the apparatus and placed in a small dish containing 0.2 mM Ca – Langendorff solution. Any remaining arterial tissue is removed from the heart. The heart is then minced gently but quickly. The minced tissue and solution are transferred to a 50 ml eppendorf tube and placed in the water bath. They are left in the bath only long enough for the tissue to settle to the bottom. The solution is decanted into a beaker and saved. To the tissue remaining in the eppendorf tube, 10-12 ml of 0.2 mM Zero Ca – Langendorff solution is added. The tube is placed in the water bath and agitated gently every few minutes. After five minutes, the supernatant is decanted into the first of the five effendorf tube and the process if repeated for the rest of the tubes. When finished, the cells are checked under a microscope. The cells are then refrigerated at 20°C and allowed settle to the bottom on the tubes.

2.2 Messenger RNA isolation by RNeasy Mini Kit (From Qiagen®, #74104)

When ready, cells are removed from the refrigerator and mixed by slowly agitating each tube. The cells are then combined and equal volumes are distributed into two balanced tubes. The cells are spun down in a slow-speed centrifuge at the lowest rotation rate available (<100 rpm) for two minutes. The supernatant is discarded and 175 μL RLN solution (50mM Tris-Cl, pH 8.0; 140mM NaCl; 1.5mM $MgCl_2$; 0.5% Nonidet p-40) is added to the pallet. Cells are mixed with RLN solution by flicking the bottom of the tube. The content is transferred to a new 2 ml tube and incubated for five minutes on ice. The

mixture is then spun in a microcentrifuge in the cold room for two minutes at 2100 rpm. In these experiments, the rest of the procedure is carried out in the cold room. The supernatant is transferred to a new tube. To the supernatant, 600 μL RLT buffer with β-Mercaptoethanol (10 ml RLT + 100 μL β-Mercaptoethanol) is added and vortexed. Then, 430 μl of 100 percent Ethanol is added and mixed by pipeting. From the mixture, 700 μL is placed on a RNeasy column in a 2 ml collection tube. The column is centrifuged at 10,000 rpm for 30 seconds. The supernatant is discarded and the process is repeated with the remaining sample. Next, 700 μL buffer RW1 is pipeted into the RNeasy column, and centrifuged at 10,000 rpm for 30 seconds to wash. The RNeasy column is then transferred into a new 2 ml collection tube. Buffer RPE (500 μL) is pipeted into the RNeasy column, and centrifuged at 10,000 rpm for 30 seconds to wash. Then 500 μL buffer RPE is pipeted into RNeasy column, and centrifuged at 14,000 rpm for two minutes to dry the RNeasy membrane. The RNeasy column is placed in a new 1.5 ml collection tube and 30-50 μL of Rnase-free water is pipeted directly onto the RNeasy membrane. The RNeasy column is then centrifuged for one minute at 10,000 rpm to elute. The last step is repeated if the expected mRNA yield is more than 30 μg. The Optical Density (OD) is checked on a spectrophotometer by diluting two μL of the sample with 98 μL of Rnase-free water. The mRNA ratio = Abs at 260nm/ Abs at 280 should be between 1.8- 1.99. The mRNA concentration in μg mRNA/ml = Abs at 260nm * 40* 100(dilution factor). The extracted mRNA is stored in the -80 °C refrigerators.

2.3 RT-PCR

The frozen mRNA is thawed by warming between the hands. After labeling aliquoted tubes, 20 μL of thawed mRNA is placed into each tube. If using internal standard mRNA, serial dilutions are prepared as required. The RT "Cocktail" is prepared according to Table 2.1 below (all materials should be stored at –20°C but thawed before use).

Table 2.1 RT Cocktail

Cocktail*	Volume needed for 40 μL of reaction mixture (μL)	Volume for 5.5 samples (μL)
25 mM $MgCl_2$	8	44
10x PCR Buffer	4	22
10 mM dNTP	16	88
Rnase Inhibitor	2	11
Oligo d(T)	2	11
Rnase-free H_2O	2	11
Mulv RT	2	11
Random Hexamers	2	11
Total	38	209

* All cocktails prepared assuming starting with 0.5 μg/μL RNA

Internal standard dilutions are denatured at 65°C for 5 min. Five PCR tubes are prepared for each mRNA sample and then 19 μL of cocktail is placed in each tube, with one of the dilutions of the internal standard in each tube. Each tube is labeled and placed in the PCR reaction plate and covers are snapped on. Automatic RT cycle is run according to the time and temperature indicated in Table 2.2.

Table 2.2 RT Cycle

Cycle	1	2	3
Temerature (°C)	42	95	4
Time (min)	60	5	hold

Upon completion of the cycle, the tubes are removed. Prior to beginning PCR, the PCR "Cocktail" is prepared by adding materials in the amount specified below in Table 2.3.

