Aneurysm Simulation and ELISA Detection in Rabbits

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Abstract

An aneurysm is defined as a “widening of an artery, developing from a weakness or destruction of the medial layer of the blood vessel”. Those who are deemed “at risk” for developing an aneurysm should be screened for Unruptured Intracranial Aneurysms (UIAs). Once a patient is diagnosed, they must determine the risk of rupture of the UIA and what management method is their best option. In order to determine a more precise noninvasive detection method and evaluate the risk of UIAs, alternate non-imaging techniques should be explored. Blood testing provides a more accurate, more convenient, noninvasive detection method. UIAs could be detected by testing the specific antibodies and antigens in the blood. By mimicking hypertension with manipulation of blood pressure as well as vascular wall repair inhibitors in the rabbit samples, it was hypothesized that there will be an increase in antibodies produced which will readily bind to the AFHYESQ peptide. Our results show a positive correlation between increase in blood pressure and increase in titer, which indicate more presence of bound antibodies, showing a more prevalent immune response to the AT1R. An ELISA assay was used for all serum samples, which can then be expanded to other models for continuous sampling.

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1. Introduction

An aneurysm is defined as a "widening of an artery, developing from a weakness or destruction of the medial layer of the blood vessel" (DeBakey 2016). Although it is believed that aneurysms occur in 6% of the population, the number is most likely to be far greater due to the large number of undiagnosed aneurysms. Overall the process of detection and management of unruptured intracranial aneurysms (UIAs) is a complicated one, but a rewarding one if diagnosed early. However, the diagnosis and treatment techniques depend on the detection of the aneurysm, which currently are not accurate for small aneurysms (Jerman 2016).

Those who are deemed "at risk" should be screened for UIAs. Once a patient is diagnosed, they must determine the risk of rupture of the UIA and what management method is their best option. If an aneurysm is detected, the risk of rupture must be weighed against the risk of surgery for the patient before a management plan can be created. If the surgical path is chosen, there are still various options to choose from depending on size, location and the risks the patient is willing to take. Currently, the most common practice for detecting an aneurysm is noninvasive imaging. This includes using Magnetic Resonance Angiography (MRA) and Computed Tomography Angiography (CTA) to visually determine the presence and size of the UIA. Invasive imaging using Digital Subtraction Angiography (DSA) is also used to diagnose aneurysms, but it is less common because of the associated risks. Unfortunately, the most common recommendation is conservative management, or observing the change in size of aneurysms using the aforementioned detection methods.

In order to determine a more precise noninvasive detection method and evaluate the risk of UIAs, alternate non-imaging techniques should be explored. Blood testing provides a more accurate, more convenient, noninvasive detection method. UIAs could be detected by testing specific antibodies and antigens in the blood, that may appear in response to hypertension, a common cause of aneurysms. By mimicking hypertension with manipulation of blood pressure as well as vascular wall repair inhibitors in the rabbit samples, it was hypothesized that there will be an increase in antibodies produced which will readily bind to the AFHYESQ peptide, the epitope of angiotensin type I receptor. An

ELISA assay was used for all serum samples, which can then be expanded to other models for continuous sampling.

2. Review of Literature

An aneurysm is defined as a "widening of an artery, developing from a weakness or destruction of the medial layer of the blood vessel" (DeBakey 2016). An aneurysm can occur in the aorta or any other major artery when the weakened vessel wall becomes enlarged due to increasing pressure. While most patients with unruptured intracranial aneurysms (UIAs) experience no symptoms, those that do will vary in severity and location. Patients with aortic aneurysms will exhibit no symptoms until the UIA is 5-6cm in diameter. Chest aneurysms will put pressure on the windpipe and bronchi, leading to issues with breathing and can cause pain all the way up to the neck and shoulder areas. Aneurysms located in the abdomen can cause pain ranging from the abdomen and back down to the groin and upper thigh region (DeBakey, 2016). Intracranial aneurysms can cause symptoms that include headache, seizure, focal deficit, subarachnoid hemorrhages (SAHs) and cranial nerve palsy from the pressure (Nasr & Brown, 2016). Although the exact causes for aneurysms are still unknown, researchers have discovered potential risk factors as discussed below. Those who are deemed "at risk" should be screened for UIAs. Once a patient is diagnosed, they must determine the risk of rupture of the UIA and what management method is their best option.

2.1. Potential Risk Factors for Unruptured Intracranial Aneurysms

Aneurysms occur in an average of 6% of the population worldwide (Ahmed, 2014). Although the causes of UIAs are still unknown, the results of various studies have determined possible risk factors. One potential risk factor is genetics (Rustemi et al., 2015). For example, the genetic condition Adult Polycystic Kidney Disease (APKD) can increase the chance of developing an aneurysm, as 10-15% of patients with this condition are diagnosed with aneurysms (Wardlaw & White 2000). Other genetic diseases that have been linked to aneurysms are: Type IV Ehlers-Danlos Syndrome, Pseudoxanthoma elasticum, Hereditary Hemorrhagic telangiectasia, Neurofibromatosis type I, α1-

Antitrypsin Deficiency, Microcephalic Osteodysplastic Primordial Dwarfism and coarctation of the aorta and bicuspid aortic valve (Wardlaw & White, 2000; Nasr & Brown, 2016). Researchers have found that females are three times more likely to develop aneurysms compared to males (Nasr & Brown, 2016**)**. Ethnicity has also shown to play a role; those with Finnish and Japanese backgrounds are, respectively, 3.6 and 2.8 times more likely to harbor an aneurysm compared to those of North American or other European descent (Nasr & Brown, 2016**)**. Having multiple family members with UIAs or subarachnoid hemorrhages (SAHs) was also determined to be a risk factor for UIAs (Wardlaw & White, 2000). Researchers have also discovered that smoking, alcohol consumption, cocaine/amphetamine use, oral contraceptives, hypercholesterolemia, increased age and hypertension are risk factors for UIAs (Wardlaw & White, 2000). Contradicting results from various experiments led researchers to not have a decisive answer as to the causes of aneurysms, but only possible risk factors. However, other studies have shown that previous SAHs have no effect on the risk of developing another aneurysm (Sonobe et al., 2010). Sonobe et al. also argue that the risk for UIAs does not increase with age, but rather that UIAs are more common in those under fifty years old.

2.1.1. Link between Humans and Rabbit Model

Rabbits are used in research as a model for human aneurysms by using one of their carotid arteries. Procedures designed for aneurysm treatments, such as coiling, clipping and other interventions, can be optimized in this rabbit model (Kang, 2010). However, the "aneurysms" created within the carotid artery are very stable and usually don't change in size over time, unlike those in humans. To simulate a more realistic human model of aneurysms in the rabbit, some common risk factors need to be introduced in order to make this model more relevant for pre-clinical experiments. One such way is to induce hypertension (Zeng, 2011). A sustained blood pressure increase in rabbits mimics human hypertension, which may result in a higher probability of aneurysm growth. This probability of aneurysm growth makes the rabbit "aneurysm" more realistic.

2.1.2. Hypertension and Aneurysms

Hypertension is a consistent increase in blood pressure that contributes to "the interaction between arterial hemodynamics and vascular wall biomechanics," which is considered an important factor of aneurysm ruptures (Lee, Zhang, Takao, Murayama & Qian, 2013). Studies have shown that hypertension plays a role in the formation of aneurysms, and is twice as likely to occur in people who have at least one aneurysm than those who do not have aneurysms (Lee et al., 2013). Hypertension and hypertensive disorders have been linked to "activating autoantibodies to the Angiotensin Type I Receptor (AT1R)," (Li et al., 2015). An autoantibody is the antibody produced as a body's response to its own tissues (Li et al., 2015). This means that these antibodies could be an indicator for hypertension, which could lead to detection of aneurysms.

2.2. Detection Methods

Individuals who exhibit one or more of the risk factors mentioned previously should undergo screening for aneurysms, as most are found incidentally while imaging for an unrelated event. The early diagnosis and correct classification of aneurysms is valuable to prevent fatal events (Jerman, Pernus, Likar, & Spiclin, 2016; Rand, Uberoi, Cil, & Tsetis, 2013). Various methods of detection for aneurysms exist, ranging from noninvasive procedures such as magnetic resonance angiography (MRA) and computed tomography angiography (CTA) to invasive techniques such as digital subtraction angiography (DSA). Unfortunately, the detection methods currently available are not 100% accurate. This accuracy level could be achieved through a blood test; however, there is not an established protocol for a blood test or marker to diagnose aneurysms, yet (Ahmed, 2014).

2.2.1. Noninvasive Imaging

Noninvasive imaging is used to identify and characterize aneurysms (Hwang, Kwak, Han & Chung, 2011). Two separate studies conducted by Rustemi et al. and Wardlaw and White determined the sensitivity of MRA and CTA to vary between 76%-98% and the specificity to vary between 85%-100%. However, Rustemi believes that the

accuracy increases with the size of the aneurysm and when the patient displays symptoms. One study showed comparable results of MRA run at 3 Tesla with DSA. However, the accuracy for aneurysms smaller than 3mm was still only 88.1 – 93.2 % (Li et al., 2009). Yet, it is believed that the accuracy of noninvasive imaging will increase along with the size of the aneurysm (Rustemi et al., 175). Other uncommon noninvasive imaging techniques include: Multislice Computed Tomography (MSCT), Color Duplex Ultrasound (CDU), Contrast-Enhanced Ultrasound (CEUS), 3D X-Ray Rotational Angiography (3D-RA) and Transcranial Doppler (TCD). MSCT has high sensitivity and specificity, but it is still not as specific as DSA (Rand, 2013). Each of the others comes with its own unique advantages and disadvantages, but overall, data collected by using noninvasive imaging techniques are "variable and sometimes even contradictory as a result of varied quality of noninvasive imaging" (Rustemi et al., 175). As a result of the varied sensitivity and specificity of noninvasive imaging techniques, many diagnosticians rely on DSA and use MRA/CTA more frequently for follow up imaging.

