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The Effects of Smoking on the Calcification of the Aortic Valve

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The Effects of Smoking on the Calcification of the Aortic Valve

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By

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Introduction

Calcific Aortic Valve Disease(CAVD) is responsible for 28,000 deaths and 48,000 hospitalizations annually in the United States. It is one of the most detrimental forms of cardiovascular disease due to the fact that it increases the chances of death by other cardiovascular disorder by 50% (18). Characterized by thickening and mineralization of the aortic valve, this degenerative valvular heart disease is broken up into two stages; aortic sclerosis and aortic stenosis. The disease begins as aortic sclerosis, which is marked by valve thickening without decrease of blood flow. Over time, it progresses to aortic stenosis due to the valve being calcified enough to restrict motion and calcium nodules are become visible as shown in figure 1 (6, 10).

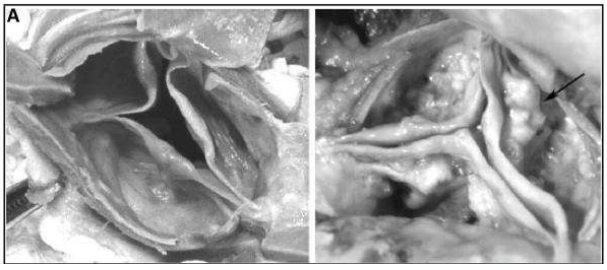

Figure 1. The progression of CAVD results in the formation of calcifying nodules as indicated by the arrow below (10). A healthy aortic valve is seen on the left while a severely diseased valve is on the right.

In the most severe cases, this disease is even known to result in bone-like formations (6). Along with valve thickening and mineralization, the disease is marked by transformations in cell phenotypes, the inflammatory responses, oxidative stress responses and the accumulation of lipoproteins. Originally, this disease was thought to be brought on by age and deterioration of the valve but these rapid stress responses when the valve is disrupted by biochemical and mechanical stress has led to its classification as an active disease. (8, 14). To understand the disease, one first has to understand the composition of the valve.

The aortic valve has a trileaflet structure (figure 1). The outer layer of the valve is lined with valve endothelial cells (VECs) which maintain anti-thrombogenicity while the inner layer is composed of valvular interstitial cells (VICs) that provide the strength and elasticity for the valve as well as repairing the valve extracellular matrix (10, 14). The VICs are broken up into three layers as well; the fibrosa on the aortic side, spongiosa in the middle and the ventricularis on the ventricular side as shown in figure 2 (10).

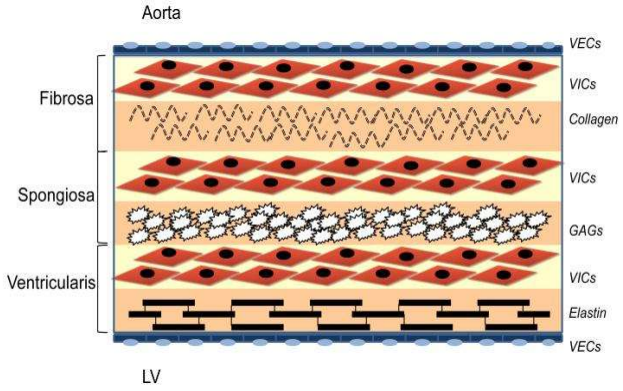

Figure 2. The organization of the aortic valve is important to the function of VICs and VECs. The fibrosa is also made of collagen that provides the strength for the VICs. Glycosaminoglycans (GAGs) in the spongiosa are a lubricant for the fibrosa and ventricularis layers. The ventricularis layer decreases strain with elastin fibers (10)

Due to the fact that VICs are much more abundant and that calcification has been known to begin in the fibrosa *in vivo*, most research to explore the disease is based on the VICs. This is also due to the fact that the role of VECs in the calcification process is not as well understood compared to VICs.

The most important trait of VICs is that they can change their phenotype and differentiate based on the response to the type of stress being experienced by the valve. VICs can differentiate into myofibroblasts, smooth muscle-like cells, and osteoblasts, cells that precede bone formation (19). The presence of either one contributes to the stiffening of the valve (6).

Once the cells have been differentiated into osteoblast as an osteogenic response, they begin to produce a bone morphogenetic protein 2 (BMP-2) which uses the Smad and Wnt/ -catenin pathway to produce runt-related transcription factor 2 (RUNX2) for the release of osteocalcin and osteopontin (8, 9, 10, 19). This pathway is shown in figure 3.

