Georgia Institute of Technology

Department of Biomedical Engineering

Effects of Serotonin and Cyclic Stretch on Aortic Valves

A Thesis
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Abstract

Aortic valve (AV) disease is highly prevalent and a major source of mortality and morbidity. AV disease involves extensive extracellular remodeling of the aortic valve. Studies show that chronic use of anti-depressants, and adverse mechanical stimuli are individually correlated to AV degeneration through remodeling. There appears to be a synergistic mechanism between cyclic stretch of the AV and the disruption of 5-HT reuptake mechanism, a signaling pathway involved in various cellular functions. The objective of our study is to use an *ex vivo* system to understand the role of the combination of stretch and 5-HT reuptake mechanism in remodeling in AV disease. Fresh porcine AV were exposed to uniaxial cyclic stretch using a stretch bioreactor at 10% and 15% stretch for three days and concurrently treated with: normal media, 5-HT only, 5-HT with four sub-receptor inhibitors or 5-HT with a 5-HT transporter antagonist. A sircol collagen quantitative assay was used to quantify changes in the collagen content, as collagen is one of the hallmarks of valvular disease and remodeling. Additionally, immunohistochemistry was used for semi-quantitative analysis using collagen degradative marker and synthetic marker for analysis. While the addition of 5-HT significantly up regulated Heat Shock Protein-47**,** a common collagen molecular chaperone, 5-HT_{1D} and 5-HT_{1A} receptor inhibitors were most effective in down regulating HSP-47. Collagen levels of the 5-HT transporter correlated with the levels of matrix metalloprotease-1 degradation marker. Investigation of the sub-receptor and transporter parts of the 5-HT pathway illustrate that both the degradative and synthetic pathways are involved in the increase of collagen due to 5-HT. The results provide clues for design of novel pharmacological treatments to counter 5-HT induced valve degeneration, through blocking specific 5-HT receptors.

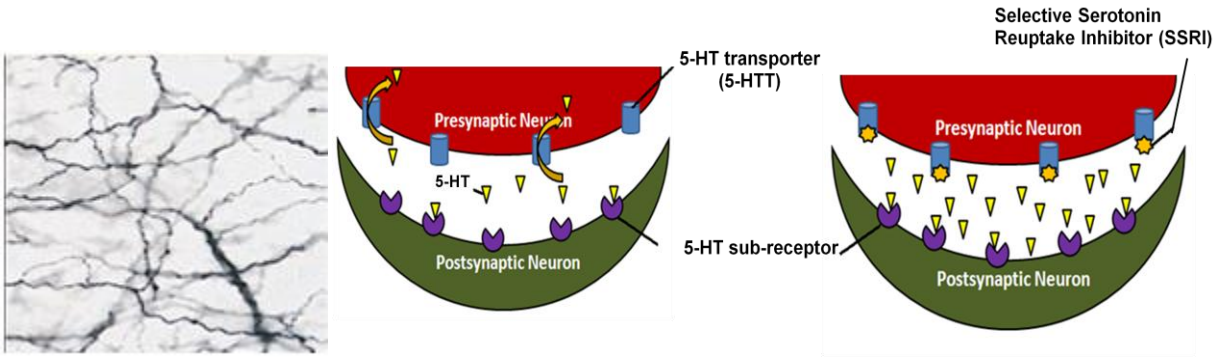
Key words: synergistic, reuptake, antagonists, degeneration

Section 1: Introduction

Aortic valves (AV) play a crucial role in controlling blood flow regulation from the left ventricle to the aorta. During systole, the AV opens to allow blood into the aorta while during diastole it closes to prevent the blood from flowing backwards. During blood flow regulation, various mechanical stimuli such as fluid shear stress, pressure, bending, and stretch act on aortic valves[1]. Recent studies show that altered levels of such forces acting upon the leaflets of the aortic valve may cause serious biological and pathological changes such as inflammation, calcification, stenosis, and ultimate failure[2]. While certain amounts of mechanical stimuli, such as stretch, are required for regulation of AV pathobiology[3], elevated levels of stretch may alter the proteolytic enzyme expression and activity in cells, leading to accelerated valve degeneration and disease progression[4,5,6]. Calcific AV disease is the third most common cardiovascular disease in the United States of America[7]. This disease leads to aortic stenosis, causing the leaflets to harden, and preventing the AV from opening and closing efficiently. This, in turn, prevents proper blood flow regulation between the heart and the rest of the body, leading to ultimate heart failure.

In previous studies of the mechanobiology of porcine aortic valves under normal hemodynamic conditions, the valves have been observed to go through a continuous renewal in order to maintain their native phenotypes.[8] Aortic valve leaflets exposed to hypertensive cyclic pressure reflected an increase in collagen and sulfated glycosaminoglycan.[9,10,11] Additionally, the cyclic stretching of porcine aortic valves showed an increase in collagen content through either the increase in collagen synthesis or decrease in degradation of collagen.[6] While all of these studies have demonstrated the effect of different mechanical forces on porcine aortic valves, further research is necessary to understand the mechanistic pathways behind these biological changes. The effect of numerous drugs and endogenous mediators in

addition to external stimuli such as stress, pressure, bending, and cyclic stretch of an aortic valve have been of constant interest to researchers.



a. b.

Figure 1: The image in (a) depicts the nerves in the aortic valve that was stained for the presence of acetylcholine, a common neurotransmitter involved in the nervous system and peripheral pathway. The black lines represent the acetyl choline in the nerves. The two images in (b) illustrate the serotonin (5-HT) pathway in the heart[12].

In order to gain a better understanding of possible causes of valve disease and the mechanistic pathways behind the changes, molecular remodeling is a very important aspect for understanding possible novel therapy to prevent the progression of valvular disease. Serotonin or 5-hydroxytryptamine (5-HT) is an endogenous vasoactive neurotransmitter that is heavily involved in regulation of various functions in the nervous system and gastrointestinal tract[13]. In a study conducted by Chester et al in 2008, the immunohistochemical characterization of choline acetyl transferase (an important enzyme involved in the synthesis of acetylcholine which is an important and common neurotransmitter of the nervous and peripheral systems) in a cross section of a valvular aortic valve reflected the presence of nerves in the aortic valves as can be seen in Figure 1. As a neurotransmitter, serotonin is present in the nerves of the AV. A native level of serotonin is present in a healthy human body that is needed for regular functioning of the gastrointestinal tract and nervous system. When the level of serotonin drops

below these native levels, a person goes into clinical depression. With the lower levels of serotonin, the psychological functions of the nervous system are negatively affected.

As a therapeutic approach to depression, doctors prescribe specific antidepressants (e.g. Zoloft®, Prozac®, etc.) that are selective serotonin reuptake inhibitors (SSRIs). These antidepressants prevent the reuptake of serotonin by the presynaptic neurons of the aortic valve. The inhibition of 5-HT re-uptake results in a higher concentration of serotonin in the body and counters the depressed levels of serotonin that result in depression. In a study by Connolly HM *et al*[10], the overproduction of 5-HT was observed to lead to valvular disease in patients with carcinoid tumors. Similarly, elevated circulating levels of serotonin have been reported to result in multiple cardiovascular pathologies such as cardiac valve fibrosis, systemic hypertension, and pulmonary hypertension.[8] Some of these studies have concluded that by blocking the reuptake of serotonin into the presynaptic neuron, SSRIs can lead to valvular disease[14]. Studies have also reported that intake of fenfluramine-phentermine, appetite suppressants that also act as SSRIs, have been observed to lead to valvular disease[10,15]. The hallmarks of valve fibrotic lesions include excessive cellular proliferation, and increased extracellular matrix synthesis[16, 17] resulting in abnormal thickening of the valve cusp leading to changes in valve mechanical properties. This irregular thickening of the valve cusp can ultimately lead to geometrical changes in the valve and valvular insufficiency[15, 18].

Recent studies indicated that these pathologies can occur via 5-HT-receptor and 5-HT transporter (5-HTT) mediated mechanisms. The presence of distinct subsets of 5-HT-receptors in heart valves further reinforces the fact that valvular cells have the potential to respond to 5-HT[16,17]. The 5-HT $_{2A}$ and 5-HT$_{2B}$ receptor subtypes are key mechanosensitive 5-HT sub-receptors that modulate the valve biosynthetic response to elevated serotonin levels [18].