2.2.2. Invasive Digital Subtraction Angiography (DSA)

Although digital subtraction angiography is invasive, it produces the most reliable results when diagnosing a patient with an aneurysm. The procedure includes injecting contrast dye into the individual's blood vessels while taking time-controlled x-rays. DSA plays a large role when diagnosing small aneurysms-those less than 5mm-due to the large inaccuracy when detecting UIAs this size using MRA/CTA (see figures 1, 2 and 3) (Rustemi et al., 2015).

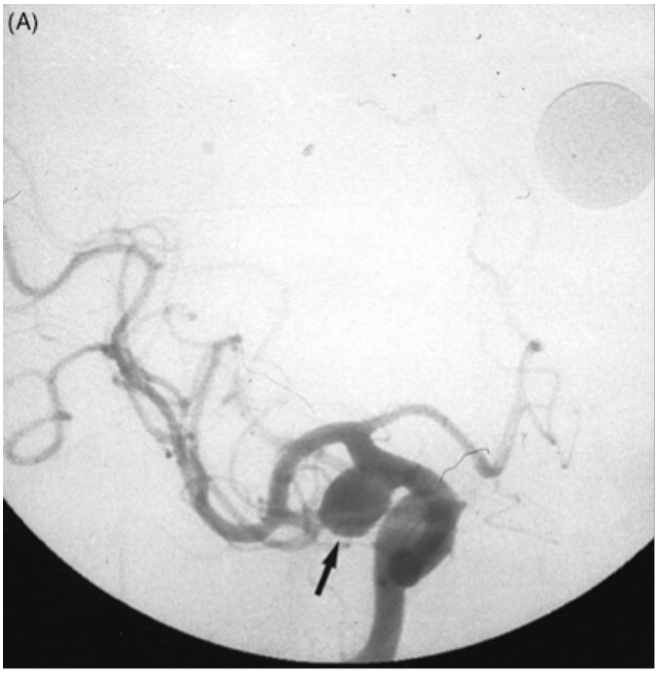


Figure 1: Imaging of an aneurysm using Digital Subtraction Angiography

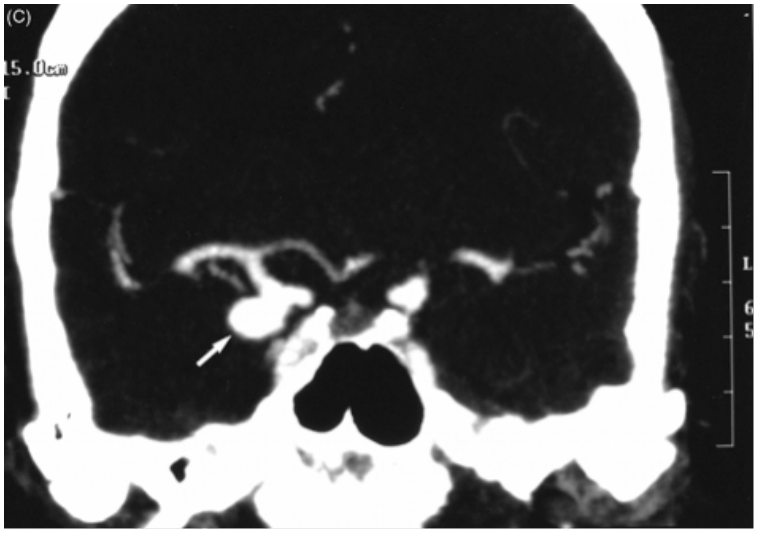


Figure 2: Imaging of an aneurysm using Computed Tomographic Angiography

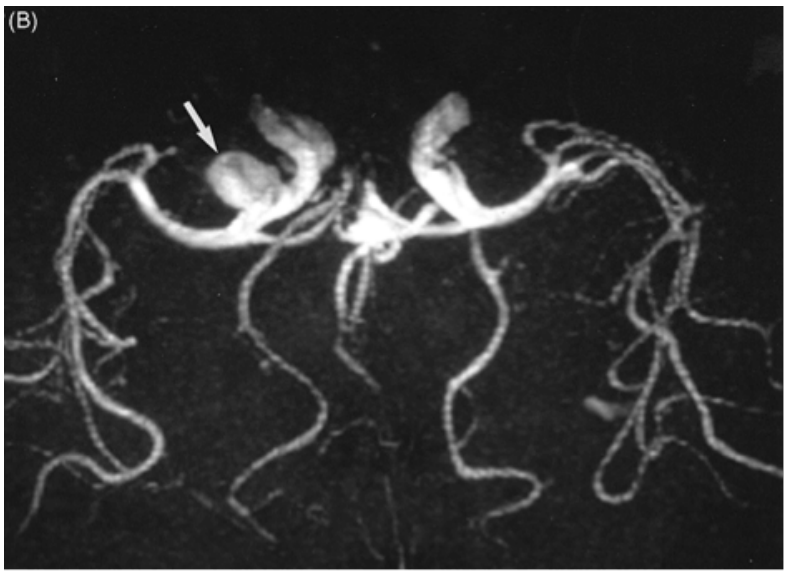


Figure 3: Imaging of an aneurysm using Magnetic Resonance Angiography

Bone subtraction significantly improves the rate of detection of an aneurysm as it improves the visualization of intracranial arteries, at or near the skull base (Sarikaya & Sarikaya, 2011). Digital subtraction angiography detects more multiple UIAs and false positives compared to noninvasive imaging. The detection of false positives is extremely important because the diagnosis of an aneurysm comes with both negative economic and psychological effects for the patient and their family. However, once an aneurysm has been detected using DSA, noninvasive imaging can be used for further follow-ups.

2.3 Treatments

Once an aneurysm is detected, the physicians involved: neurosurgeons; endovascular surgeons; and the family physician, must determine the best course of treatment for their patient based on the risk of rupture of the UIA. Aneurysms that increase in size and those already greater than 7mm have an increased risk of rupture (Sengupta, 2015). The definition of aneurysm growth is different depending on the type of aneurysm: small or large. Small aneurysms (smaller than 5mm) that increase in size by more than 1mm are considered to have grown in size. Large aneurysms (larger than 5mm) that increase in size by more than 2mm are considered to have grown. This growth leads to considering surgical methods because the rupture rate of growing UIA increases to 3% per year compared to stable UIA with a rupture rate of 0.1% yearly (Nasr &

Brown, 2016**).** Large aneurysms, those larger than 7mm should be treated, while those less than 5mm should only be monitored with frequent follow-up imaging (Jerman et al., 2016). The rupture risk will also increase if a patient has had a previous SAH from another UIA (Sengupta, 2015). A study showed that the rupture rate per year of a small aneurysm in patients with a previous SAH is 0.5%, compared to 0.05% in patients without a previous SAH (Wardlaw & White, 2000). Locations of the aneurysm within the brain, such as in the posterior circulation artery and anterior and posterior communicating arteries, are also believed to increase the rupture rate (Nasr & Brown, 2016). If the risk of rupture is higher than the surgical risk, surgery, rather than observation or treatment is recommended. Choosing the correct treatment path is crucial because if the aneurysm were to rupture, the bleed or its complications can be fatal.

2.3.1. Conservative Management

There are various treatments and management methods available to patients with UIAs. Observation is a logical choice for patients whose aneurysms are smaller than 7mm (Sengupta, 2015). Elderly patients or those with other illnesses that may increase the risk of surgery are also advised that observation may be their best management method (Sengupta, 2015). Patients who do not want to take this risk of surgery may also choose this method. Observation includes frequent imaging of the aneurysm, at least annually for a minimum of three to five years (Nasr & Brown, 2016). The conservative management approach may also include modifying risk factors by making changes to the patient's lifestyle by controlling the blood pressure and/or ceasing smoking if these factors apply in the situation (Nasr & Brown, 2016). Medical management of the aneurysm can also be an option, such as antiplatelet or anticoagulation medicines, aspirin, antihypertensive drugs, antithrombotics and statin therapy (Nasr & Brown, 2016). Angiotensin converting enzyme (ACE) inhibitors and angiotensin receptor blockers have also shown to be effective in decreasing the rupture rate in animals by decreasing the elastin degradation (Nasr & Brown, 2016). But, this effect has not been validated in human studies as of yet. However, once the risk of rupture outweighs the risk of surgery, observation is no longer a logical option.

2.3.2. Endovascular Surgery

Endovascular surgery (EV), using coils and stents to relieve pressure on the weakened walls of the artery, is less invasive but more costly (DeBakey, 2016). Most UIAs can be coiled, but wide neck UIAs need stent assistance and require additional pre/post treatments (Nasr & Brown, 2016). Coils are reliable and have instantaneous detachments (see figure 4), but cannot grip the vessel wall in straight arteries and may sometimes still allow blood flow in these and in wide-necked aneurysms (Henkes & Weber, 2015).

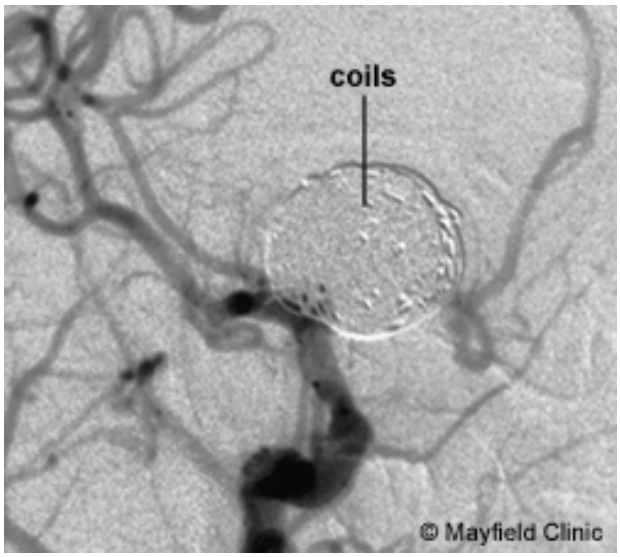


Figure 4: An angiogram showing coils filling an aneurysm

As a result, scientists created stents to assist coiling procedures. Stents help hold the coils in place, while still allowing blood flow through the artery, but not through the aneurysm (see figure 5) (Ringer, 2016).