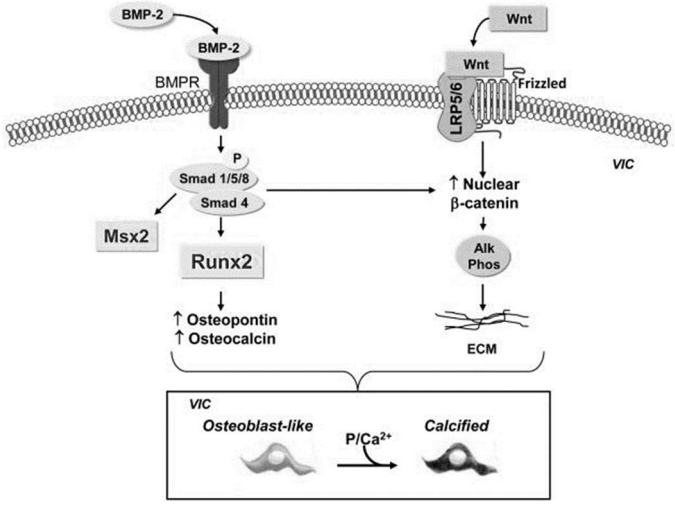

Figure 3. The Smad and Wnt/ β-catenin pathway induced by BMP-2 is the main way that bone producing proteins are released for the formation of calcifying nodules (10). BMP-2 is brought about by TGF-β1.

As shown in figure 4, Osteopontin is also the result of macrophages that enter the cell (6). Other cytokines that are produced as an inflammatory response include TGF-β1 and interleukin-6 (IL6) that are released from T-lymphocytes that enter the cell as an inflammatory response (6, 5). IL6 is also known to accumulate with oxidized low density lipoproteins (ox- LDLs) (18).

LDLs are present in the plaque-like lesions that occur during the beginning stages of the disease and are used as a form of diagnosis. These plaque-like regions are the result of LDLs joining with macrophages (white blood cells), that have differentiated from monocytes (white blood cells), to form foam cells (10, 17). This accumulation of LDLs will also be accompanied by the presence of lectin-like oxidized low density lipoprotein receptor-1 (LOX-1), which

represent an oxidative stress (9). A visual of these interactions was displayed in a study by Freeman et al (Figure 4).

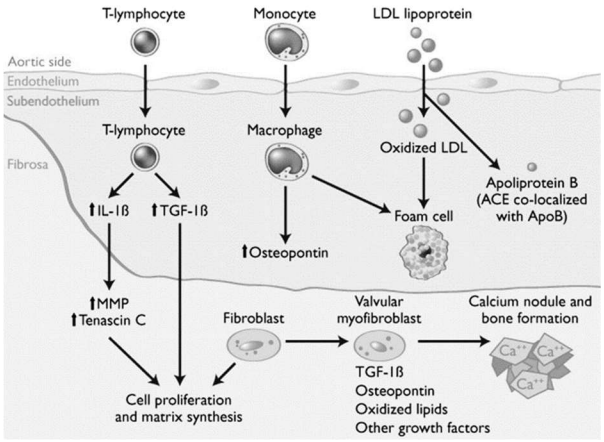

Figure 4. CAVD is characterized by the release of inflammatory factors, lipid accumulation and differentiation of VIC fibroblast in to osteoblast to produce bone formation (6).

All of this research reinforces the idea that CAVD is actually a multifaceted disease that requires the interaction of many different pathways and biological responses. LDL accumulation is not only caused by calcification.

Smoking cigarettes causes annually causes 140,000 premature deaths from cardiovascular disease and one of the main mechanisms it uses is it increases the amount of LDLs (2). This is due to the fact that cigarette smoke release over 4000 chemical substances and the three major toxins in cigarette smoke that can lead to the same type of physiological changes needed for calcification are nicotine, carbon monoxide and oxidant gases (1, 2). Nicotine, the addictive component of the smoke, leads to much of the mechanical stress that causes the phenotypical changes for VICs and VECs (14). When a person smokes, the nicotine induces the release of catecholamines from neurons and adrenal to increase heart rate, blood pressure, myocardial contractility and vasoconstriction (2,3). When the veins become affected by vasoconstriction, it means that the blood has to be pushed faster through a smaller diameter vein and then leads to

the mechanical stress that is so detrimental to VICs and VECs (2). Aiding the detrimental effects of nicotine is the carbon monoxide that induces hemodynamic effects (1). The main hemodynamic effect is that it causes an increase of red blood cell mass due to the fact that carbon monoxide binds with a higher affinity to hemoglobin than oxygen so more red blood cells are made to try and make up for the lack of oxygen (2, 3). These two factors work together to make it harder to pump blood through the veins both by decreasing vein diameter and increasing the blood viscosity. (2, 9). This means that nicotine induces both biochemical and mechanical stress. Another thing increased by nicotine is the presence of LDLs, which is a sign of sclerosis (13).