Table 2.3 PCR Cocktail

Cocktail*	Volume needed for 45 μL of reaction mixture (μL)	Volume for 16 samples (μL)
25 Mm $MgCl_2$	2	32
10x PCR Buffer	4	64
10 mM dNTP	1.6	25.6
Taq DNA Polymerase	0.25	4
Distilled H_2O	35.15	562.4
Total	43	688

Then, 43 μL of PCR cocktail, 1 μL of the 3' and 5' primers and 5 μL of mRNA sample (Product from RT) are placed in a new PCR tubes (Total =50 μL). The primers are short nucleotide sequences 10-20 base pairs long that bind a specific DNA sequence. DNA polymerase will recognize the bound primer as the start point of transcription and will synthesize a copy of the original sequence. Each tube is labeled and placed in the PCR reaction plate with the covers snapped securely. One of the following automatic PCR cycle is run based on the gene amplification desired.[7]

AT1 primer sequence

5'- cagcttggtggtgattgtc

3'- gccatcggtattccatagc

Table 2.4 Cycle for AT1

Cycle	1	2 (63X)			3
Temperature (°C)	94	94	57	72	72
Time (min)	2.45	.45	.45	.45	7

AT2 primer sequence

5'- tagtctctctcttgccttgg

3'- ctgaccttcttggatgctct

Table 2.5 Cycle for AT2

Cycle	1	2 (70X)			3
Temperature (°C)	94	94	63	72	72
Time (min)	5	.45	1.45	2.10	10

GAPDH primer sequence

5' - ttcaccaccatggagaaggc

3' - ggcatggactgtggtcatga

Table 2.6 Cycle for Housekeeping Gene GAPDH

Cycle	1	2			3
Temperature (°C)	95	95	61	73	73
Time (sec)	6	20	30	30	5

The Housekeeping (GAPDH) gene serves as a positive control in these experiments. Due to its abundance in the cell it is easily transcribed. Therefore, the absence of the Housekeeping signals that an error in either process or technique has taken place. In order for the RT-PCR technique to work, the error has to be found and corrected.

An agarose gel (1.2 %) is prepared by adding 1.2 grams of Agarose into 100 ml of 1X TEA buffer. The solution is boiled so that all of the Agarose dissolves. When all the agarose is dissolved, the solution is cooled until comfortable to touch. Then, the solution is poured into the casting unit and 2 μL of Ethidium Bromide is added (Toxic, wear gloves when handling it). The lane comb is put in place and the gel is allowed to solidify. Enough 1X TAE running buffer is added to cover the gel completely. The PCR tubes are removed from the PCR system. A piece of parafilm is set up and labeled in a similar way as the tubes. At each position, two μL of loading dye and 12 μL of corresponding sample are placed. Before loading samples into wells, the dye and the sample are thoroughly mixed on the parafilm. While preventing air bubbles from entering, the sample is pipeted off the parafilm. The tip is placed into a well and slowly the sample is inserted. The power source is connected and the gel is run at 100V for 45 minutes. Once completed, the gel is removed immediately so the buffer does not fade the bands. An appropriate UV light imaging system is used for viewing the bands on the gels.

2.4 Cell Attachment and Flow Chamber Procedure

2.4.1 Materials

The following items are required to carry out the cell attachment and flow experiments.

35 mm Tissue Culture Dish Polystyrene (Corning Glass Works, Corning, NY, 14831)

Becton Dickinson (B-D) Falcon Cell Culture Inserts pore size 1 μm (Fisher Scientific Company, Pittsburgh, PA 15219)

Inverted-stage microscope

Objective lens (6.3 x, 10 x, 22 x, 40 x)

Cardiomyocytes obtained from cell isolation procedure

Computer with interface to imaging system

Flow chamber deck with gasket and tube fittings (GlycoTech, Rockville, MD 20850)

Syringe Pump Model '33' (Harvard Apparatus Inc. Holliston, MA 01746)

Syringes to fit on the pump (10cc, Becton Dickinson & Co, Franklin Lakes NJ 07417)

Silastic tubing for syringe and flow chamber (ID 1/8" OD 3/16" Fisher Scientific Company, Pittsburgh, PA 15219)

Vacuum pump

Display monitor

Video recorder (SVHS)

SVHS videocassette

Video Camera

Culture dish restraint (designed internally to prevent culture dish movements while on the microscope stage)

2.4.2 Procedures

Upon isolating cardiomyocytes by the methods described previously, approximately 3ml of Langendorff solution containing cardiomyocytes is placed on a 35 mm culture dish. Equal volume is also placed on a cell culture insert. The culture dishes are covered and allowed to incubate at room temperature for three hours. The interval of three hours was most suitable for attachment. Intervals shorter than three hours were not sufficient for cell attachment and intervals longer than three hours did not translate into stronger attachment. Overnight incubation was also attempted; however, most cells did not survive the incubation period and attachment of remaining cells was not improved significantly.

Prior to starting assembly for the flow chamber, 0.9% saline solution of pH 7.4 is prepared and placed in 37°C water bath. Next, the flow apparatus is assembled as follows. The tubing is attached to the syringe and filled with 0.9% saline solution that will be used as the flow medium in these experiments. All the air is evacuated from the syringe and the tubing. Then, the syringe is secured to the pump by following the instructions in the pump's user manual. Next, the inlet, the outlet and the vacuum lines are connected to the flow chamber deck and the vacuum is turned on. The culture dish containing cardiomyocytes is secured to the inverted-stage microscope by the culture dish restraint. Next, the pump is turned on and the desired fluid rate and syringe size are selected. It is also recommended that the pump be calibrated when initially received. Again, before placing the flow chamber deck into the culture dish all air bubbles that may have entered the apparatus are evacuated. At this point, the gasket is placed onto the

deck and the pump is run for a few seconds so that there is a small amount of medium in the flow path; this ensures that no bubbles are produced when the deck is placed into the culture dish. Next, excess medium is removed from the culture dish. This will also remove unwanted debris and dead cells. Then while the vacuum is engaged, the chamber deck is slowly lowered into the culture dish. The culture dish and flow chamber are then secured in place with the microscope stage clamps and the flow is initiated. The microscope is turned on and image acquisition is started at the desired magnification.