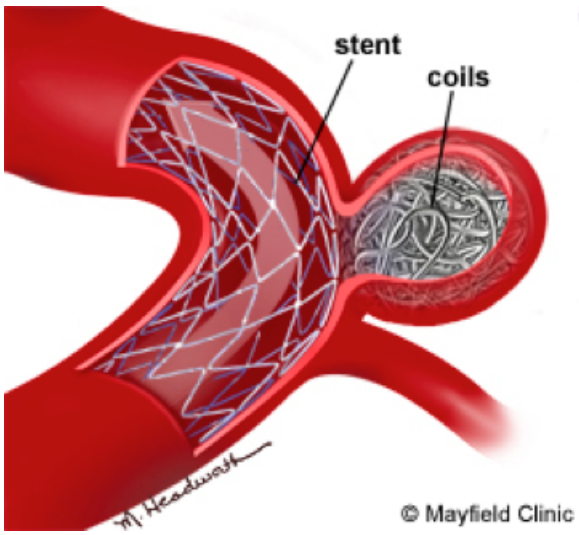


Figure 5: An image of a stent-assisted coil

However, stents also come with their own limitations. EV has been shown to produce worse outcomes compared to neurosurgery including a 2% mortality rate and only 82% obliteration rate (Sengupta, 2015). Obliteration rate refers to the percent of aneurysms that are completely eliminated and do not return. The most common complication of EV surgery (10%-50%) is the occurrence of an endoleak (EL), or blood flow within the aneurysm sac that lies outside of the endograft/stent (Rand, 2013). Endovascular surgery has also resulted in more rebleeds post surgery compared to clipping (Molyneux, A. & International Subarachnoid Aneurysm Trial (ISAT) Collaborative Group, 2002). While some aneurysms may be suited for EV surgery, others may respond better to neurosurgery.

2.3.3. Neurosurgery

Neurosurgery and the "clipping" process, while more invasive than EV options, is less expensive (Sengupta, 2015). Clipping is most useful for middle cerebral artery (MCA) bifurcation aneurysms and very small or oddly shaped IA where EV surgery would be difficult (Nasr & Brown, 2016). The procedure includes a craniotomy, and placing a clip at the neck of the aneurysm to prevent blood flow into it (see figure 6) (Zuccarello & Ringer, 2016).

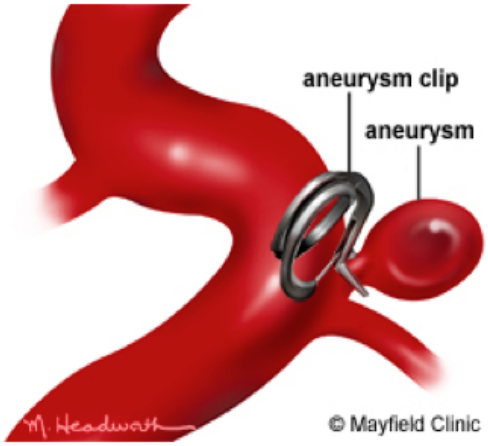


Figure 6: An image of a clipping procedure

Unfortunately, risks come with every surgery. Clipping has shown to have a 1% mortality rate and a 95% obliteration rate (Sengupta, 2015). During aneurysm clipping, the patient is at risk for stroke, seizure, bleeding and an imperfectly placed clip (Zuccarello & Ringer, 2016). If an aneurysm has only been partially clipped, patients need follow up tests to determine if the aneurysm is growing or not. Due to the various risks and limitations there is no correct detection method or solution for aneurysms, as treatment plans will vary depending on the location, size and patient's history.

2.4 Assays to Determine Treatments

Due to the lack of 100% effective detection, diagnosis, and treatment protocol, scientists are researching and developing more accurate methods for aneurysm detection. Current methods are either invasive with very high complication risks, or non-invasive but inaccurate and unable to detect most small aneurysms (Rustemi et al., 2015). The goal is to determine a detection method that is noninvasive with high accuracy because as previously discussed; the most important factor of aneurysm treatment is early detection. Currently, researchers are using chemical assays to test as possible non-invasive molecular imaging detection techniques. Common assays include cell based AT1R activation assay, contractility assay, and enzyme-linked immunosorbent assay (ELISA) (Li et al., 2015). These assays use blood chemistry analysis and detection of antibodies to detect hypertension.

2.4.1. ELISA

ELISA assays detect antibodies (see Figure 7) (Li et al., 2015). It is important for diagnosis of infectious diseases, but can also be used to determine changes in blood chemistry and antibody levels in the blood that are indicative of hypertension, which can be indicative of aneurysms (Nahar, Bora, Sharuma, & Kannoujia, 2012).

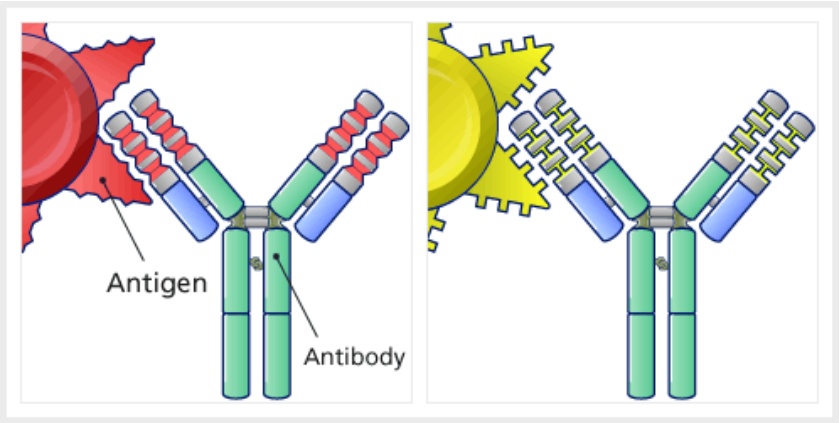


Figure 7: An image of an antibody binding to epitope on antigen

The experimental procedure for ELISA involves coating microtiter plates with peptide followed by incubation, blocking any unbound surface, binding with an antibody, and absorbance reading (Nahar et al., 2012). The procedure itself determines whether the peptide used, in this case AFHYESQ (see Figure 8), can bind to the specific antibody in the blood, immunoglobulin (IgG) (see Figure 9).

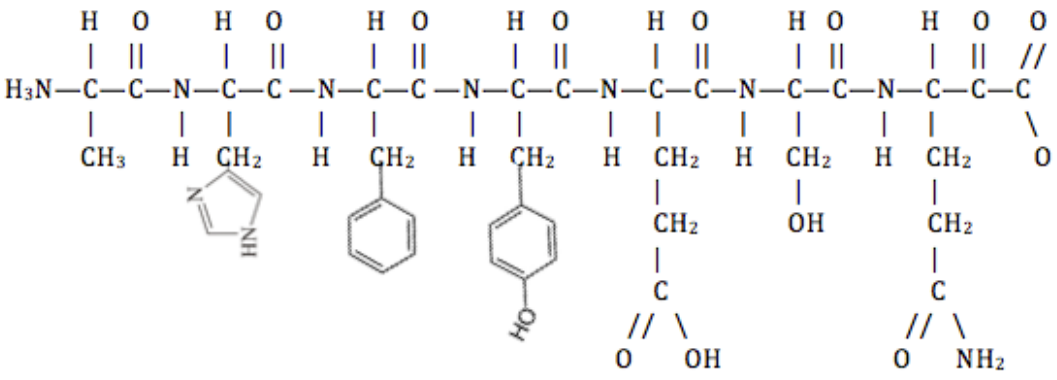


Figure 8: Molecular structure of the peptide AFHYESQ

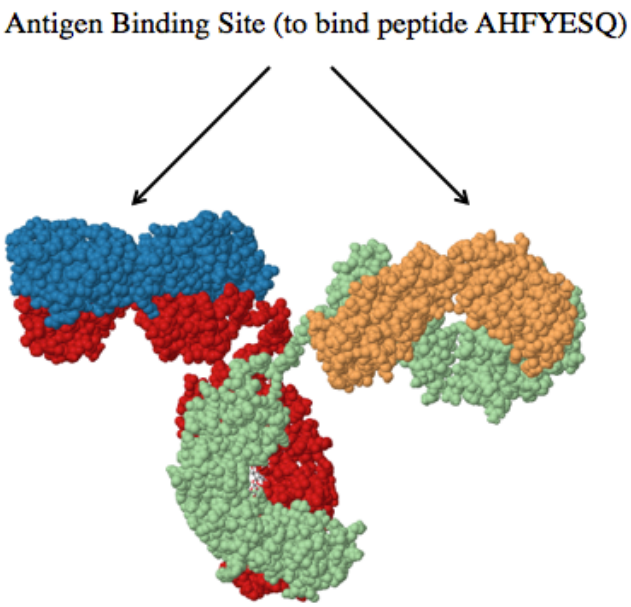


Figure 9: An image of the tertiary IgG structure

The amount of antibodies bound to the antigen is determined by using 1-Step ABTS solution and reading the absorbance at 405 nm, as this is the solution's maximum wavelength (see Figure 10).

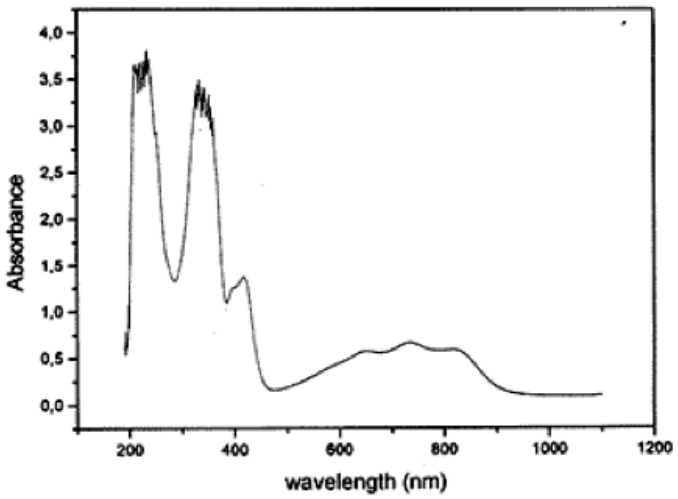


Figure 10: Absorbance spectrum of 1-Step ABTS solution, with a maximum wavelength in the visible spectrum at 405 nm

Although this is a very accurate procedure, ELISA tends to take a long time (approximately 18 hours). Further research has been conducted to determine whether ELISA can be performed more efficiently when under pressure (PELISA), heat (HELISA), or microwave mediated (MELISA). All three of these have been shown to have comparable results as ELISA but reduce the time of the assay to 10 minutes, 3 hours, and <5 minutes, respectively (Nahar et al., 2012). This eliminates the need for overnight incubation (Kumar & Nahar, 2009). Although these types of ELISA differ in experimental time, all of these types of ELISA assays are credible antibody titers. Titers test the amount of antibodies in blood samples. Using this information, we will be able to carry out our own ELISA to determine the levels of antibodies in hypertensive rabbit samples.