The current study focuses on understanding the stretch-sensitivity of 5-HT transporters and sub-receptors and their involvement in 5-HT reuptake mechanisms that modulate valve remodeling. The focus of the study is on individuals suffering from depression, a very common disorder, and who are on chronic medication, such as dietary supplements, that disrupt the reuptake mechanism. The central hypothesis of this research is that aortic valve cusps respond to elevated levels of 5-HT via the knock-down of stretch-sensitive 5-HT reabsorption by increasing extracellular matrix synthesis and altering mechanical properties. Alterations of the extracellular matrix can stiffen the valve and results in stenosis, or lead to malcoaptation and regurgitation, leading to poor heart function. The clinical importance of this study lies in testing whether 5-HT has such life-threatening effects so that patients who are taking medications, such as Paxil® or Zoloft®, that increase 5-HT levels in the blood, may be prescribed different medicine that will not harm them in these ways.

Section 2: Methods and Materials:

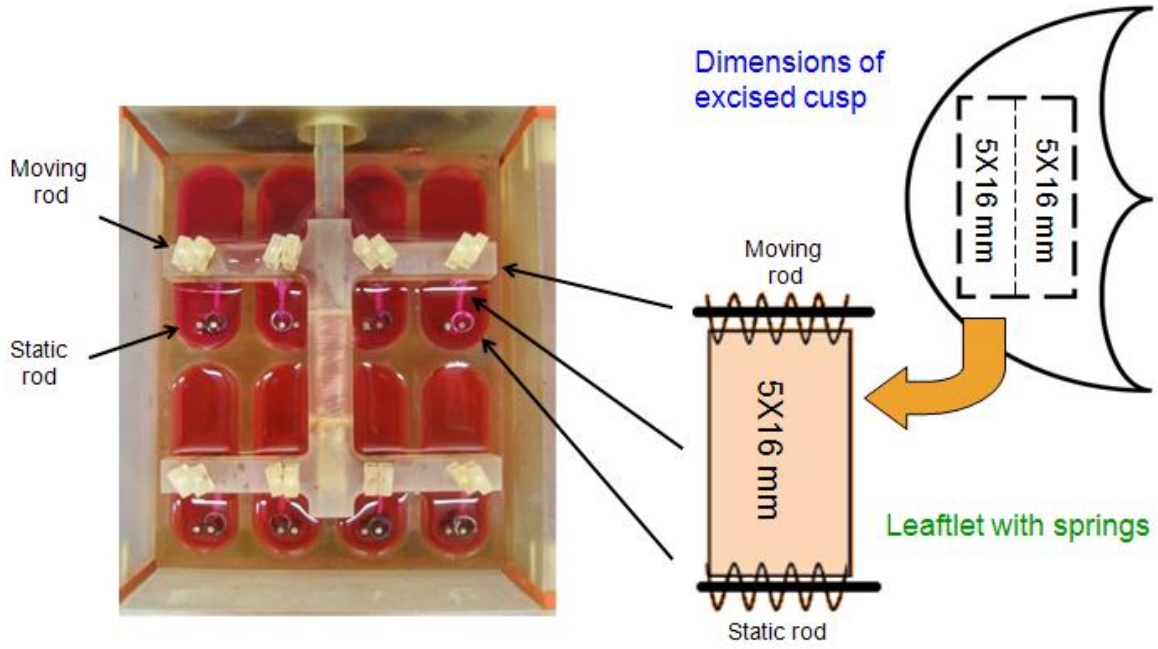


Figure 2: Porcine aortic valves obtained from slaughterhouse. Multiple 5 x 16mm sections were obtained and coupled to a stretch bioreactor with the use of springs.

Fresh porcine aortic valves were obtained from a local abattoir (Holifield Farms, Covington, Georgia) following on-site dissection of the hearts within 10 minutes of slaughter. The valves were then transported to the laboratory in sterile, ice-cold Dulbecco's Phosphate Buffered Saline (PBS). Upon arrival to the laboratory, a rectangular section of tissue with an aspect ratio of 16x5 millimeters was isolated from the central region of each valve cusp. Circumferentially-oriented tissue sections were extracted to be studied. These samples were then randomly assigned to a treatment group and placed into a stretch bioreactor where the tissue was coupled to the moving and static rods using springs as shown in Figure 2.

The Stretch Bioreactor

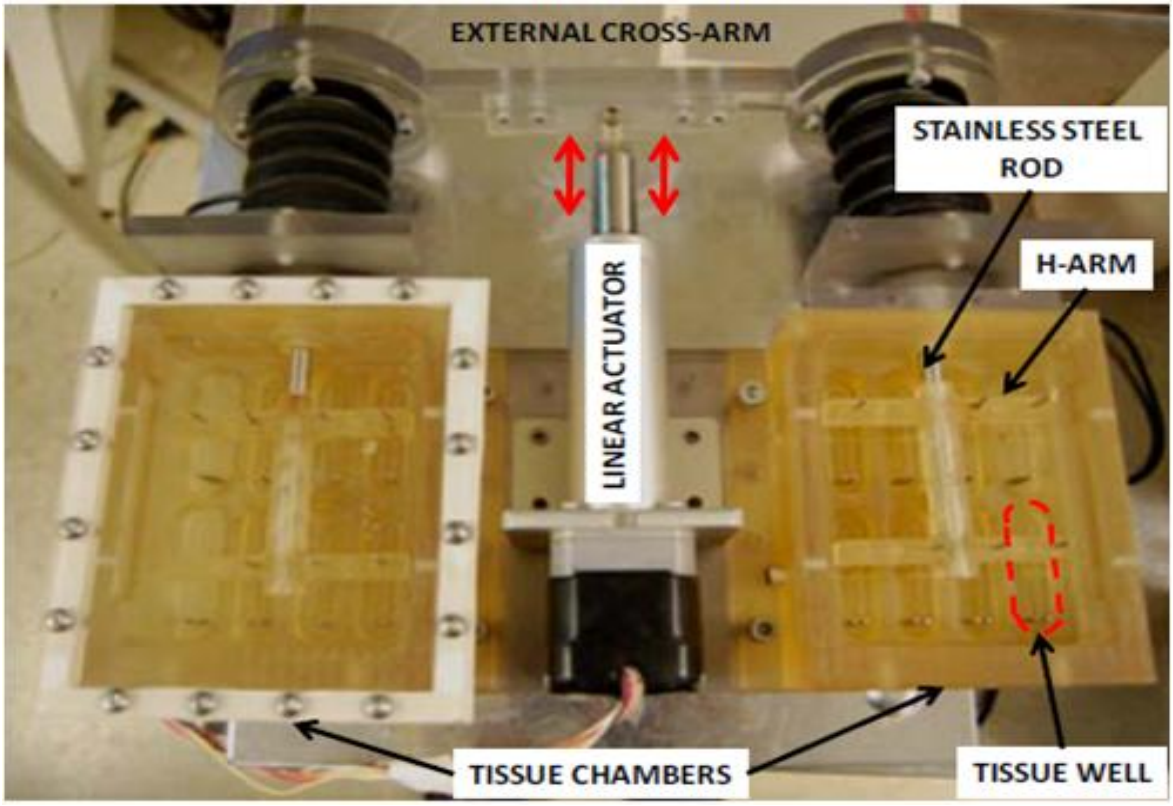


Figure 3: *Ex Vivo* tensile stretch bioreactor used in this study. The eight wells on both sides were coupled to the linear actuator (motor) through which a waveform specific for 10% or 15% stretch was input.

The main function of the stretch bioreactor that can be viewed in Figure 3 was to mimic the hemodynamic stretch forces present in the native environment of an aortic valve. Siprogrammer was used to input different stretch levels in the linear actuator that is coupled to the bioreactor. The input waveform varied according to the stretch level and the relationship varied, as can be observed in Figure 4, as a linear function of time. Circumferential cyclic 10% or 15% stretch levels were used as experimental conditions.