A similar procedure is used for the culture inserts. After incubating the cells for three hours, the membrane from the culture insert is cut out and placed on the inverted flow chamber deck. Next, air bubbles that may have entered between the deck and the membrane are carefully removed. While holding the deck and membrane in the inverted position, a clean 35 mm culture dish is placed on the deck. Once the flow chamber, the membrane and the dish are secured onto the microscope deck, the flow and image capture are initiated. A diagram of the general set up is presented below.

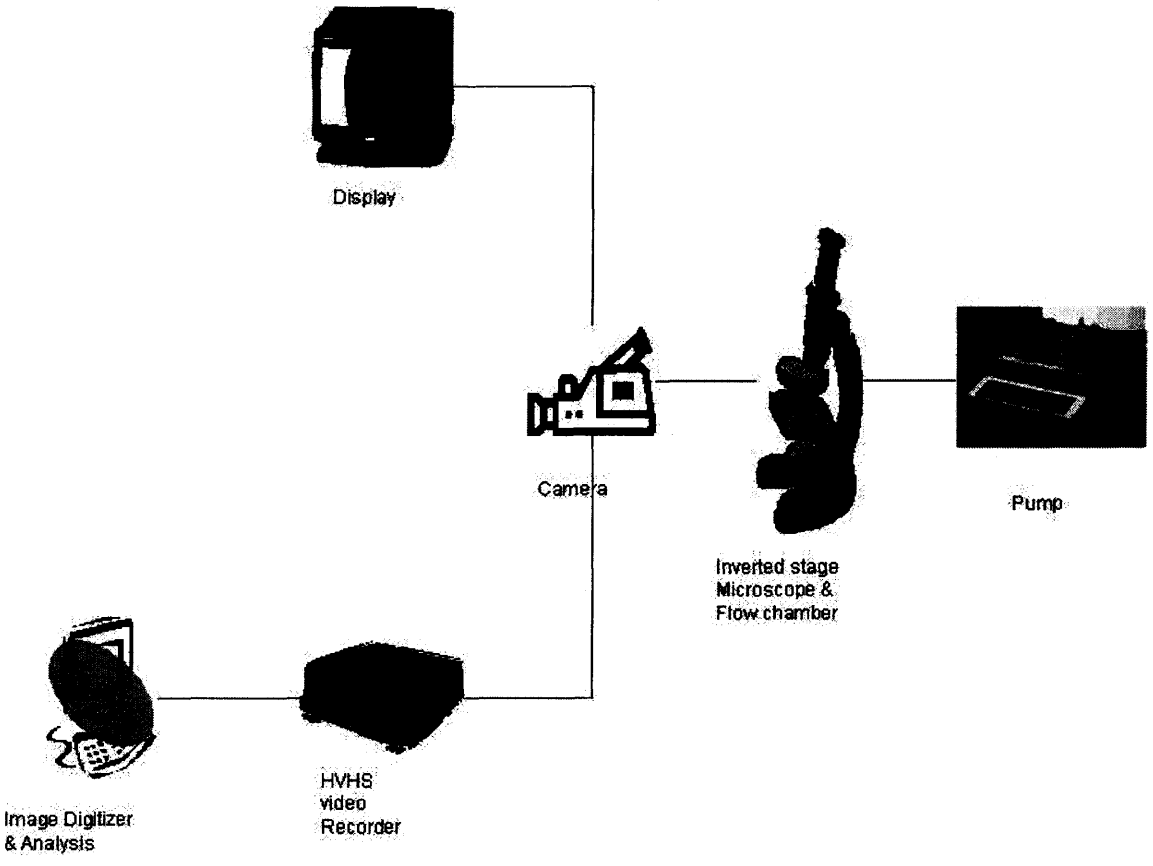

Figure 2.2 General diagram for flow experiments

CHAPTER 3

RESULTS

3.1 mRNA Extraction and RT-PCR

To extract mRNA from cardiomyocytes, the Quigen® Mini Kit was utilized. Cardiomyocytes were obtained from Sprage-Dawley rats through the cell isolation method described before. Through the use of the reagent provided in the kit, mRNA was consistently extracted in the range of 2.8 μg/ml. Upon isolation, the mRNA was stored at -80° C to prevent degradation.

Next, RT-PCR was carried out with receptor specific primers and receptor specific PCR cycles. Figure 3.1 below shows gel electrophoresis results of the AT1 amplified signal. A single strong band corresponding to AT1 in the range of hundreds of base pairs was obtained. Similarly, the AT2 gene was also amplified. Using AT2 specific primers and PCR cycle a single strong band for AT2 was obtained as seen in Figure 3.2. In Figure 3.2 a smeared band corresponding to the AT1 gene can also be seen. The AT1 signal is smeared because an AT2 specific PCR cycles was used during the amplification process that lead to fragmented copies of the AT1 gene.