3. Methodology

This project was conducted from August 31, 2016 until March 2017 on the campus of UMass Medical School (UMass) in the Radiology Department under the supervision of Dr. Alexei Bogdanov and his staff. This project involves running ELISA on blood samples from rabbits with induced high blood pressure. Angiotensin should be present in these samples, which promotes aldosterone, a hormone, and raises the blood pressure (Li et. al, 2015). The angiotensin type one receptor (AT1R) is a receptor for autoantibodies, specifically those that would be produced if the body were hypertensive (Li et. al, 2015). The ELISA plates were coated with a peptide containing the AT1R epitope, which will determine whether the AT1R antibodies that are produced in these immunized rabbits actually activate AT1R. The plates were then blocked, and washed. Dilutions of the rabbit blood samples were plated and observed spectroscopically in order to titer and to see the levels of antibodies. It was tested and checked that the antibodies from the blood samples actually bound specifically to AT1R. As the group tested for the immune response regarding antibody levels, the veterinarians at UMass tested for the physical response of increased blood pressure.

3.1 ELISA

This project focused on six rabbits (541, 544, 705, 706, 707 and 708) with induced high blood pressure as a result of being immunized with AT1R. Samples from Rabbits #541, 544 and 705 were used as practice for learning how to run the experiment. The project then focused specifically on Rabbits #706, 707 and 708 for analysis and discussion purposes. Blood samples were taken by Dr. Bogdanov's staff pre-immunization (0 weeks), and post-immunization at 2 weeks, 4 weeks, 6 weeks and 8 weeks. Each blood sample was divided into four aliquots of about equal volume ranging from 100 μL to 200 μL.

Stock solutions of HEPES buffer saline (HBS) and sodium bicarbonate were created for use throughout the project. HBS (10x 20mM) was created using 26.0 g of HEPES and 43.4 g NaCl in 500 mL of dH_2O. The pH was adjusted to 7.4 using HCl. Sodium bicarbonate (10 mM) was created using 0.420 g in 500 mL of dH2O.

The next step was to create the peptide solution to be used to coat the bottoms of the well plates for each ELISA. Each well required 10 μg of peptide per mL, therefore a 0.1 mg/mL stock solution was made. To coat the plates, the solution was diluted by a factor of 10, using 2.5 mL of the peptide solution and 22.5 mL of sodium bicarbonate. The solution (100 μL) was placed into each well and the plate was incubated for 1 hour at 37 ^{o}C, and then 4^{o}C until it was ready to be used.

Before blocking the plate, the peptide solution was flicked out. The plate was then blocked using a solution containing HBS and albumin. Albumin (14.2 g) was added to 13.9 mL of 10X HBS and was diluted to 150 mL with dH2O. This solution (300 uL) was added to each well and allowed to sit at room temperature for two hours. The plate was then placed in the freezer at -5 ^{o}C, until the next step was taken.

The next step was to wash the plate. First, the blocking solution was flicked out in a similar fashion to the peptide solution. Next, a 0.05% solution of tween 20 / PBS was created using 500 mL of PBS and 0.25 mL tween 20. Each well was washed three times with 300 μL of the solution and then stored in a freezer at -80 ^{o}C until ready to be used again.

The plate was taken out of the freezer and allowed to defrost. A solution (50 mL) of HBS containing 1% horse serum was created and placed in a tube. Next, 180 μL of

buffer solution was placed into each well A-G, 1-12 of a serial dilution plate (see Table 1).

	Serum Sample #1			Serum Sample #2			Serum Sample #3			Serum Sample #4		
	1	2	3	4	5	6	7	8	9	10	11	12
A	1:10											
B	1:100											
C	1:1000											
D	$1:10^4$											
E	$1:10^5$											
F	$1:10^6$											
G	$1:10^7$											
H	Blank											

Table 1: Guide for 1:10 serial dilutions for ELISA protocol

Then, 20 µL of rabbit serum from Rabbit #541 pre-immune was placed into wells A1-A3. Serial dilutions were created down the column of the plate, using 20 µL each time from the previous well. When transferring the solution to the following well, the new solution was mixed 10 times using a pipettor. This process was repeated for Rabbit #541 post-immune, Rabbit #544 pre-immune and Rabbit #544 post-immune in columns A4-A6, A7-A9 and A10-A12 respectively. After each serial dilution was made, 100 µL from each well was transferred onto the peptide coated plate in the corresponding wells. The solutions were transferred from the most dilute to the least dilute. Row H1-12, was used as a blank and contained only 100 µL of HBS. This plate was then allowed to incubate at 37 ^{o}C for 2 hours, and stored at 4 ^{o}C until ready to be used again.

The plate was then washed using 0.05% tween 20/HBS solution. Tween 20 (0.269 mg) was weighed and added to 500 mL of 1X 20 mM HBS. The solution (300 µL) was added to each well and flicked out. This process was repeated four times.

Immediately following the last washing, 100 µL of the secondary antibody solution, rabbit IgG, was added to each well. This solution was created using 1% bovine serum albumin (BSA) with HBS. BSA (0.128 mg) was added to about 12.8 mL of HBS and vortexed. The plate was then allowed to incubate at room temperature for 1 hour. Next, the plate was washed 3 times with the 0.05% Tween 20/HBS solution and incubated at 4 ^{o}C until further use.

1 Step ™ ABTS substrate for use with horseradish peroxidase (150 µl) was added to each well and incubated at room temperature on a rotating plate for 1 hour and 30 minutes. The plate was then analyzed using a SpectraMax M5 at 405 nm.

3.2 Changes to Original Protocol

Because the original plates created were not specifically designed for ELISA, new plates, specifically for ELISA, were used to continue the rest of the experiments after the first trial of rabbits 541 and 544 pre- and 2 week post-immunization. To create the peptide solution, a 0.1 mg/mL solution was created using 4 mg of peptide, 800 µL of water and 39.2 mL of Sodium Bicarbonate. A new 10% Tween 20 Stock was created using 2.3 mL of Tween 20 and 20.7 mL of H_2O. A 0.05% solution was made to wash the plates using 2.5 mL of the stock in 500 mL 1X HBS. An extra washing step was added in between flicking the peptide solution out of the plates and adding the blocking solution. Changes were also made to the procedure regarding plating of the secondary antibody solution. Immediately following the last washing, 100 µL of the secondary antibody solution, Rabbit IgG, was added to each well. This solution was created using 5.2 µL secondary antibody solution (Rabbit IgG) 1% Bovine Serum Albumin (BSA) with HBS. BSA (0.128 g) was added to about 12.8 mL of HBS and vortexed. The plate was then allowed to incubate at room temperature for 1 hour.

Due to the high absorbance values gathered on plates containing rabbit serum from #541, #544, #705 and #706, higher dilutions of serum were used for trial 1 of rabbits #707 and #708 and all other following trials. The first dilution began at $5x10^{-2}$ and was diluted 10-fold until $5x10^{-8}$ (see Table 2).

	Serum Sample #1			Serum Sample #2			Serum Sample #3			Serum Sample #4		
	1	2	3	4	5	6	7	8	9	10	11	12
A	1:20											
B	1:200											
C	1:2000											
D	$1:2x10^4$											
E	$1:2\ x10^5$											
F	$1:2\ x10^6$											
G	$1:2\ x10^7$											
H	Blank											

Table 2: Guide for 1:20 serial dilutions for ELISA protocol

3.3 Future Direction

Samples for Rabbit #706, #707 and #708 were chosen to be further analyzed. These rabbits were chosen due to vitality of the rabbits, as well as availability and consistency of samples from pre-immunization (0 week) and 2, 4, 6, 8 weeks post-immunization. The samples for 4 weeks post-, 6 weeks post- and 8 weeks post-immunization were assayed in a similar fashion and then analyzed. All samples were analyzed in the SpectraMax M5 to determine the absorbance values after a 30 minute color development period. Using these absorbance values, we were able to create trend lines. A nonlinear regression with a three parameter approximation was run to find titers. The three parameters were: the top, bottom and EC50 (mid-titration point).

3.4 Human Aortic Cells

Human aortic cells were obtained from and provided to us from UMass Medical School. An ELISA assay was performed on these cells, similar to that of the rabbit blood serum samples, to determine if the antibodies present in the rabbit serum solutions bound specifically to the antigens on human aortic cells. The first step was to create a 1X HBS solution using 270 mL of H2O and 30 mL of 10X HBS. The slide containing the human aortic cells was washed for 5 minutes in a container with 40 mL of 1X HBS. Next, the slide was fixed with 2% formaldehyde by adding about 80 μL to each of the eight sections on the slide. The slide was then washed in 1% BSA for five minutes. This was repeated three times. After, the slide was placed in a container with 40 mL of Albumin BSA blocking solution for 30 minutes.

The addition of the first antibody, serum from Rabbits #707 and #708, had to be diluted 1:200 in blocking solution. Rabbit serum (1.5 μL) and blocking solution (298.5 μL) were combined in a microfuge tube. Serum (about 80 μL) was placed onto each well and incubated in a humidity chamber for one hour. A diagram of the wells can be seen in Figure 11.