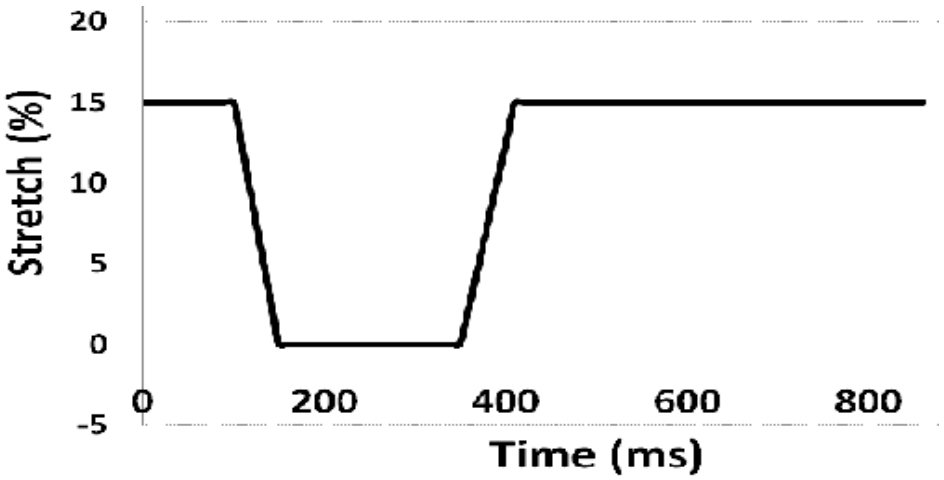


Figure 4: The linear relationship of the input waveform as a function of time.

Each of the wells were filled with a media called Dulbecco's Modified Eagle medium (DMEM), a base medium used to incubate the tissue that was to be stretched. This media was comprised of various components such as ascorbic acid, antibiotics, HEPES buffer, non-essential amino acids, fetal bovine serum albumin and water. The exact amounts of each component can be found in the appendix A. The media was buffered to a pH value of 7.4 and filtered prior to addition to the experimental samples.

Table I*. Experimental treatments*

Experimental group	5HT concentration	Pharmacological blocker	Concentration
Fresh control	-	-	-
Stretch control (10% and 15%)	-	-	-
5HT	$1x10^{-5}$ M	-	-
Inhibit 5-HT_{1A}	$1x10^{-5}$ M	WAY-100635	$3x10^{-6}$ M
Inhibit 5-HT_{1D}	$1x10^{-5}$ M	BRL14472	$1x10^{-5}$ M
Inhibit 5-HT_{2A}	$1x10^{-5}$ M	Ketanserin	$5x10^{-5}$ M
Inhibit 5-HT_{2B}	$1x10^{-5}$ M	SB204741	$1x10^{-6}$ M
Inhibit 5-HTT	$1x10^{-5}$ M	Paroxetine	$1x10^{-6}$ M
Inhibit 5-HTT	$1x10^{-5}$ M	Paroxetine	$1x10^{-5}$ M

The effect of two different concentrations of Paroxetine, a 5-HT transporter silencing RNA, on the aortic valve leaflets that were being stretched was tested by adding the aforesaid concentrations to the normal DMEM media. Additionally, the receptor part of the serotonin pathway was studied to understand which serotonin sub-receptors were highly involved in the collagen synthesis pathway. The control group consisted of fresh valves that were preserved immediately after slaughter. The concentrations and pharmaceutical names of the different experimental groups are provided in table I. The chemical structure of the agents in table I are provided in Appendix C.

Experimental Duration

The entire bioreactor was placed in an incubator with 5% CO_2 and experiment was run for 72 hours. Media in the bioreactor was checked to ensure that p^H remained at the physiological value of 7.4, as the changes in p^H may induce unwanted responses such as apoptosis or contamination. If p^H levels varied, CO_2 levels were checked and adjusted to 5%, but if the media discoloration occurred, the media was replaced.

Quantification of Collagen Content

Total pepsin soluble collagen content was determined using the commercially available Sircol Collagen assay kit (Biocolor, UK) colorimetric assay. This kit enables to use a quantitative dye-binding method to determine the total quantity of pepsin-soluble collagen in the samples. The amount of collagen was then normalized by the dry weight of the sample. The protocol and its details are provided in appendix A.

Immunohistochemical Analysis of Collagen

At the conclusion of an experiment, samples were embedded in an embedding media called Optimal Cutting Temperature (OCT) and snap frozen. Tissue sections of 5-7 μm were obtained using a cryostat. Immunohistochemistry analysis was done on tissue sections for the expressions of lysyl oxidase (a common collagen cross-linking marker), heat shock protein-47 (an important molecular chaperone involved in the maturation of collagen molecules), and prolyl hydroxylase (an enzyme that is involved in the production of collagen). Images of the slides were taken using a fluorescent microscope. A custom-written MATLAB program was used to quantify the area of immunopositive staining per image field normalized by the number cells in that image field. The protocol and its details are provided in the appendix B.

Statistical Analyses

A minimum sample size of at least 6 for each treatment group was obtained. Error bars indicate the standard errors. A paired t-test was used and a value of $p<0.05$ was considered to be statistically significant.

Section 3: Results

The results from the study reflected interesting and significant aspects about the serotonin pathway. The results are divided based on the part of the mechanistic pathway that was focused on.

Serotonin Transporter Results

The first marker that was experimented with was collagen type I and it is shown in Figure 5. Addition of 5-HT led to increase in collagen type I expression and the addition of 5-HT + 5-HTTI led to increase in collagen type I as well. At the physiological stretch level, there is a significant increase in the collagen type I content in both the 5-HT and 5-HTT inhibitor groups compared to the normal group. A significant difference, however, is not observed between the 5-HT and 5-HTT inhibitor groups.

To understand whether the increase in collagen type I content was due to decreased degradation of collagen or increased synthesis of collagen, the expression of several common markers was investigated. Matrix Metalloprotease-1 (MMP-1) is the degradative marker that I examined. As can be seen in Figure 6, the increase in collagen type I correlated with a decrease in MMP-1 expression. High levels of remodeling corresponded to lower levels of collagen type I present, whereas low levels of remodeling reflected higher levels of collagen type I present.

For the synthetic pathway of collagen, enzymes involved in the synthesis of molecules and cross-linking of newly synthesized molecules are very important expression that can provide critical information about a pathway. Prolyl-4-hydroxylase (P4H) is the common collagen synthesizing enzyme that I investigated in this study. No significant alterations were observed for the P4H results as seen in Figure 7. Lysyl oxidase (LOX) is the common collagen cross-linking marker that I studied in this experiment which also did not reflect statistically significant alterations in the presence of the

pharmacological blockers as shown in Figure 8. However, a measurable increase with an increasing stretch level was observed.

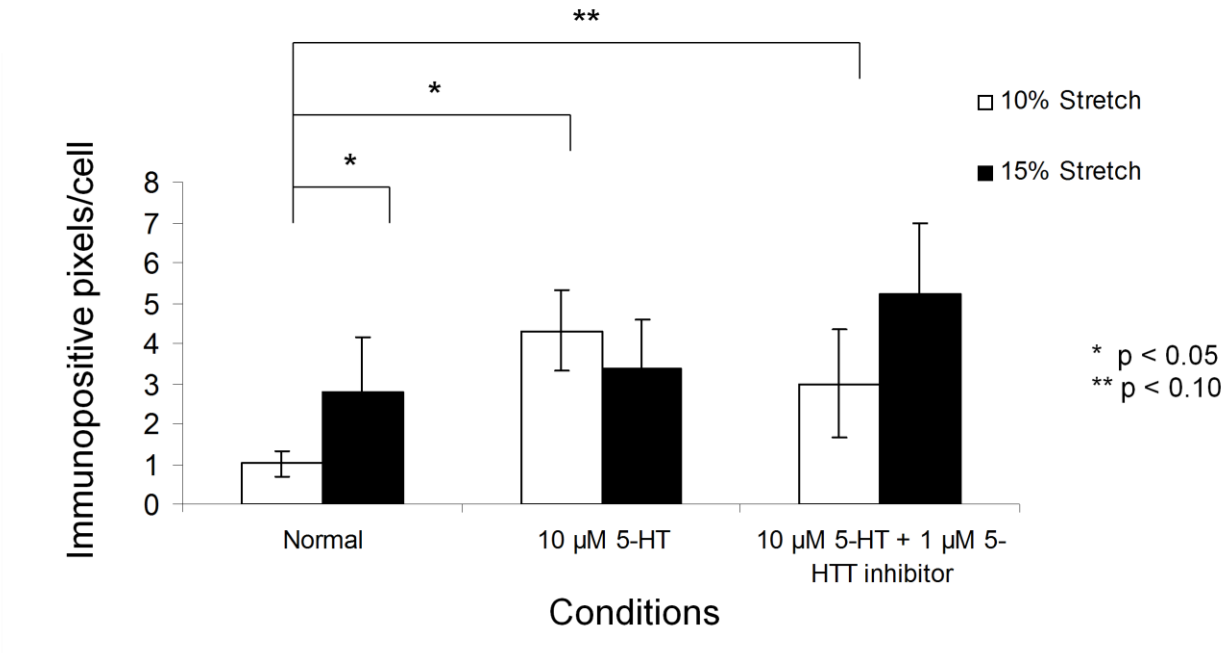


Figure 5: Quantification of collagen type I expression in the 5-HT transporter pathway