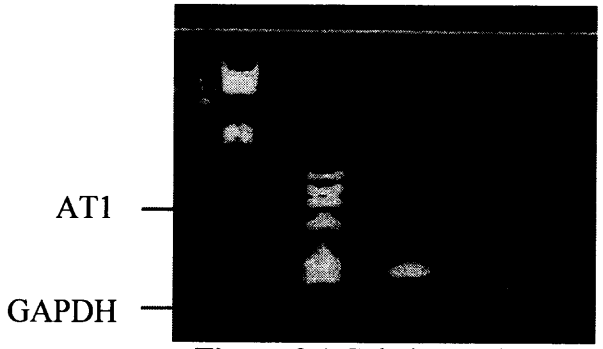

Figure 3.1 Gel electrophoresis of AT1 gene

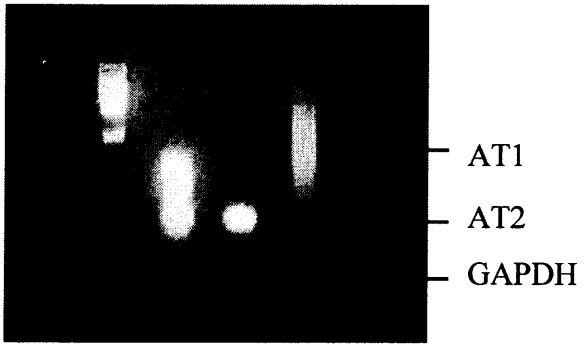

Figure 3.2 Gel electrophoresis of AT2 gene

3.2 Cardiomyocytes

Prior to looking at cell attachment, a brief introduction to cardiomyocytes is presented here. Figure 3.3 below presents both healthy and dead cardiomyocytes. The four cells toward the right of the figure show the characteristic rectangular morphology and striations of a live cardiomyocyte. The figure also shows a dead cardiomyocyte towards the left that is characterized by the loss of both the striation and the rectangular morphology.

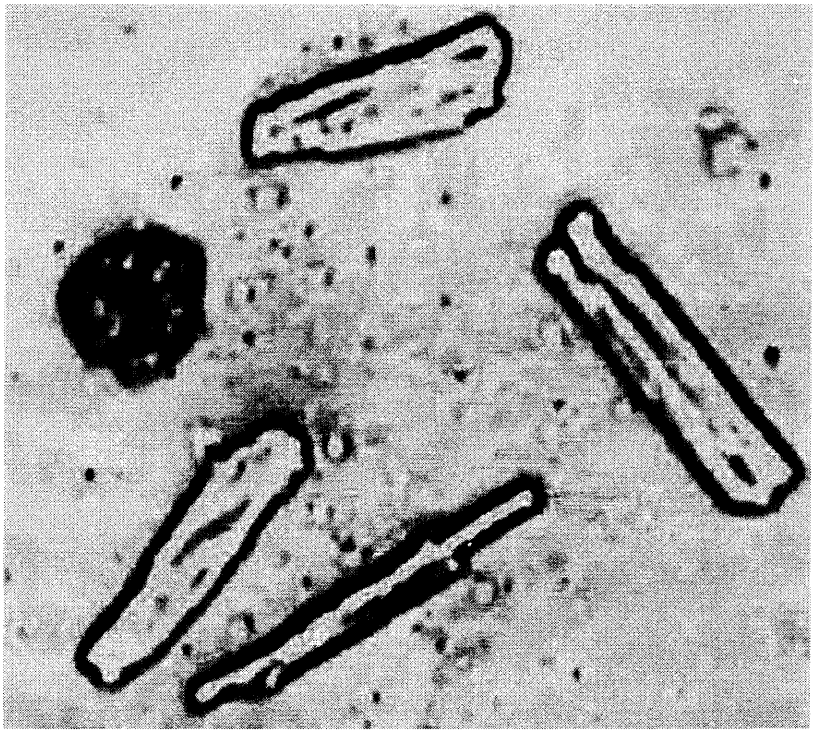

Figure 3.3 Cardiomyocytes

Another characteristic of a live cardiomyocyte is its ability to contract. In Figures 3.4(a-c), a cell undergoing contractions is presented. Figure 3.4a shows the cell in its resting stage, fully extended. As the contraction continues, the cell begins to shorten. Figure 3.4b shows the cell in its contracted state. After contracting fully, the

cell begins to elongate again and reaches the resting state in Figure 3.4c. For illustration purpose, a line has been drawn on these figures representing the resting state. With the aide of the line, it becomes easy to observe the cell contract and fully elongate again.