When nicotine is introduced into the body, the LDL concentration greatly increases and these lipoproteins join with macrophages to form plaques much like CAVD (2). This means that it elicits an inflammatory response and it has been found that smoking decreases the use of one of the main defense mechanisms against calcification; nitric oxide release (15). Nitric oxide is known to decrease myofibroblast activation, osteoblastic differentiation and VIC calcification as shown in a study by Richards et al. This defensive mechanism is known to be released by VECs *in vivo* but there is no way to experience the same inhibition *in vitro* (15). The inhibition of nitric oxide release is also due to the oxidant gases released by the cigarettes that are known to oxidize LDLs and have been shown to degrade nitric oxide (2). These gases are also known to release peroxynitrite, which enhances the cellular oxidative stress response (1). The fact that smoking influences the valve in this way shows that an increase in LOX-1, IL-6 and TGF- β1 due to the increased presence of LDLs can be expected. These factors would then suggest upregulation of osteopontin, osteocalcin and RUNX2. The exact mechanisms by which smoking activates these processes are not fully understood but it can be concluded that the detrimental effects of both smoking and CAVD result in similar responses. The focus of this Honors project is thus to

identify if smoking will elicit a pathological response in vitro to better understand the pathways induced and to identify ways to mitigate smoking-related valve disease. It is hypothesized that cigarette smoke will lead to an inflammatory response, osteogenic-like response, and oxidative stress.

This objective was met by studying the changes in LOX-1, TGF-β1, IL-6, osteopontin, osteocalcin and RUNX2 expression when VICs were cultured in a variety of (0.5% and 1% Marlboro red and silver) cigarettes extracts. These cigarettes were smoked for 4 hours per day for five days with the percent representing the amount of cigarette smoke relative to the amount of air. The mRNA was then isolated from these cells and transcribed to cDNA for analysis with real time polymerase chain reaction (RT-PCR) which was used to test for the presence of osteopontin, osteocalcin, RUNX2 and IL-6 genes (15,17, 18). Another set of VICs were cultured so that the protein could be isolated for western blot and were used to test for the presence of LOX-1, TGF-β1, osteopontin, osteocalcin, and RUNX2 expression. The expression for all was compared to cells that were not cultured in the smoking extracts.

This study was conducted in the hopes of providing a starting point for other members of the lab to further analyze the connection between smoking and CAVD. It was also hoped that the smoking extracts will accelerate the pathological response so that the pathways could be analyzed in a faster manner. The need to find an answer to this problem is great not just because of the risk of having CAVD but also due to this lack of knowledge that the only treatment for severe calcification is to have a surgery for a bioprosthetic valve replacement or a transcatheter aortic valve replacement. These replacement valves though, have a 30-60% chance of degeneration after 15 years due to high biochemical stress (10). Without the surgery though,

there is only a 50% survival rate two years after the diagnosis (6). This disease is a detrimental disease with only a temporary fix.

Methods and Materials

VIC Culture

Fresh porcine hearts (3-6 months old) were obtained from Braunschweig Meat Processing (Neosho, MO) and Cockrum's Custom Meat Processing (Rudy, AR). The hearts were then transported to our laboratory in cold Dulbecco's Phosphate Buffered Saline (dPBS; ThermoFisher, Waltham MA) supplemented with 1% antibiotic/antimycotic solution. Hearts were immediately dissected aseptically to reveal the aortic valve leaflets. Left, right and noncoronary leaflets were pooled and washed 2 times in Hank's Balanced Salt Solution (HBSS; ThermoFisher). Cells were isolated using collagenase digestion as described by Butcher et al and Gould et al (4,7). To briefly explain, valve endothelial cells (VECs) were removed by incubating the leaflets in collagenase solution (60U/ml) for 30 min and discarding the digestate. Valve interstitial cells (VICs) were then isolated by incubating the leaflets in collagenase solution (60U/ml) at 37°C for 2 hours with frequent agitation. The digestate was spun down in centrifuge at 250g for 5 minutes at 4°C and re-suspended for cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 50U/mL penicillin, 50U/mL streptomycin, and 10mM HEPES (all ThermoFisher). Fresh DMEM was exchanged every two to three days. Cells from passages 2-7 were used in all subsequent experiments and were cultured in a 6 well plate. The smoking extract were then added to the plates in such a way that each of the 4 types of smoking extracts had its own well. 50µL of each smoking extract was added for every trial. The fifth well was treated with dimethyl sulfoxide (DMSO) and 6^{th} was not treated with anything as a negative control. The plates were then incubated for 6 hours, 24 hours, 48 hours after which time, the mRNA was isolated from the plates. Protein was only isolated from the 24 hour and 48 hour time points.