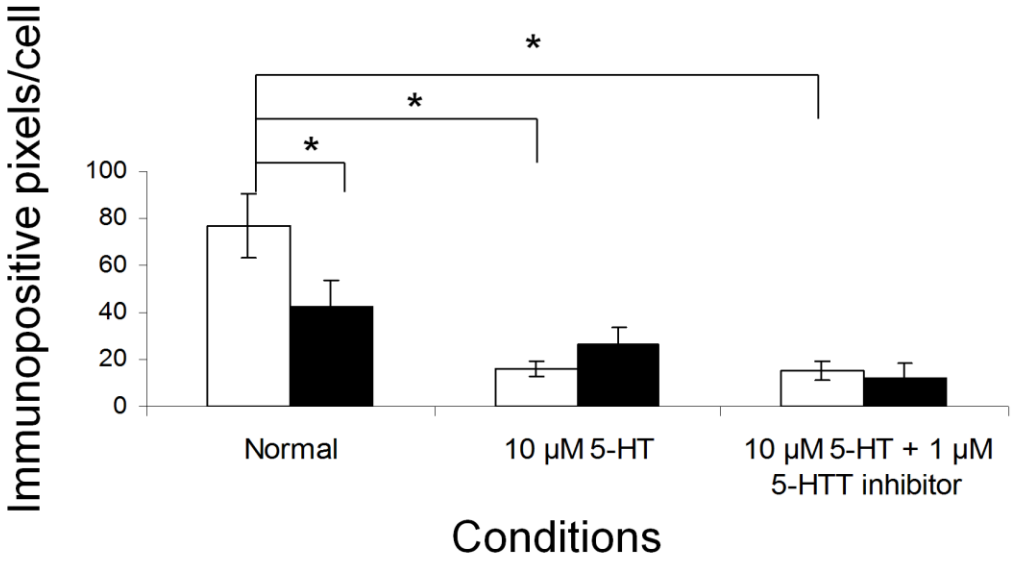


Figure 6: Expression of Matrix Mettaloprotease-1 in the 5-HT transporter pathway

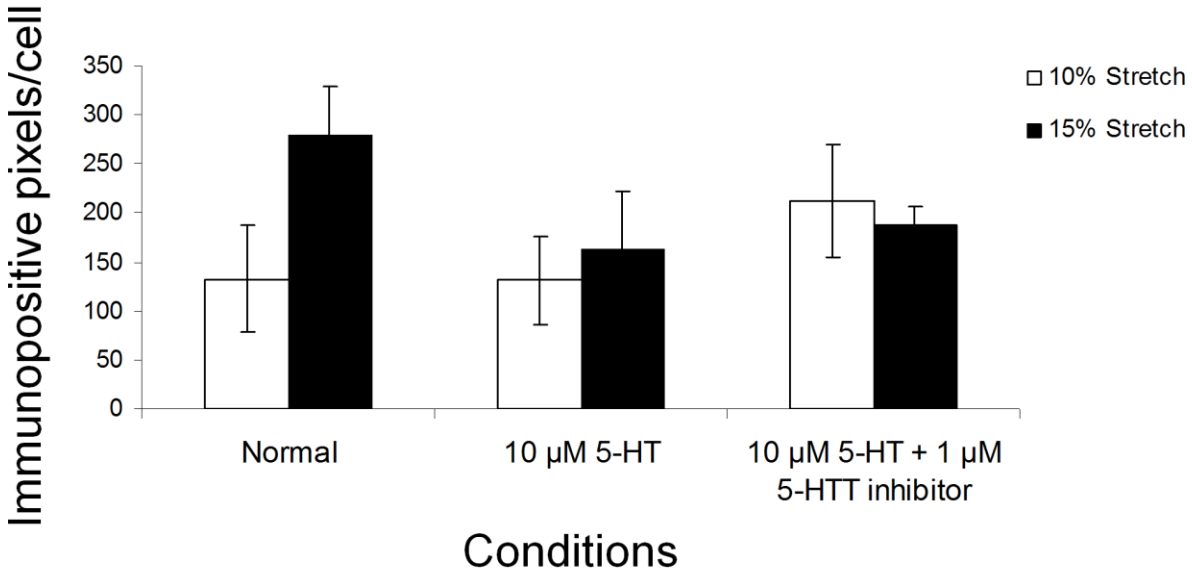


Figure 7: Quantification of Prolyl-4-hydroxylase in the 5-HT transporter pathway

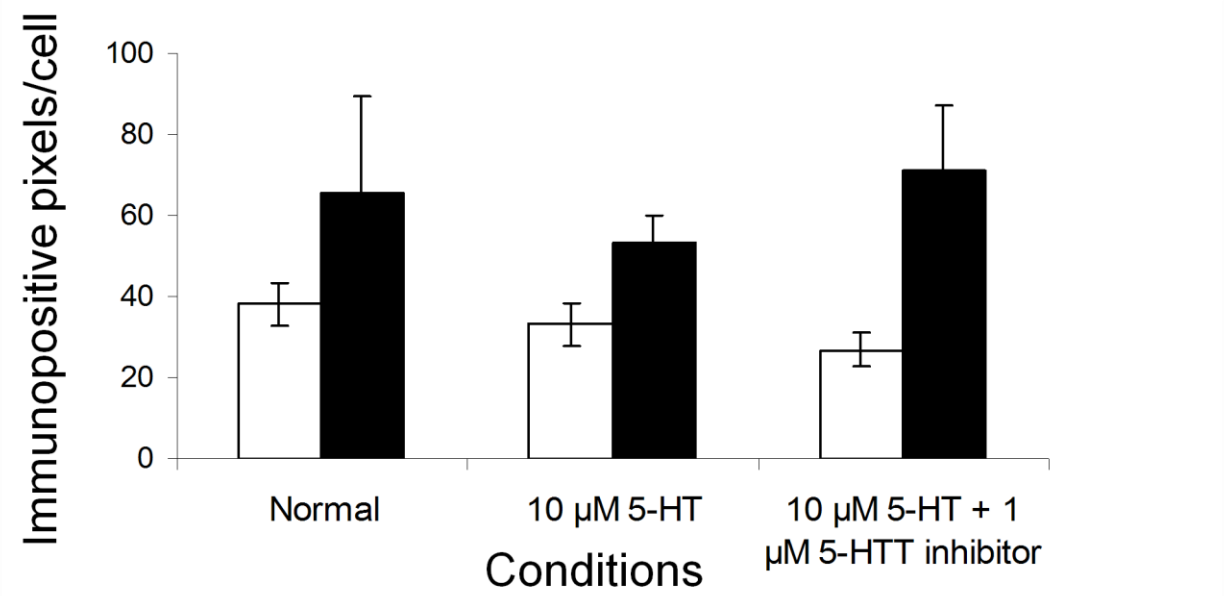


Figure 8: Expression of Lysyl Oxidase in the 5-HT transporter pathway

Serotonin Sub-Receptor Results:

For the serotonin sub-receptors, a quantitative assay was first used to observe any differences in the quantitative level of pepsin soluble collagen and to determine the effect of the sub-receptors both in the presence of serotonin and individually for two main sub-receptors. As can be observed in Figure 9,

the addition of 5-HT, resulted in a significant increase in the level of pepsin soluble collagen in the experimental samples. Increasing the stretch level from 10% to 15% stretch also reflected a significant change in the level of pepsin soluble collagen. The presence of two of the most common inhibitors in the absence of serotonin reflected how the inhibitors maintained the native collagen level in the tissue. In comparison to the normal group, the level of collagen decreased in the presence of both the inhibitors tested.