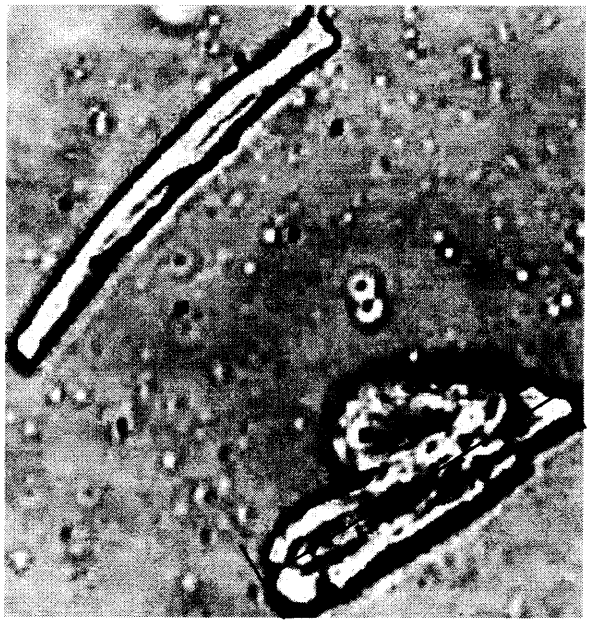

Figure 3.4a Cardiomyocyte fully extended

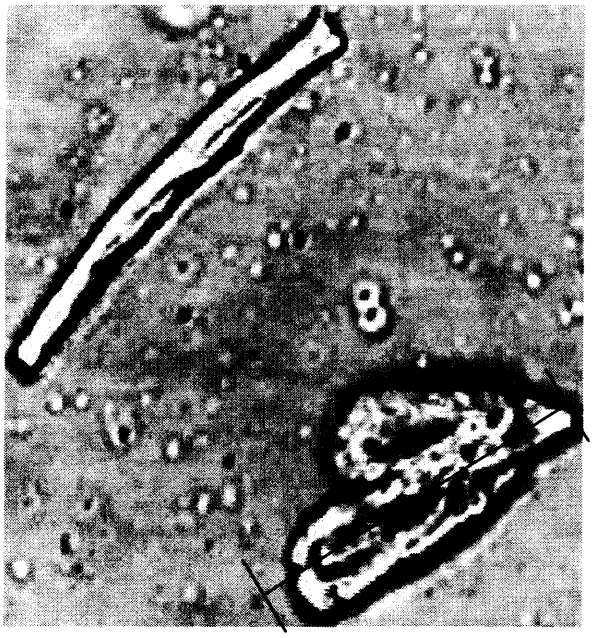

Figure 3.4b Cardiomyocyte in contraction

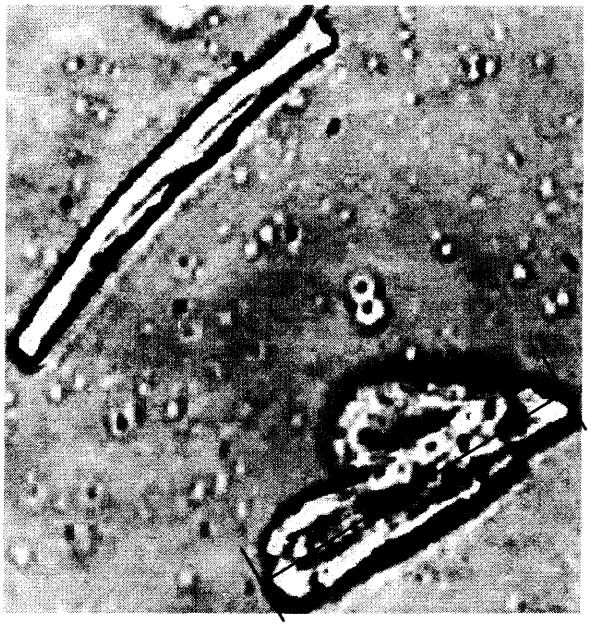

Figure 3.4c Cardiomyocyte in extension

3.3 Cell Attachment

In order to conduct flow experiments, first the cardiomyocytes need to be attached to the culture dish. Two surfaces were selected to for this purpose. The first was a polystyrene culture dish from Corning. Cells were incubated in the culture dish for three hours and allowed to settle to the bottom and bind to the surface. Figure 3.5 shows a cardiomyocyte bound to the polystyrene surface of the culture dish. Cells were also incubated in the cell