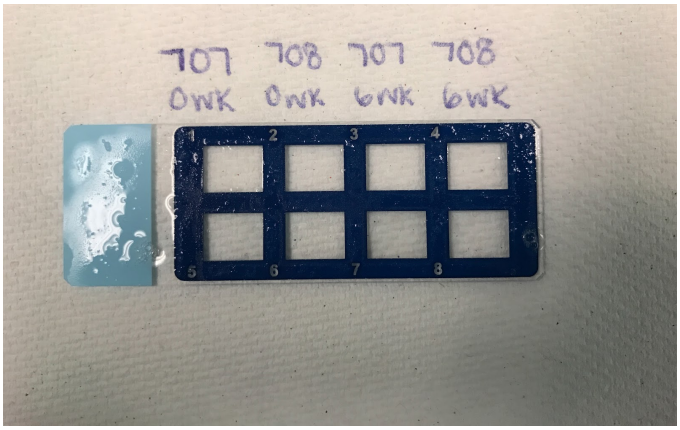


Figure 11: A picture of a slide containing human aortic cells, divided into eight wells, with each well labeled with

Rabbit #707 and #708 pre-immune serum was used as the control, while 6 week post-immune was used as the experimental group.

The slide was then washed again three times in a container, for five minutes each, using 40 mL 1% BSA solution. The rest of the ELISA procedure including adding the

secondary antibody, washing and microscopy was performed by the staff at UMass due to time constraints of the project.

4. Results

This project included multiple ELISAs that were run using various blood samples collected from six rabbits at varying time points pre- and post-immunization with angiotensin. The hypothesis was that if the rabbit was injected with AT1R, it would have both an immune response, indicated by antibodies bound to the ELISA plate, as well as a blood pressure response, indicated by hypertension. The experiment was slightly altered over the course of the project to ensure the highest quality results were obtained. The results of the first plate tested can be seen in Figure 12.

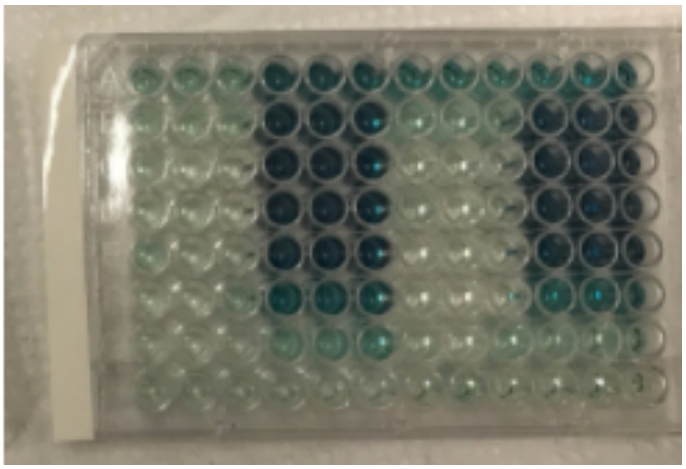


Figure 12: Picture of ELISA plate containing rabbit serum from Rabbit #541 (columns 1-6) and #544 (columns 7-12) 30 minutes post

The darker colored solution represents a higher number of antigens (AFHYESQ) bound to the IgG antibody. These serial dilutions began with a 1:10 serum dilution, and 1:10 dilutions carried out down each column. Pictures of all other ELISA plates can be seen in Appendix A. Due to the dark colors at the lower dilutions, the absorbance levels were very high. To correct this issue, and bring the absorbance values back within linear proportionality to antibody concentration, a 1:20 dilution was made to begin the serial dilutions, but 1:10 dilutions were still carried out down each column (see Figure 13).

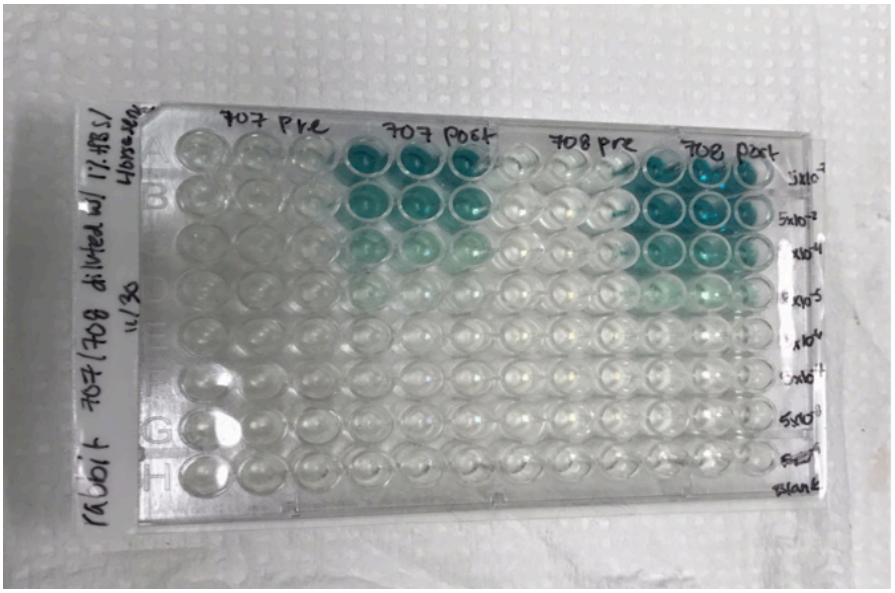


Figure 13: Picture of ELISA plates containing rabbit serum from weeks 0 and 2, from Rabbit #707 (columns 1-6) and #708 (columns 7-12) 30 minutes post

The lighter solution colors allowed for more appropriate absorbance values to be collected. The columns containing pre-immunization (columns 1-3 and 7-9) rabbit serum do not change color from the original clear solution due to the lack of antibodies. The columns containing 2 week post-immunization (columns 4-6 and 10-12) rabbit serum change from a clear solution to a blue-green indicating the presence of antibodies in response to the injection of AT1R.

Rabbit #706

To determine the number of antibodies made in response to these injections, titers were calculated based on the graph of log(dilution) vs. absorbance for Rabbit #706, 707, and 708 (Figures 14, 16, and 18, respectively).

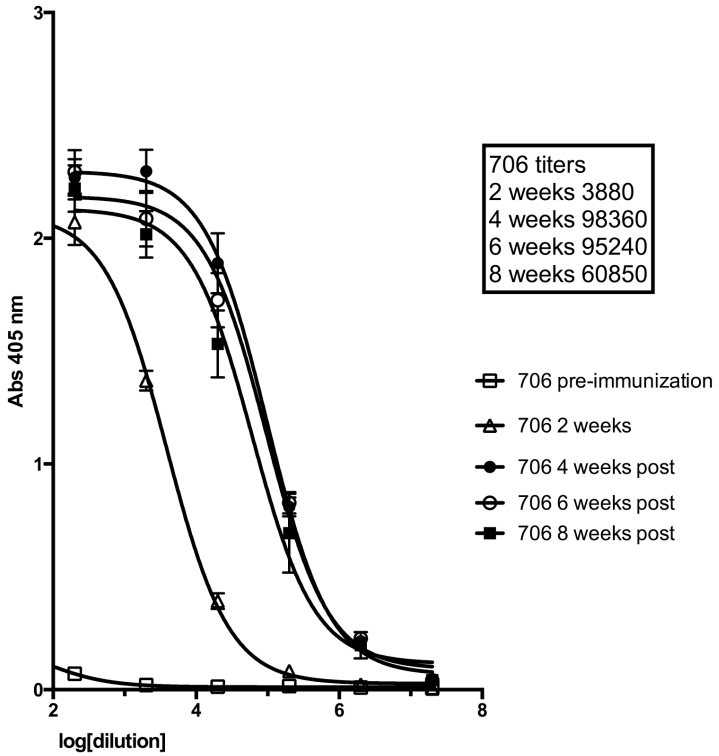


Figure 14: Graph of log(dilution) vs. absorbance values for Rabbit #706

The titers for rabbit #706 increase drastically as the post-immunization time increases from 2-4 weeks. From 4-6 weeks the titers remain fairly stable, and then decrease again 8 weeks post-immunization. The higher titers indicate more presence of bound antibodies, showing a more significant immune response to the AT1R. Based on this graph, we can see that between 4 and 6 weeks is when the rabbit displayed the highest immune response. Typically, we would expect for the titer to continue to increase for a longer period of time. While the titers were being tested by the group members, veterinarians were simultaneously testing the blood pressures of each of the three rabbits. The results for Rabbit #706 can be seen in Figure 15 below.

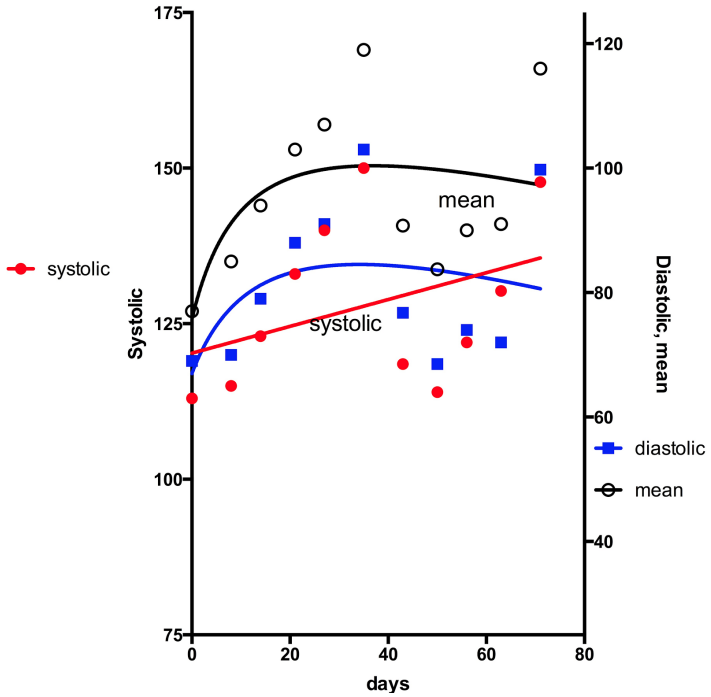


Figure 15: Blood pressure readings of Rabbit #706 over 80 days

This figure depicts the change in blood pressure for rabbit #706 from pre-immunization (0 weeks) to 8 weeks post-immunization. The diastolic pressure, which is the arterial relaxation blood pressure, is shown in blue. The systolic pressure, or blood pressure while the heart is in full contraction, is shown in red. These curves, as well as the mean curve, increase with time, thus, blood pressure increases with the presence of antibodies (immune system response). As blood pressure increases, hypertension intensifies, and chances of inducing an aneurysm, similar to ones seen in humans, increase. The titer results for Rabbit #707 can be seen in Figure 16.