For the sub-receptor part of the serotonin pathway, the two major biological markers that were tested to understand the mechanistic pathway of collagen accumulation were the synthetic markers, heat shock protein-47 (HSP-47) and LOX. As shown in Figure 10, the addition of 5-HT led to an increase in the expression of Heat Shock Protein 47 at both 10% and 15% stretch. In the presence of any of the experimental sub-receptors, the HSP-47 expression decreased but to different extents. The most effective sub-receptor inhibitors were the $5\text{-}HT_{1D}$ and $5\text{-}HT_{1A}$ sub-receptors at 10% and 15% stretch respectively. Figure 11 illustrates no significant changes for the expression of LOX, a common collagen cross-linking reagent.

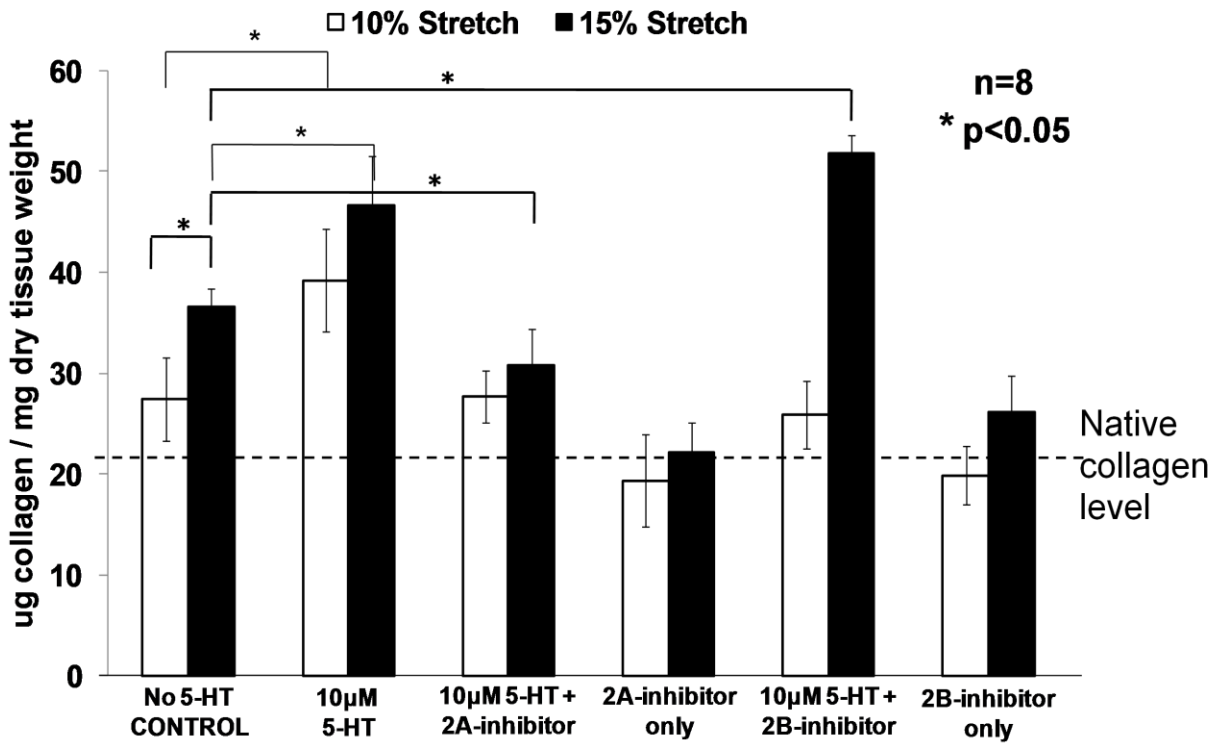


Figure 9: Sircol Collagen Assay Quantitative Assay results for some sub-receptor inhibitors

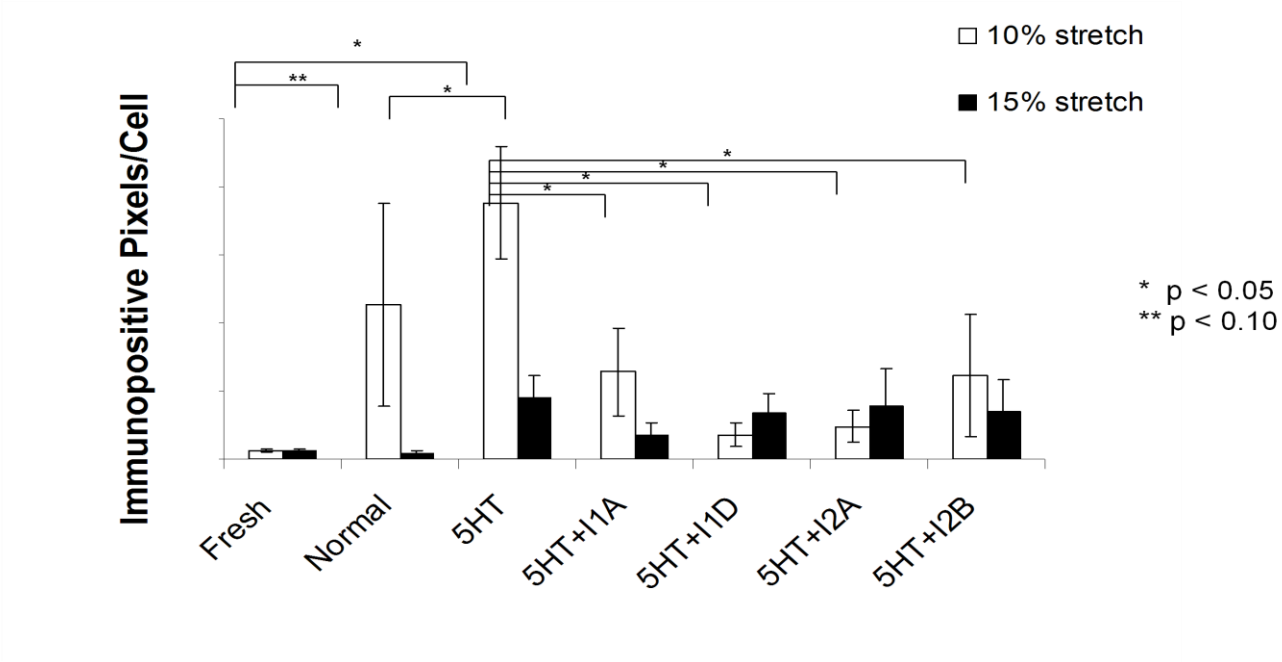


Figure 10: Expression of Heat Shock Protein-47 expression in the 5-HT sub-receptor pathway

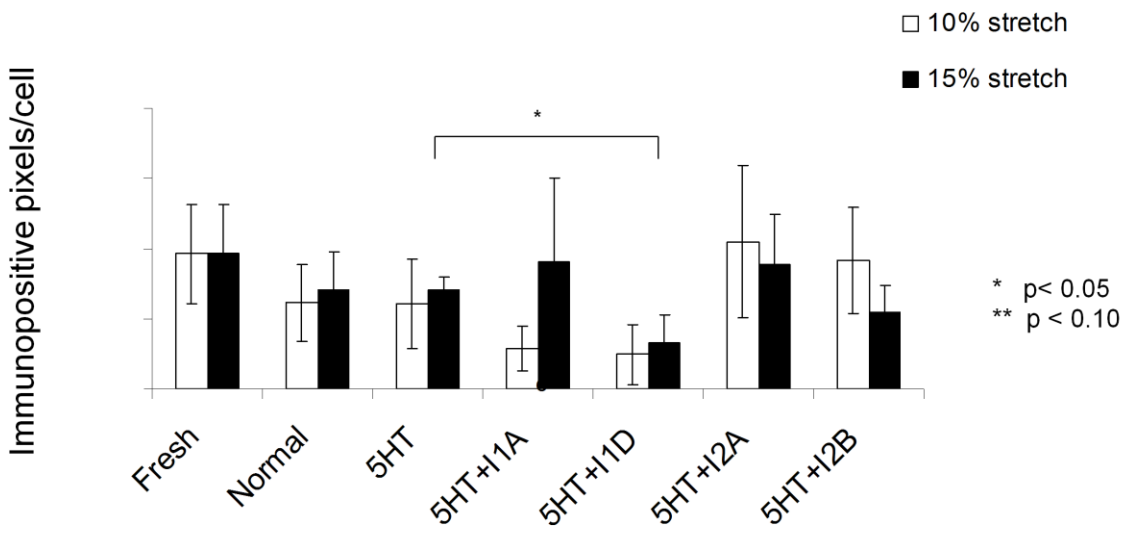


Figure 11: Quantification of Lysyl Oxidase in the 5-HT sub-receptor pathway

Section 4: Discussion

Collagen Type I is the most abundant collagen in the human system. Due to this reason, the experimental samples were analyzed for the expression of collagen type I and as shown in Figure 5, the level of collagen type I increased significantly with the addition of serotonin and the serotonin transporter inhibitor at both the 10% and 15% stretch levels. A statistically significant difference was, however, not observed between the serotonin only and serotonin + transporter inhibitor samples. This could imply that the dosage or concentration of serotonin transporter inhibitor was not significant enough to cause a considerable change in the collagen type I level.