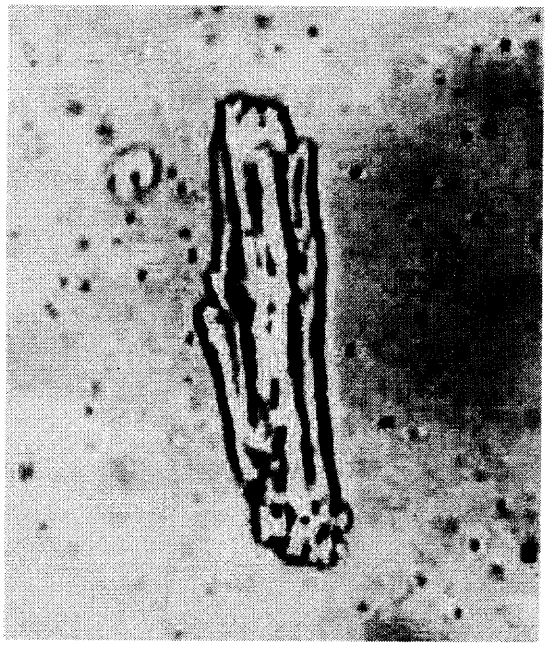

Figure 3.5 Cardiomyocyte attached to polystyrene surface

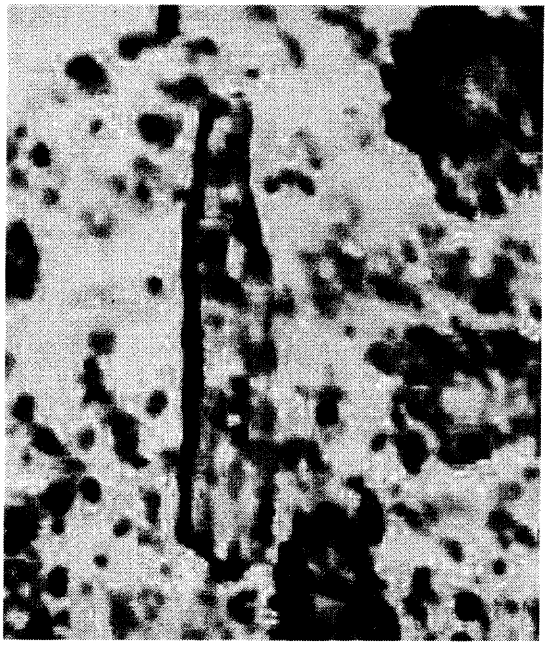

Figure 3.6 Cardiomyocyte attached to the membrane

culture inserts from Becton Dickinson. Cells were incubated and allowed to settle onto the membrane of the insert. Figure 3.6 shows a cardiomyocyte attached to the membrane of the culture insert. Once the cells had attached to the surface, the flow chamber apparatus was set up according to the procedures presented in the methods section of this paper. Upon completing the set up procedure, flow was initiated and cells were monitored and recorded on videotape.

3.4 Shear Stress

3.4.1 What is shear stress?

In a laminar flow chamber, the shear stress (force/area) acting on a fluid element by adjacent fluid elements is opposed by the drag force caused by the intramolecular attractive forces of the fluid (viscosity μ). The shear stress in a flowing fluid is maximal at the walls. Since the cardiomyocytes are attached to the walls of the flow chamber, they are subject to the maximal shear stress.

3.4.2 Calculation of shear stress at the wall

Based on the dimensions of the slit and the velocity of the medium, shear stress is calculated as described by Bird et al.[25] For laminar flow in a slit of dimensions **B** (thickness), **W** (width) and **L** (length), a steady state momentum balance on the fluid yields equation 3.1 relating shear stress τ_{xz} as a function of pressure difference Δ**P**. Then Newton's law of viscosity, equation 3.2, is substituted for τ_{xz} and the resulting relationship is integrated to give the velocity distribution (V_z) in the slit, equation 3.3. Since flow is the measured quantity, flow is calculated as average velocity $\langle v_x \rangle$ times slit area. The average velocity is obtained by integrating the velocity distribution over the cross-sectional area of the slit to give equation 3.4. From equation 3.4, $\Delta P/L$ (which is not measured) can be eliminated from equation 3.1 to give the shear stress in terms of the flow (**Q**), which is the measured quantity. This is given by equation 3.5 below.

$$(3.1)\quad \tau_{xz} = \left(\frac{\Delta P}{L}\right) B$$

$$(3.2)\quad \tau_{xz} = \frac{-\mu\, dv_x}{dz}$$

$$(3.3)\quad V_Z = \frac{(\Delta P)\, B^2}{2\mu L}\left[1 - \left[\frac{x}{B}\right]^2\right]$$

$$(3.4)\quad Q = \frac{2(\Delta P)\, B^3\, W}{3\mu L}$$

$$(3.5)\quad \tau_{xz} = \frac{3Q}{2B^2W}$$

The flow chamber is formed by sandwiching a rubber gasket between the flow chamber deck and the surface of the culture dish as illustrated by Figure 3.7. A rectangular slit is cut in the gasket to provide the path for the fluid to move through the chamber. Along with the flow chamber, four gaskets of varying width and thickness were provided. In these experiments, a single gasket of width 0.25 cm and thickness of 0.010 inch was utilized. By using the formulas above, shear stresses through the chamber were calculated. The shear stress values for the two flow rates used in these experiments are provided in Table 3.1. As specified above, the thickness of the path (**B**) was assumed to be 0.010 inch and width (**W**) was assumed to be 0.25 cm. For ease of calculation, viscosity (μ) of the medium was assumed to equal one centipoises, the viscosity of water. For this thesis, experiments were conducted with flow rates of 10 ml and 20 ml per hour.

At 10 ml/hr flow rate, the cells remained attached for two minutes and nine seconds on the polystyrene surface and fourteen minutes and eleven seconds with the membrane from culture inserts. For experiments with flow rate of 20 ml/hr, the longest duration that cells remained attached was nineteen minutes and twenty seconds on the polystyrene surface. No experiments were conducted at 20ml/hr using membrane from culture inserts.