Rabbit #707

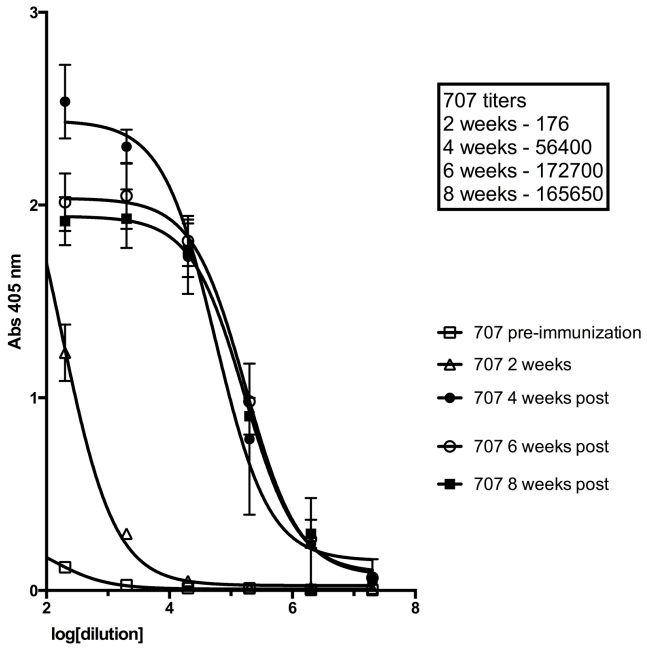


Figure 16: Graph of log(dilution) vs. absorbance values for Rabbit #707

The titers for Rabbit #707 also increase drastically as the post-immunization time increases from 2-4 weeks and 4-6 weeks. From 6-8 weeks the titers remain fairly stable. Based on Figure 15, it can be seen that the highest titers were seen at 6 and 8 weeks post-immunization. Typically, this is what we would expect for the immune response. The blood pressure response was analyzed next, and can be seen in Figure 17.

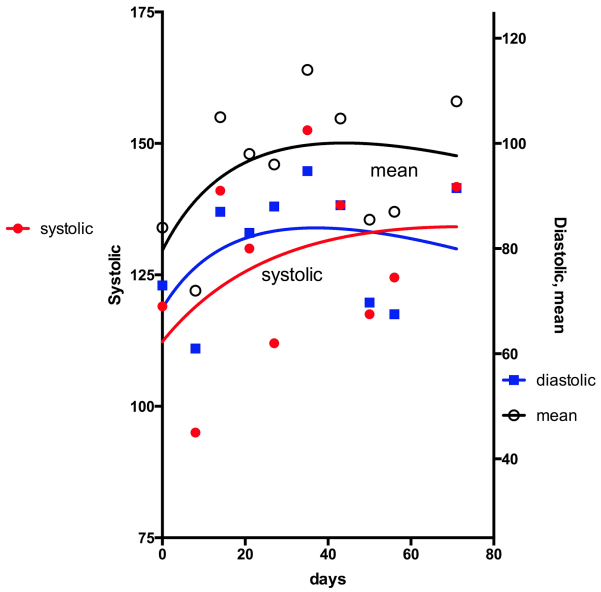


Figure 17: Blood pressure readings of Rabbit #707 over 80 days

Figure 17 depicts the change in blood pressure for rabbit #707 from pre-immunization (0 weeks) to 8 weeks post-immunization. Similar to that of Rabbit #706, the systolic, diastolic and mean blood pressure curves all increase with time.

Rabbit #708

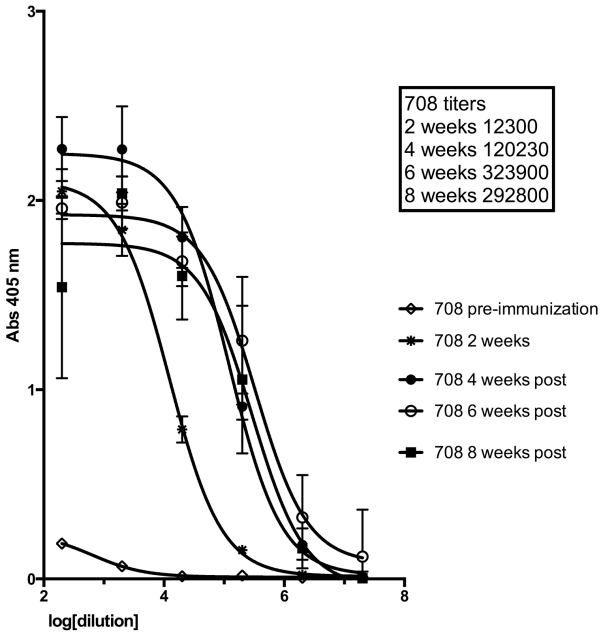


Figure 18: Graph of log(dilution) vs. absorbance values for Rabbit #708

The titers for Rabbit #708 follow the same trend as Rabbit #707. The values increase from 2-6 weeks and then remain fairly stable. The highest titers were seen at 6 and 8 weeks post-immunization. This was consistent with what was expected for the immune response. The blood pressure response can be seen in Figure 19.

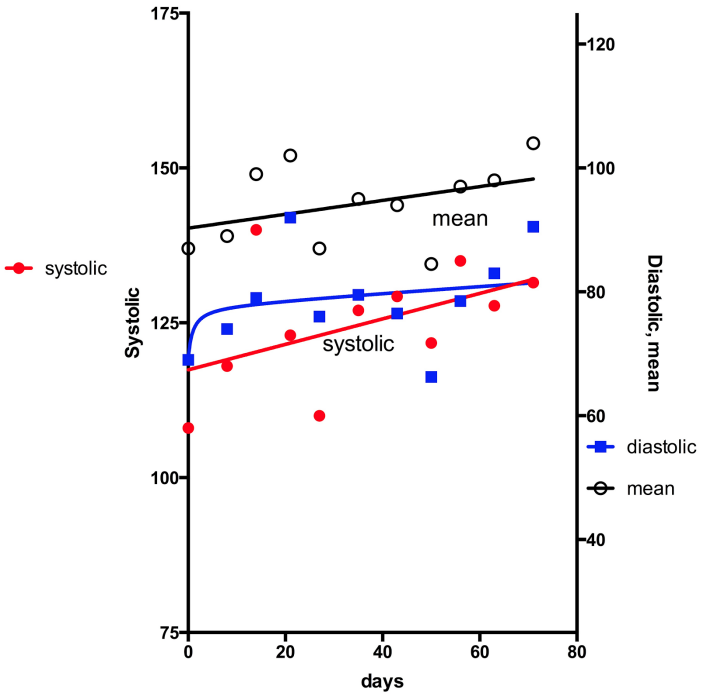


Figure 19: Blood pressure readings of Rabbit #708 over 80 days

Figure 19, representing the change in blood pressure for rabbit #708 from pre-immunization (0 weeks) to 8 weeks post-immunization, follows the same trend as that of Rabbit #706 and 707. As time increases, overall blood pressure (including diastolic and systolic) increases. After all tests and analysis was run, the titer results for all three rabbits were compiled into a table and compared (see Table 3).

Immunization	Rabbit 706	Rabbit 707	Rabbit 708
2 weeks	3880	176	12300
4 weeks	98361	56246	120234
6 weeks	95244	172747	323910
8 weeks	60848	165647	291793

Table 3: Titer Values of Rabbit #706, 707 and 708 at 2, 4, 6, and 8 weeks post-immunization

Table 3 compares the titers of each rabbit for each week post-immunization. Rabbit #706, overall, showed the lowest titers, meaning it did not have as great of an immune response towards the AT1R injections. Rabbit #708 had the highest titers, with its highest occurring at 6 weeks post-immunization. This indicates that Rabbit #708 had the strongest immune response towards the injections. As the titer increased for all three rabbits, so did the blood pressure. This shows that as hypertension was induced, blood pressure did indeed increase. Continuing this timeline, the ELISA assays showed an increase in the amount of antibodies present, indicating a higher immune response to the AT1R. These antibodies were particularly high affinity antibodies because those that did not bind as well would have been washed away due to the repetition of washing during the assay.

Although blood pressure did increase, the blood pressure for all rabbits began at a high blood pressure initially. This could be due to low accuracy of the blood pressure measurements in the rabbits, as it was difficult to obtain a baseline blood pressure. The positive trend for both immune system response and blood pressure response was observed post-immunization.

5. Conclusion

As seen in Figures 14, 16, and 18, the titers in each rabbit sample increased over time. The titers stabilized around the 6 and 8 week time points (Table 3). The higher titers indicate more presence of bound antibodies, showing a more prevalent immune response to the AT1R. Our results show a positive correlation between increase in blood pressure and increase in titer (Figures 15, 17, and 19). As blood pressure increases, hypertension intensifies, and chances of inducing an aneurysm, similar to ones seen in humans, increases.

Replication of this experiment could be done with a few changes. Firstly, experiments could begin with rabbits with lower baseline blood pressures to be able to observe a larger overall change in blood pressure over time. Future experiments could include testing on human aortic cells to check whether the antibodies, produced as a result of the AT1R immunizations tested in the rabbits, bind to the real AT1R receptor on these human cells. If the antibodies in the rabbit serum bind to the cells, this serves as a check on the experiment, verifying that the antibodies in the original ELISA experiment were in fact the antibodies produced as a result of the immunizations and not other naturally occurring antibodies with a similar conformation. The antibodies within the rabbit sample, if the correct ones, should be able to bind to the antigen receptors on the human aortic cells because the AT1R receptor amino acid sequence is conserved between the two mammalian species.

References

Ahmed, S. S. (2014). Systems biology in unruptured intracranial aneurysm: a metabolomics study in serum for the detection of biomarkers. *Metabolomics*,*10*(1), 52-62.

DeBakey, M. E. (2016). *aneurysm* Encyclopædia Britannica Inc.

Harris, L.J., Larson, S.B., Hasel K.W., McPherson, A. (1997) Structure of Immunoglobulin *Biochemistry* 36: 1581-1597

Henkes, H., & Weber, W. (2015). The Past, Present and Future of Endovascular Aneurysm Treatment. *Clinical neuroradiology*, *25*(2), 317-324.