In order to understand whether the increase in the expression of collagen type I with the addition of serotonin and transporter inhibitor was due to a synthetic pathway or a degradative pathway, several markers were tested to observe which pathways demonstrated clear trends. By analyzing the samples for the expression of matrixmetalloprotease-2 (MMP-1), an enzyme involved in the collagen degradation and remodeling process, the results correlated very clearly with the collagen type I results. A high level of remodeling and thus high expression of MMP-1, corresponded with a lower level of collagen type I. Similarly, a lower level of remodeling correlated with a high level of collagen type I expression in the serotonin groups. Again while the serotonin and serotonin + transporter inhibitor groups were both significantly different compared to the normal group, the groups were not significantly different from each other.

Analyzing the samples further with synthetic markers revealed insignificant differences between the normal and serotonin groups. Prolyl-4-hydroxylase, a common enzyme involved in the synthesis of collagen, and lysyl oxidase, a common collagen cross-linking reagent, did not reflect statistically significant differences between the serotonin and normal control groups. While this could imply that these may not be the specific synthetic enzyme or cross-linking reagent involved in the synthesis of

collagen, it is also possible that the increase in collagen due to synthesis is minute compared to the degradation of collagen. Therefore, the results suggest that in the 5-HT transporter part of the pathway, high levels of collagen may result from the decreased degradation of collagen and that the increased synthesis of collagen is not a contributing factor.

Investigation of the serotonin sub-receptor part of the serotonin pathway, the increase in collagen observed could be the result of a different set of proteins and biological operations. Analyzing the amount of pepsin soluble collagen present in the normal and two of the common mechanosensitive groups, the 5-HT_{2A} and 5-HT_{2B} sub-receptor groups, gave results that were very significant as shown in Figure 9. With an increase in stretch level from 10% to 15%, the amount of pepsin soluble collagen increased. This is consistent with previous studies which have shown that compared to physiological stretch levels (10% stretch), the amount of collagen and remodeling that results at pathological stretch levels (15% stretch) is higher[3]. Additionally, with the addition of 5-HT the amount of pepsin soluble collagen also increased, suggesting that the presence of a higher concentration of 5-HT can result in a higher level of pepsin soluble collagen.

In the presence of the two commonly tested sub-receptors, the amount of pepsin soluble collagen decreased but to different extents. Addition of the 5HT 2A inhibitor in the presence of 5-HT, the level of collagen decreased by a greater amount than did the level of collagen with the addition of the 2B inhibitor in the presence of 5-HT. The 2A and 2B inhibitors were experimentally tested to ensure that the drugs themselves did not have any additional effect that would alter the results of the study. With the addition of only the sub-receptors without any additional serotonin, the sub-receptors were observed to regulate and maintain the native levels of collagen as can be seen in Figure 9. These results suggest that the inhibition of mechanosensitive receptors can inhibit the pathological over expression of collagen.

In order to understand how the increase in collagen came about for the sub-receptors, a few synthetic markers were tested. In Figure 10, the samples were analyzed for the expression of HSP-47, a common collagen molecular chaperone that is involved in the folding and maturation of newly synthesized collagen molecules. The presence of 5-HT led to a statistically significant increase in the expression of HSP-47 at both stretch levels. With the addition of any of the four different sub-receptors, the level of collagen was observed to decrease by different amounts. The sub-receptors that were most effective in inhibiting the synthesis of collagen were the 1A and 1D sub-receptors at the 10% and 15% stretch levels respectively. The least effective sub-receptor at both stretch levels was observed to be the 2B, which reflected the greatest expression of HSP-47, which could mean that more molecular chaperones were present due to the greater amount of newly synthesized collagen molecules that needed to be folded. Cross-linking is evident in newly formed collagen molecules. However, analyzing the samples for the expression of LOX did not demonstrate statistically significant results. This could imply that LOX might not be the cross-linking reagent involved in this collagen pathway. Therefore, the results imply that these higher levels of pepsin soluble collagen may result in the serotonin sub-receptor part of the pathway by synthesizing new collagen molecules. In conclusion, the overall increase in collagen due to the inhibition of serotonin transporters results in a lower amount of degradation and a higher amount of collagen present. With the inhibition of these serotonin transporters, a higher level of serotonin can be taken up into the body by the 1A and 1D sub-receptors which were observed to be highly involved in the synthetic pathway of collagen.

In conclusion, the role of serotonin in the remodeling pathway can be through both degradative and synthetic pathways. Therefore, any novel therapy to prevent heart disease in patients that take anti-depressants or anti-dietary medications needs to target the 1A and 1D sub-receptors to prevent remodeling of the aortic valve leaflets that could possibly lead to aortic heart valve disease.

Appendix A: Experimental materials and reagents

For Tissue Harvesting:

Reagent	**Company**
Dulbecco's phosphate buffered saline (dPBS) 500 mL	Sigma #D5773
Ethanol/Methanol spray bottle	
1 x 6-well plate	Fisher #08-772-1B
1 x surgical scissors	McMaster
Stainless steel dissection tray (big)	Fisher/VWR
Tweezers (small)	McMaster
Cooler with ice	

For stretch experiments:

Components	**Company**
1x scalpel handle (no. 3)	Fisher #08-915-2
Sterile scalpel blades	EMS #72044-10
2x razor blades (0.009")	VWR #55411-050
Spacer rods (5 mm, 11 mm, 6mm)	McMaster
Screws and nuts for spacers	McMaster
Stainless steel dissection tray	McMaster
1x 6-well plate	Fisher #08-772-1B
Stainless steel springs (cut-to-length) McMaster	#9663K14McMaster
Tweezers (1 curved, 1 straight)	McMaster

For 1 liter of Dulbecco's Modified Eagles Medium (DMEM, Sigma#D5648) solution:

Reagent	amount
Dulbecco's Modified Eagles Medium	13.3grams
Fetal Bovine Serum	100 milliliters
Ascorbic Acid	2.5 milliliters
Sodium Bicarbonate	3.7 grams
Non-essential amino acid (100x)	10 milliliters
antibiotics (100x)	10 milliliters
HEPES buffer	25 milliliters
deionized water	volume required to fill solution to 1 Liter

Stretch bioreactor and motor:

Allen key set	McMaster
5x 10/32 screws	McMaster

Tissue processing for Sircol Collagen Assay

For snap-freezing tissue:

Reagents	**Company**
Cryovials	EMS #61800-1
Cryovial holders	EMS #61080-1
Liquid nitrogen	

Tissue processing for Immunohistochemistry

For frozen block preparation:

Reagent	**Company**
plastic molds	EMS #70180-70184
OCT compound	EMS #62550-01
Tweezers (small)	McMaster
Liquid nitrogen	IBB mechanical chase

Immunohistochemistry antibody dilutions:

Antibody	**Dilution**
Heat shock protein 47	1:100
Lysyl oxidase	1:50
Collagen type I	1:300
Prolyl-4-hydroxlase	1:400
Matrix Metalloprotease-1	1:50

Appendix B: Experimental Protocols

Day before Experiment

1. Autoclave scissors and forceps in small sterilization pouch.

2. Autoclave tray and absorption pad in large sterilization pouch.

3. Autoclave all spacers, razor blades, surgical tools, screws, nuts and springs in a small sterilization pouch.

4. Pack and sterilize stretch bioreactor in ethylene oxide (EtO) sterilizer.Alternatively, fill wells of stretch bioreactor with ethanol/methanol, cover and place under UV overnight (not preferred).