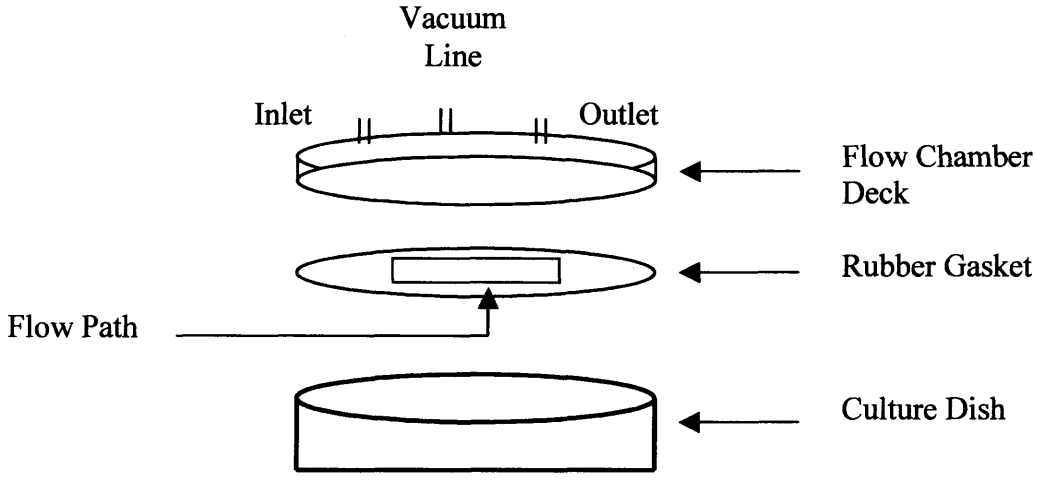

Figure 3.7 The flow chamber

Table 3.1 Shear Stresses

Flow Rate Q	Shear Stress τ_{xz}
10 ml/hour	.258 dynes/cm^2
20ml/hour	.516 dynes/cm^2

To illustrate flow through the flow chamber, Figures 3.8(a-d) are presented below. The figures present a live cardiomyocyte attached to the surface while another cell passes through the flow chamber. In Figure 3.8a the nonattached (free) cell is just entering the visible area as the attached cell remains adhered to the surface. In Figures 3.8b and 3.8c the free cell can be seen coming in contact with the adhered cell while passing though the flow chamber. Finally, in Figure 3.8d, the cell is seen exiting the visible window. The figures document the presence of flow through the flow chamber and ability of cardiomyocytes to remain attached to the surface during these experiments.