Henriquez, C., & Lissi, E. (2002, July 2). Evaluation of the Extinction Coefficient of the ABTS Derived Radical Cation. Retrieved February 21, 2017 from http://www.scielo.cl/scielo.php?script=sci_arttext&pid=S0366-16442002000400029

Hwang, S. B., Kwak, H. S., Han, Y. M., & Chung, G. H. (2011). Detection of intracranial aneurysms using three-dimensional multidetector-row CT angiography: is bone subtraction necessary?. *European journal of radiology*,*79*(2), e18-e23.

Jerman, T., Pernuš, F., Likar, B., & Špiclin, Ž. (2016). Blob Enhancement and Visualization for Improved Intracranial Aneurysm Detection. *IEEE Transactions on Visualization and Computer Graphics*, *22*(6), 1705-1717.

Kirin, K. Therapeutic Antibodies: Antibodies bind to a specific target. Web.

Kumar Kannoujia, D., & Nahar, P. (2009). Pressure: a novel tool for enzyme-linked immunosorbent assay procedure. *BioTechniques*, *47*(6), 468-472.

Lee, C. J., Zhang, Y., Takao, H., Murayama, Y., & Qian, Y. (2013). A fluid–structure interaction study using patient-specific ruptured and unruptured aneurysm: The effect of aneurysm morphology, hypertension and elasticity.*Journal of biomechanics*, *46*(14), 2402-2410.

Li, H., Kem, D. C., Zhang, L., Huang, B., Liles, C., Benbrook, A., ... & Yu, X. (2015). Novel Retro-Inverso Peptide Inhibitor Reverses Angiotensin Receptor Autoantibody–Induced Hypertension in the Rabbit. *Hypertension*, *65*(4), 793-799.

Li, M. H., Cheng, Y. S., Li, Y. D., Fang, C., Chen, S. W., Wang, W., ... & Xu, H. W.

(2009). Large-cohort comparison between three-dimensional time-of-flight magnetic resonance and rotational digital subtraction angiographies in intracranial aneurysm detection. *Stroke*, *40*(9), 3127-3129.

Molyneux, A., & International Subarachnoid Aneurysm Trial (ISAT) Collaborative Group. (2002). International Subarachnoid Aneurysm Trial (ISAT) of neurosurgical clipping versus endovascular coiling in 2143 patients with ruptured intracranial aneurysms: a randomised trial. *The Lancet*,*360*(9342), 1267-1274.

Nahar, P., Bora, U., Sharma, G. L., & Kannoujia, D. K. (2012). Microwave-mediated enzyme-linked immunosorbent assay procedure. *Analytical Biochemistry, 421*(2), 764-766. doi:10.1016/j.ab.2011.09.029

Nasr, Deena M., and Robert D. Brown Jr. "Management of Unruptured Intracranial Aneurysms." *Current Cardiology Reports*, vol. 18, no. 9, 2016., pp. 1-6.doi:10.1007/s11886-016-0763-4.

Rand, T., Uberoi, R., Cil, B., Munneke, G., & Tsetis, D. (2013). Quality improvement guidelines for imaging detection and treatment of endoleaks following endovascular aneurysm repair (EVAR). *CardioVascular and Interventional Radiology, 36*(1), 35-45. doi:10.1007/s00270-012-0439-4

Ringer, A. "Aneurysm Embolization: Coiling." *Endovascular Aneurysm Coiling.* Mayfield Clinic, April 2016. Web. 29 Sept. 2016.

Rustemi, O., Alaraj, A., Shakur, S., Orning, J., Du, X., Aletich, V., . . . Charbel, F. (2015). Detection of unruptured intracranial aneurysms on noninvasive imaging. is there still a role for digital subtraction angiography?. *Surgical Neurology International,* 6(1), 175-175. doi:10.4103/2152-7806.170029

Sarikaya, S., & Sarikaya, B. (2011). Three-dimensional subtracted CT angiogram for aneurysm detection. *European Journal of Radiology,* 80(2), 502-503. doi:10.1016/j.ejrad.2010.12.024

Sengupta, R. (2015). Unruptured intracranial aneurysms. *Neurology India, 63*(6), 826-828. doi:10.4103/0028-3886.170064

Sonobe, M., Yamazaki, T., Yonekura, M., & Kikuchi, H. (2010). Small unruptured

intracranial aneurysm verification study: SUAVe study, japan. *Stroke, 41*(9), 1969-1977. doi:10.1161/STROKEAHA.110.585059

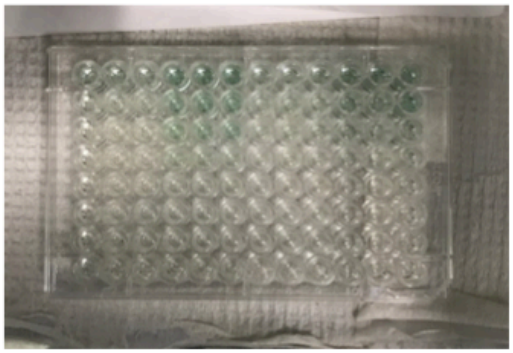
White, P., & Wardlaw, J. (2003). Unruptured intracranial aneurysms - detection and management. *Journal of Neuroradiology, 30*(5), 336-350.

Zuccarello, A. and Ringer, A. "Aneurysm Clipping." *Aneurysm Surgery; Clipping.* Mayfield Clinic, April 2016. Web. 29 Sept. 2016.

Appendix A

Pictures of well plates from each experiment conducted.

A) Trial 1, 15 Minutes post



B) Trial 2, 30 Minutes Post

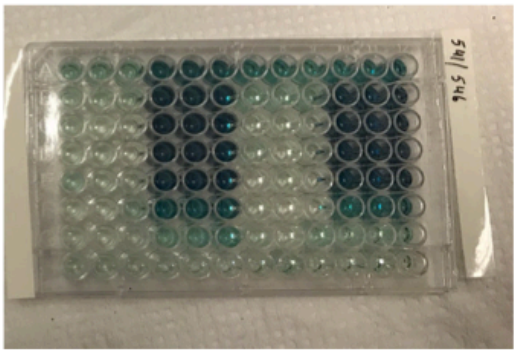
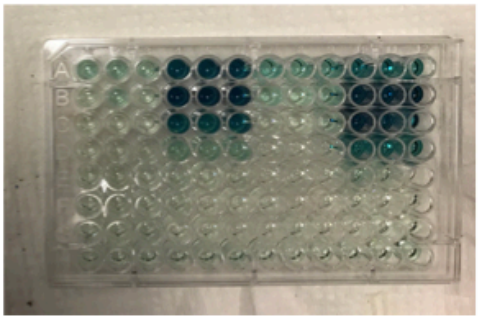
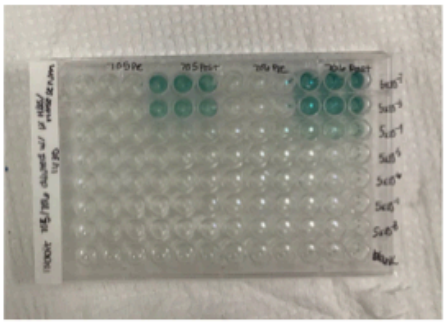


Figure 20: Picture of ELISA plates containing rabbit serum from Rabbit #541 (columns 1-6) and #544 (columns 7-12)

A) Trial 1, 15 Minutes post



B) Trial 2, 15 Minutes post



C) Trial 2, 30 Minutes post

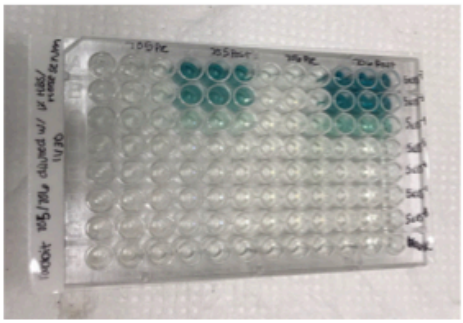


Figure 21: Picture of ELISA plates containing rabbit serum from Rabbit #705 (columns 1-6) and #706 (columns 7-12)

Appendix B

Graphs of log(Concentration) vs. Absorbance for each experiment conducted.

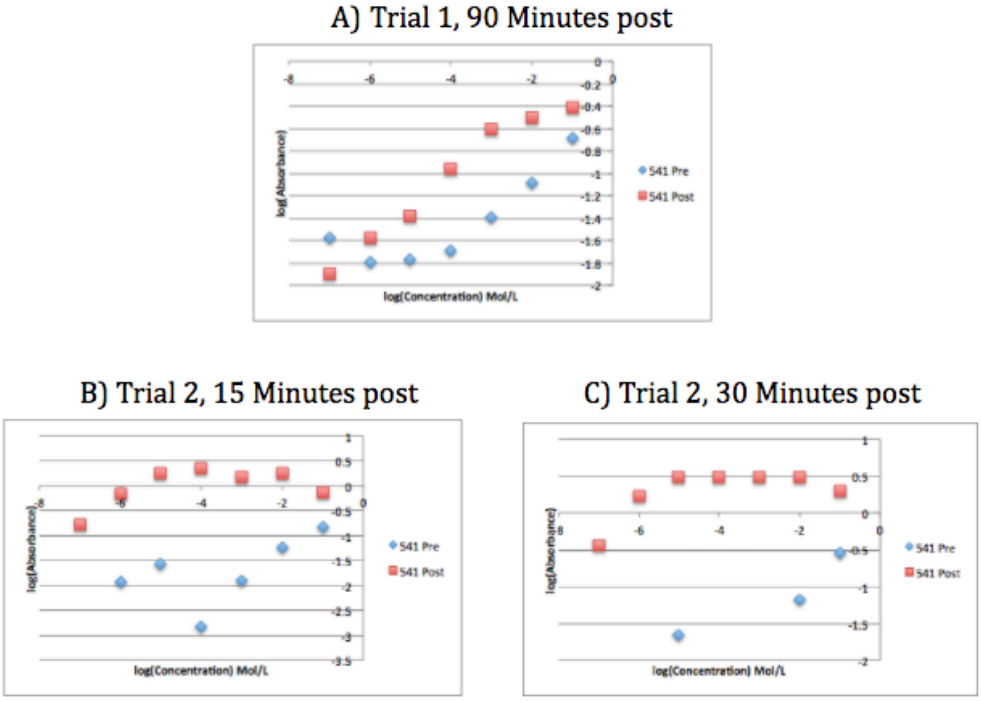


Figure 22: Graph of log(concentration) in Mol/L vs. log(absorbance) values fro Rabbit #541