5. Place all sterilization pouches under UV overnight.

6. Autoclave dPBS and put in fridge overnight.

7. On morning of slaughterhouse trip, fill cooler with ice and place dPBS bottle in it.

At slaughterhouse

1. Spray work area and tray thoroughly with alcohol.

2. Fill 6-well plate(s) with ice-cold dPBS.

3. Cut porcine heart between right and non-coronary leaflet and through the septum to reveal all three leaflets.

4. Excise each leaflet, wash in ice-cold dPBS and place in 6-well plate, keeping track of the anatomical position of each leaflet sample.

5. Repeat 3-4 for each heart.

6. After tissue collection, place 6-well plate(s) in ice and return to lab.

Experiment setup

1. Use sterile techniques and a laminar flow hood at all times.

2. Place DMEM from fridge into incubator/water bath at 37°C.

3. Using the spaced razor blades, cut out a rectangular section of valve tissue from the base/belly region of the leaflet.

4. Thread springs through each of the short ends of the leaflet section.

5. Couple linear motor to bioreactor.

6. Mount leaflets in the bioreactor and fill ~8 mL of DMEM in each well and place the bioreactor in the incubator.

7. Start the linear motor using appropriate code within Si Programmer.

8. Monitor bioreactor at least twice a day. Media should be changed every 1-3 days, or whenever it has turned yellow (due to generation of waste/metabolic byproducts).

Note: During the experimental steps, sterile techniques must be used at all times. Wearing face masks is not absolutely necessary for porcine tissue, but must be worn at all times for other tissue types (eg: human, ovine etc). Additionally, an alternative to 6-well plates is to use autoclaved plastic containers and excise out the entire aortic valve with the root and a portion of the aorta and place it in ice-cold dPBS. The individual cusps will then be excised in a laminar flow hood in the laboratory.

Stopping of experiment

1. Autoclave surgical tools and dPBS well in advance. Refrigerate dPBS.

2. On day of experiment, bring dPBS to 37ºC in incubator or water bath. Fill 6-well plate with dPBS.

3. Return stretch bioreactor to laminar flow hood.

4. Use Si Programmer to stop linear motor.

5. Remove tissue from stretch bioreactor. If there are still samples in the bioreactor

(eg: we are looking at different timepoints), immediately cover bioreactor and return to incubator and resume the linear motor.

6. Using scalpel, remove portion of leaflet that was attached to the spring.

7. Immerse leaflet in dPBS 3x to wash away DMEM. More washes may be necessary. Tissue should appear whitish and lose most of the pink color from the DMEM before proceeding to the next steps of the protocol. dPBS should not be colder than room temperature as that might result in thermal shock to the tissue samples.

Preparation of frozen blocks for histology/immunohistochemistry

1. Label plastic molds accordingly.

2. Arrange tissue samples at the base of plastic mold in appropriate orientation.

3. Fill mold with OCT compound.

4. Using microdissection forceps “stand” the tissue in the OCT compound. If en face sections are required, tissue should be placed face down in the mold.

5. Fill vacuum flask with liquid nitrogen.

6. Using long hemostat, dip OCT block in liquid nitrogen. Ensure that the block is not submerged (liquid nitrogen should NEVER contact the OCT directly). Make sure liquid nitrogen does not fall on the OCT compound as it will cause bubbles and cracks to develop in the block.

7. OCT blocks will start to solidify and turn opaque. Keep blocks in liquid nitrogen until just a small circle (approximately 5mm in diameter) of OCT on the top face of the block is left to be solidified. This will ensure that the block does not crack.

8. Place frozen blocks in -80°C freezer.

Snap-freezing of tissue for Sircol Collagen Analysis

1. Label cryovials accordingly.

2. Place one tissue sample per cyrovial and snap vials into cyrovial holder.

3. Place entire cryovials into liquid nitrogen (in the dewar).

4. Transfer cryovials to -80°C freezer. Alternatively, cryovials can be maintained in liquid nitrogen, but care should be taken to ensure that the liquid nitrogen level is always topped up.

Pulverization of Cardiac Tissue

1. Place mortar and pestle, funnel, cryovials into Styrofoam box filled with ~ 2 L+ of liquid N2.

a. This is to ensure everything is at the same temp and that nothing will stick to the mortar and pestle (i.e. frozen grounded-up valve tissue).

2. Immerse tissue (you only need ~ 1 g for any biochemical study) into the liq. N_2 for at least 1 minute.

3. Using the tongs, bring the mortar out and leave it half filled with liquid N2. Pull out the pestle and tissue and then in a twisting and pulsating downwards motion, push down on tissue, using one hand to hold the mortar down.

a. Don’t push too fast or too hard because some liq. N2 and bits of tissue will spill out.

4. Continue grinding and pulverizing until the heart tissue is a very fine granular powder. This may take a few minutes.

a. At times the grinding takes so long that more liq. N2 will have to be added to the mortar.

b. Use a small plastic cup and be careful when pouring it in (else tissue might spill out).

c. Add enough liq. N2 to make a nice tissue slurry as this will be important in later steps. 238

5. When done grinding heart tissue up, use tongs to get the cryovial and funnel. Set the cryovial onto the test tube rack and sit it upright. Insert the small hole of the funnel into the mouth of the cryovial.

6. Using your gloves, quickly pour the tissue slurry into the funnel-cryovial. If there is tissue left in the mortar, use the spatula (dip it into liq. N_2 first) and then scrape the remaining bits in. Finally, use the spatula and tap the side of the funnel so that the rest of the tissue funnels into the cryovial.

Sircol Collagen Assay

Reagent	**Company information**
Pepsin	Sigma#P7000
Acetic acid 0.50 M	Sigma #242843
Sircol collagen assay kit	Accurate Chem #CLRS1000

Procedure:

1. The tissue is first digested by using pepsin dissolved in 0.5 M acetic acid (buffer) for 48 hours at 37 °C. During this period, the tissue needs to be stirred. The ratio of pepsin added to dry tissue weight is 1:3.
2. Reagent blanks (100 µL buffer), collagen standard (double aliquots containing 5, 10, 25, 50 µg) and test samples (10-100 µL) are then prepared. The contents of all tubes are then adjusted to 100 µL with the 0.5 M acetic acid buffer.
3. Approximately 1 mL of Sircol Dye reagent is then added to the solutions and mixed using a mechanical shaker for 30 minutes.
4. Tubes are then transferred to a micro centrifuge and spun at > 10,000 xg for 10 minutes.
5. Tubes are then inverted to drain the unbound dye.
6. Approximately 1 mL of alkali reagent is added and the solution mixed.
7. Approximately 200 µL aliquots of samples are taken from the tubes and put into a 96-well multi-well plate.
8. The plate is then placed in a microplate reader, and the absorbance is read at 540 nm.

Immunohistochemistry

General Protocol:

Slides were first thawed to room temperature, and permeabilized in acetone at -20°C for approximately 5 minutes followed by a few 5 minute washes in phosphate buffered saline (PBS, Sigma). Sections were then blocked using either 10% animal serum in 10% Bovine Serum Albumin (BSA)/ 1 molar PBS for 30 minutes. The choice of animal serum to use as the blocking agent depended on the species that the secondary antibody was raised in. This step is to ensure that there is no non-specific binding of the secondary antibody to the section. Following the blocking step, the slides were then incubated in primary antibody in 10% blocker overnight to ensure efficient staining. The sections were then saturated in biotinylated secondary antibody (Vector laboratories) in 10% Bovine Serum Albumin (BSA)/ 1 molar PBS for 1 hour. Subsequently, Avidin-D Texas red (Vector Laboratories) fluorochrome was applied to the sections after the secondary antibody was washed off, for 1 hour. The slides were washed in 1 molar PBS for approximately 15 minutes between the different antibodies. The sections were then counterstained with 0.25 μgmL-14',6-Diamidino-2-phenylindole (DAPI; Sigma) for 5 minutes, coverslipped and stored at 4 o C. Slides were subsequently imaged under a mercury lamp using a FITC/DAPI filter. Images were then analyzed using the ImageJ program by setting the threshold for the DAPI stains and biological markers at constant values throughout all of the images analyzed for the different markers.