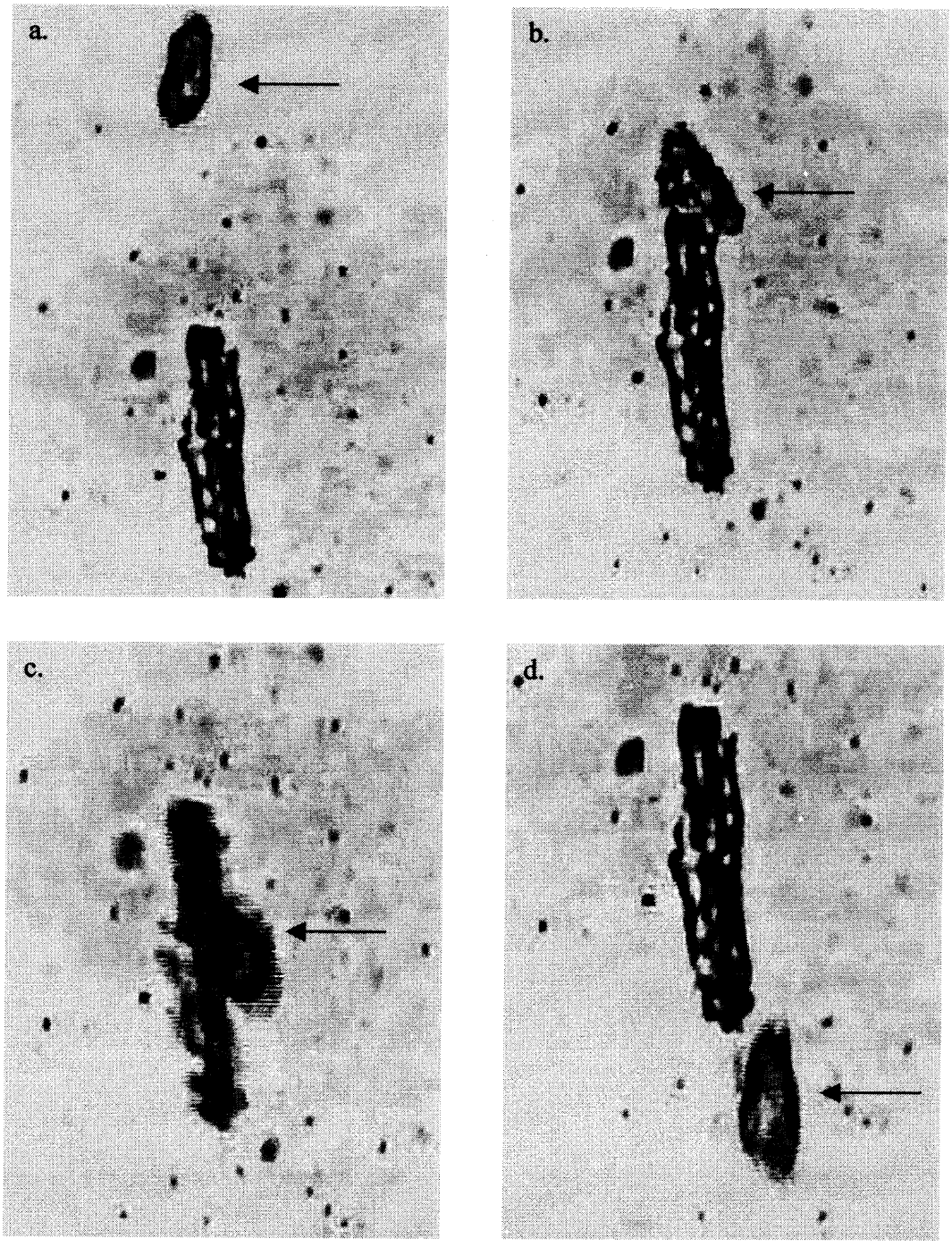

Figure 3.8a-d Fluid movement through the flow chamber

CHAPTER 4

DISCUSSION

This laboratory is the first to consider the effects of shear stress on cardiomyocytes in connection with regulation of the genetic expression of AT1 and AT2 receptors. This paper reports the preliminary findings and suggests future direction of research. As reported previously by others, cardiac hypertrophy is mediated by Angiotensin II through Angiotensin receptors AT1 and AT2. In the current research, procedures were developed that will allow observation of the effects of shear stress on regulation of the AT1 and AT2 receptor genes.

The current study reports on two significant achievements in completion of this task. First, in order to show regulation of AT1 and AT2 receptors, the ability to use the molecular biology techniques of RT-PCR were developed. The rat cardiomyocytes were successfully isolated, messenger RNA was extracted from these cells and the ability to consistently carryout RT-PCR to amplify AT1 and AT2 mRNA signals was demonstrated.

The other achievement includes the ability to attach live cardiomyocytes to a culture dish used for flow experiments. In order to administer shear stress through the use of the flow chamber, it was necessary to first successfully attach cardiomyocytes to the culture dish surface and prevent them from washing away with the flow medium. Through an incubation procedure, this laboratory has demonstrated the ability to attach cardiomyocytes to two different surfaces. The polystyrene surface of the culture dish

from Corning and the membrane of cell culture inserts from B-D were appropriate for cell attachment.

However, changes can be suggested to yield better results. In the flow chamber experiments, application of higher shear stresses is desired. However, there are several hurdles in accomplishing this task. The flow chamber provides some challenges due to its unstable construction. The components of the flow chamber including the flow chamber deck, the rubber gasket and culture dish are held together by vacuum. The slightest movement tends to throw off the equilibrium and causes suction to overwhelm the system and suction away the content of the chamber. The other challenge in utilizing the flow chamber is the entry of air bubbles. Even in the most careful techniques, it is impossible to completely eliminate the entry of air bubbles. The slightest movement of the chamber causes air bubbles to enter the flow chamber as the airtight junctions between the gasket and flow chamber deck are temporarily disturbed. Once an air bubble enters the flow path, it is impossible to extract it. It is also unrealistic to continue with the experiment as the presence of the air bubble in the flow path prevents shearing of the cells. The bubble also interferes with normal flow of the medium through the flow path.

The other challenge is the attachment of the cells to the surface. In order to increase the shear stress through the chamber, it is necessary for the cells to remain attached. If the cells are not adequately attached to the surface, they tend to flow away with the medium.

To strengthen cell attachment, two different surfaces were utilized. The first of these surfaces included the culture grade plastic (culture dish) from Corning. The results presented here showed that the plastic surface was useful in experiments utilizing flow

rates in the range of 10-20 ml/hr. Next, a culture membrane from culture inserts was utilized. The insert membrane is treated with a polymer to strengthen cell adhesion. Once again the results showed that cell attached best when flow rates of 10-20 ml/hr were administered.

As far as the challenges posed by the flow chamber, improvements will have to be made by the manufacturer. Until a better flow chamber is available, careful handling of the flow chamber is suggested. On the cell attachment side, other options remain. Some researchers have suggested using various attachment substances and serum mediums that will allow better cell adhesion.[26-28]

CHAPTER 5

CONCLUSION AND RECOMMENDATIONS

As discussed above, techniques have been developed to study the correlation between AT1 and AT2 gene regulation as it relates to the effects of shear stress on cardiomyocytes. The ability to track genetic regulation of angiotensin receptor through molecular biology techniques of RT-PCR has been shown. The ability to attach cardiomyocytes to a culture dish surface in order to subject these cells to shear stress via the flow chamber apparatus, has also been demonstrated

However, improvements can be made to take full advantage of the flow chamber apparatus. The central issue of these improvements is being able to get stronger cell attachments to the culture dish surface. This will increase the number of cells that remain attached to the dish and the length of time these attachments last. Improvements in the attachment will also allow higher flow rates to be applied during flow experiments.

Other methods for adhering cardiomyocytes have been published. Mitcheson and group have suggested a "rapid attachment" method.[26] The method is best for acute studies that will utilize the cell shortly after isolation. The culture surface is pretreated and cells are plated in a serum-free medium. The method utilizes laminin (a protein) diluted between 1-5 μl/ml in phosphate buffered saline. The culture surface is treated at least 30 minutes prior to plating cells. Other researchers have suggested use of various attachment substances including laminin, cell culture plastic, elastin, rat tailed collagen and fibronectin.[27] Use of medium containing foetal bovine or horse serum has also been suggested.[28]

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