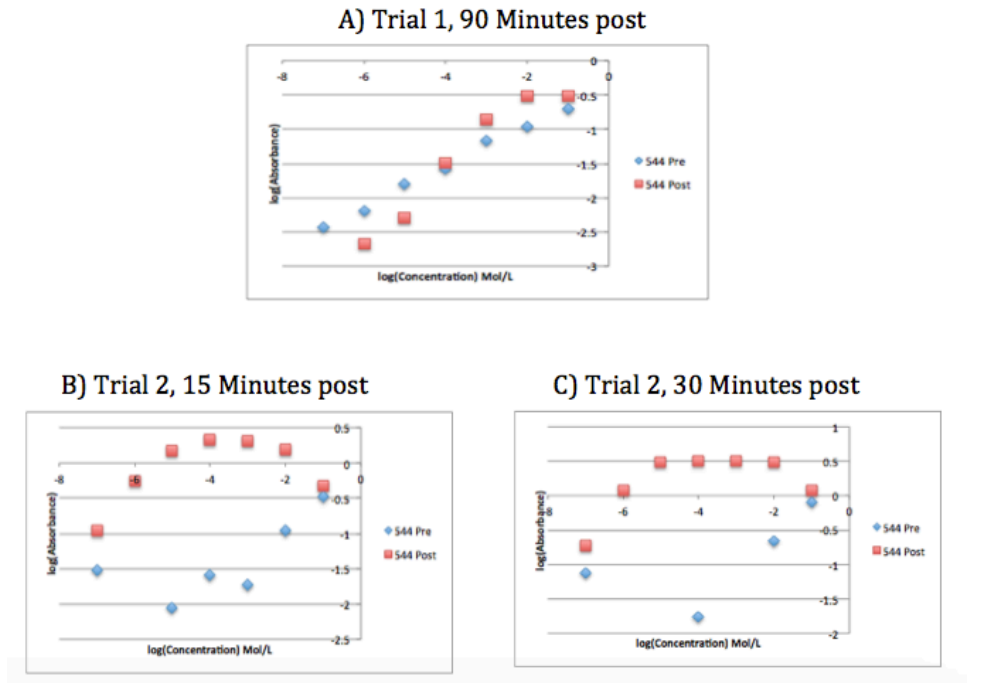


Figure 23: Graph of log(concentration) in Mol/L vs. log(absorbance) values fro Rabbit #544

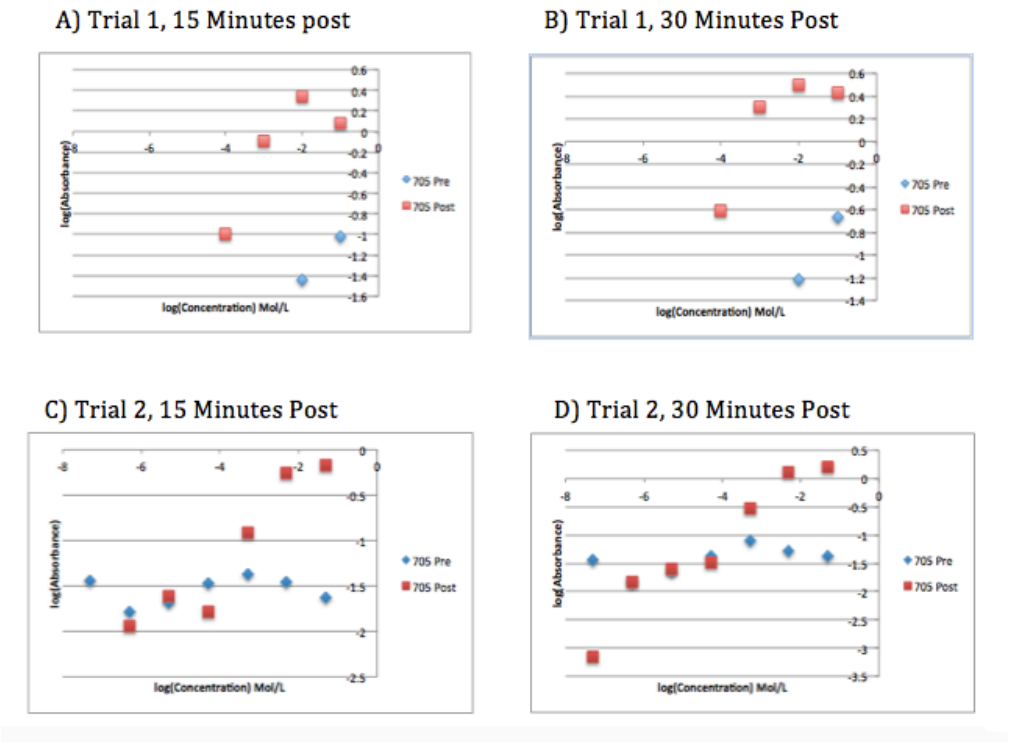


Figure 24: Graph of log(concentration) in Mol/L vs. log(absorbance) values fro Rabbit #705

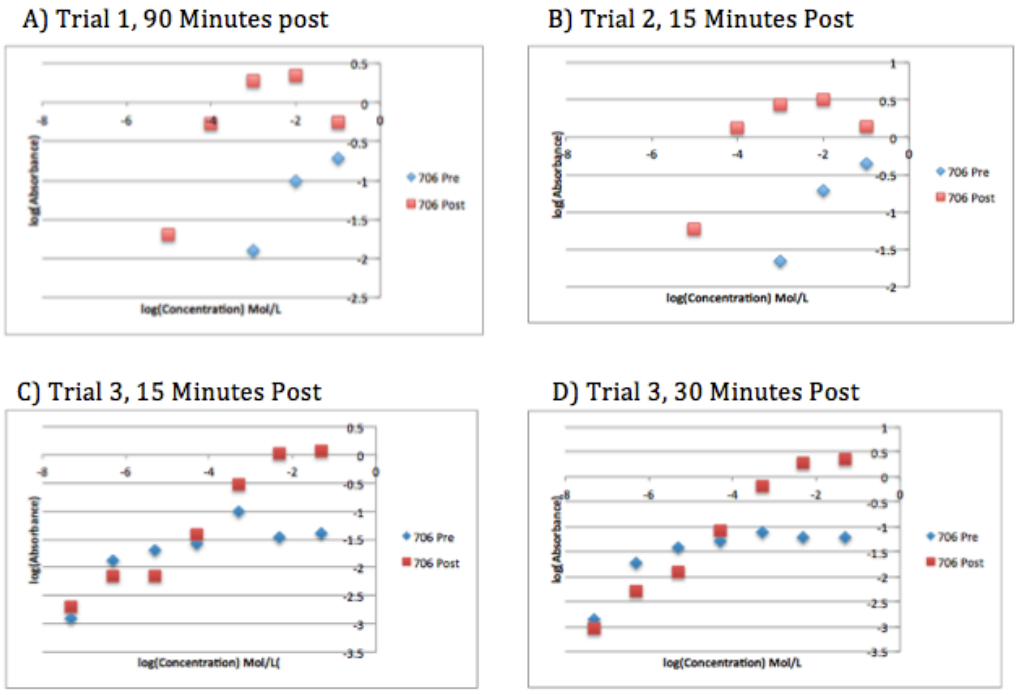


Figure 25: Graph of log(concentration) in Mol/L vs. log(absorbance) values for Rabbit #706

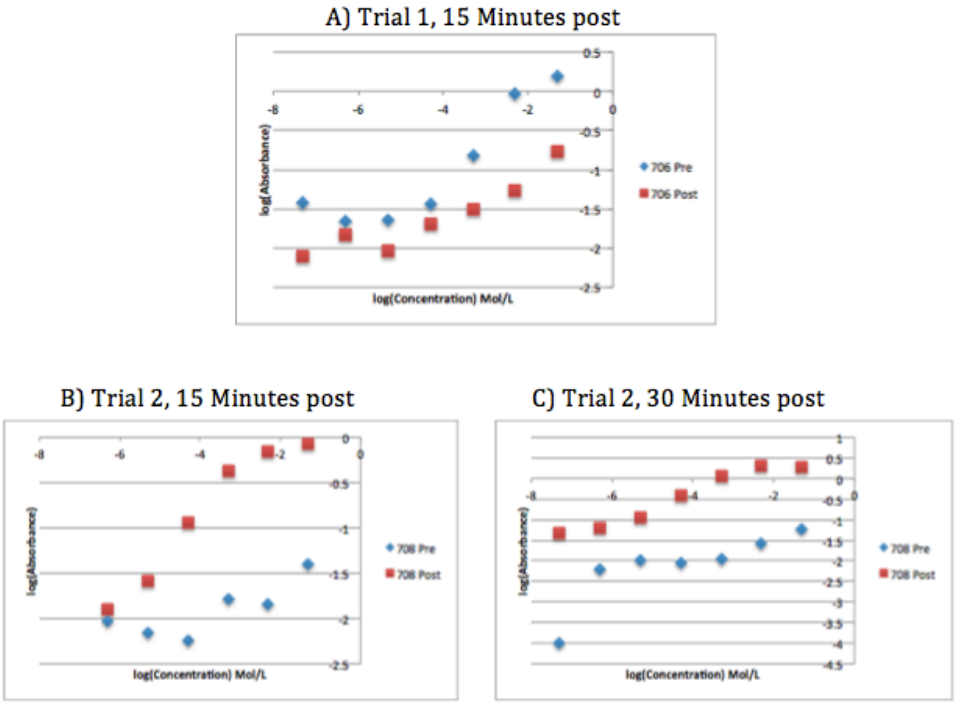


Figure 26: Graph of log(concentration) in Mol/L vs. log(absorbance) values for Rabbit #708