Appendix C

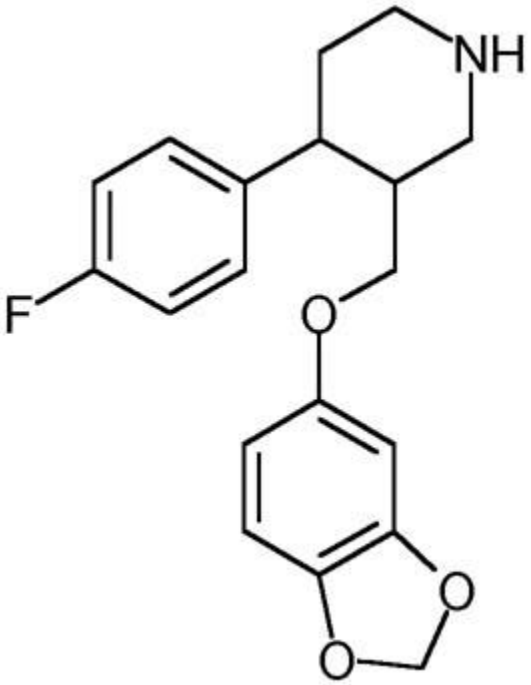


Figure 12. Structure of Paroxetine

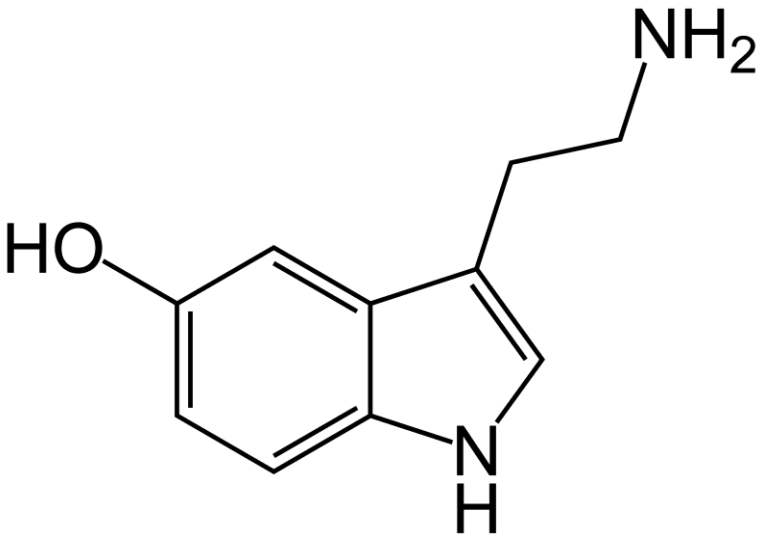


Figure 13. Structure of Serotonin (5-HT)

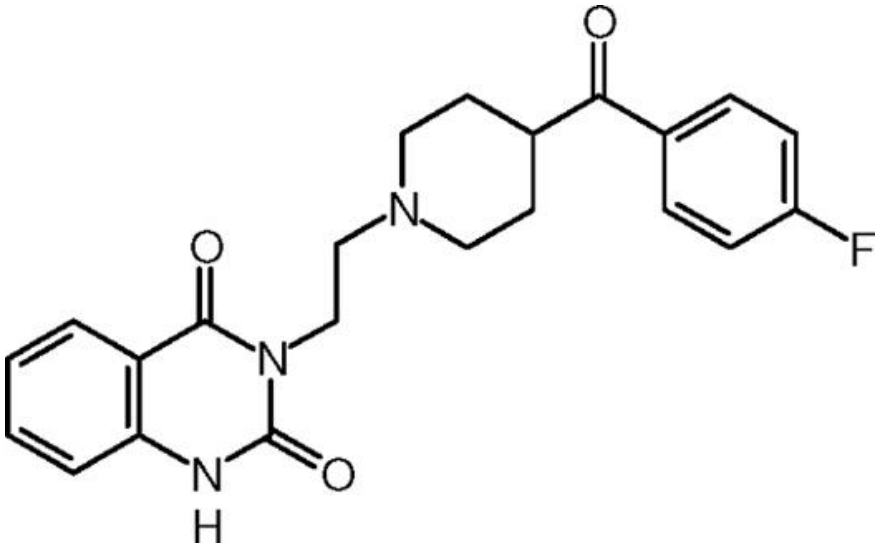


Figure 14. Structure of Ketanserin (5-HT-$_{2A}$ inhibitor)

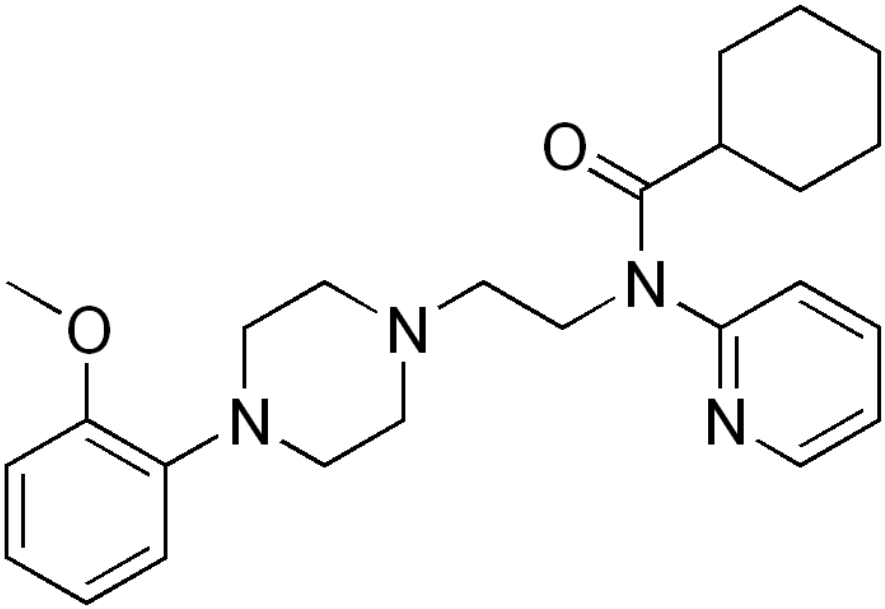


Figure 15. Structure of WAY-100635 (5-HT-$_{1A}$ inhibitor)

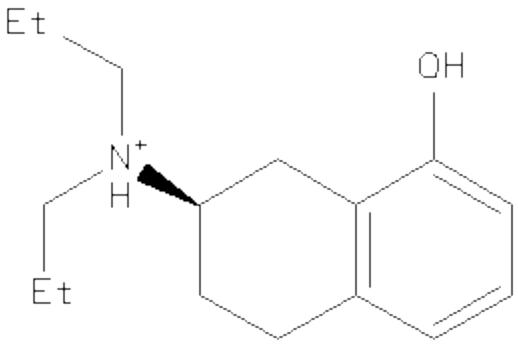


Figure 16. Structure of BRL14472 (5-HT-$_{1D}$ inhibitor)

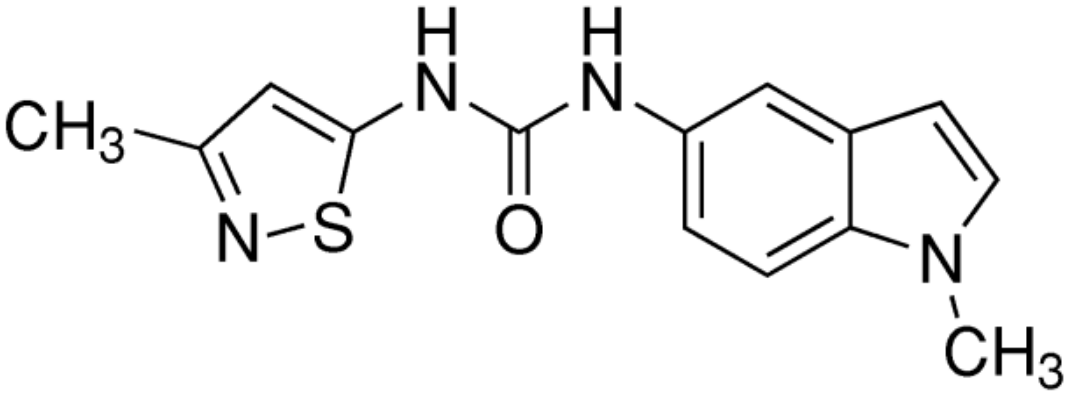


Figure 17. Structure of SB204741 (5-HT-$_{2B}$ inhibitor)

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