RT- PCR

The mRNA was isolated via a Qiagen RNeasy Minikit into approximately 100µL quantities for each well. The sample was then analyzed by first testing the 260:280 nm absorbance ratio the samples to ensure that the sample did not contain DNA and to find the concentration of each sample. A value of approximately 2 indicated that the sample was "pure" RNA. The quality of the mRNA was insured for use by a Bioanalyzer. RNA of good quality had a RNA index level of around 10; which showed that there was no degradation. Once the quality and concentration of the RNA was established, the mRNA was transcribed to cDNA by reverse transcription with 5x iScript reverse transcriptase and RNAse/DNAse free water for a total amount of approximately 0.7 µg of RNA from each sample. A Bio-Rad CFX 96 Real Time Detection machine was used for this process. Once the cDNA was made, primers for were ordered from Integrated DNA technologies (Figure 5). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the reference gene (16). PCR was performed on each sample with a final concentration of 1 ng using SYBR Green.

Gene	Forward Primer	Reverse Primer
Osteopontin	5'-GGT CTA TGG ACT GAG GTC AAA ATC TA-3'	5'-TCC GAG GAA ATA GTA TTC TGT GGC-3'
Osteocalcin	5'-CTC CAG CCA CAA CAT CCT TT-3'	5'-TTG CCT CCA GCA CTG TTT AT-3'
RUNX2	5'-GCA CTA CCC AGC CAC CTT AT-3'	5'-TAT GGA GTG CTG CTG GTC TG-3'
IL6	5'-CAC CAG GAA CGA AAG AGA GC-3'	5'-CTT TTG TCC GGA GAG GTG AA-3'
GADPH	5'-TGT ACC ACC AAC TGC TTG GC-3'	5'-GGC ATG GAC TGT GGT CAT GAG-3'

Figure 5. Primers were obtained from Integrated DNA Technologies.

Gel Electrophoresis/ Western Blot

The proteins were isolated through the use of 100 µL 4M urea/ 5mM/ 0.5% SDS/ 0.5% NP-40/ 100 mM Tris lysis buffer that lysed the cells from the plate. Cell lysate was vortexed for 20 seconds then it was centrifuged at 14500 rpm, 4°C for 5 minutes. The supernatant was separated from the cell pellet for analysis with a micro-BCA protein assay kit. The concentration

of each sample was determined by comparing the average of the control absorbance to that of the sample absorbance. The samples were then made into 40 μL sample of 30 ug of protein and combined with 10 μL of 4X sample buffer, 2 μL of β-mercaptoethanol and the remaining amount was water. They were then boiled at 95 °C for 5 minutes in a heating block. Two standards were also prepared by adding 8 μL of the standard, 10 μL of 4X sample buffer and diluted the remaining 40 μL with water. Gel electrophoresis was then run with 1X Tris/ glycine/ SDS running buffer, with 25 μL being added to each well, in a 10% polyacrylamide gel at 200 V for 45 minutes. The proteins were then transferred to a polyvinyldifluoride (PVDF) membrane in a 1X transfer buffer; made of methanol, 10x tris/glycine buffer, and water, for 1 hour at 100V in a 4°C fridge. The membrane was placed in Li-Cor blocking buffer for 2 hours to promote the binding of primary antibodies. After, the membrane was treated with a 500 μL solution of primary antibodies: rabbit osteopontin antibody (1:1000), mouse RUNX2 antibody (1:1000), mouse β-actin antibody (1:1000), rabbit LOX-1 antibody (1:1000), mouse osteocalcin antibody (1:500), and rabbit TGF- β1 antibody (1:1000), that were diluted with a 1:1 ratio of Li-Cor blocking buffer to PBS (phosphate buffer saline). The membranes were incubated for 16 hours at 4°C. The primary antibodies were then washed from the membranes by washing them 3 times with a 1:4 solution of Li-Cor Blocking buffer to water and followed by two washings with water. The membrane was then incubated with the appropriate Li-Cor secondary antibody (1:15000) by submerging the membrane in 20 ml of a solution of diluted secondary antibodies for1 hour at room temperature. The washing process was repeated for the secondary antibodies. The membranes were then analyzed using a Li-Cor Odyssey Scanner. Protein expression was quantified by obtaining the band intensity for LOX-1, TGF-β1, osteopontin, osteocalcin, and

RUNX2 and normalizing it with the intensity obtained for β-actin protein. All primary antibodies were obtained from Abcam.

Results and Discussion

As stated beforehand, the primary objective of my research was to determine if there was a connection between smoking and CAVD progression and potentially identify candidate genes/ pathways for further study. The project began with VICs from adult porcine aortic being cultured in smoking extracts for 6 hours, 24 hours and 48 hours. The first group of cells were subjected to mRNA isolation and transferred to cDNA to be analyzed with RT-PCR (Figures 6-8). These results are consistent with the 6 hour treatment but vary greatly from the expected outcome for the 24 hour and 48 hour treatment. Osteopontin, IL6, and RUNX2 are upregulated for 1% R, 0.5% R and 1% S when compared to the well with no treatment and the well with only DMSO during the 6 hour treatment (Figure 6).

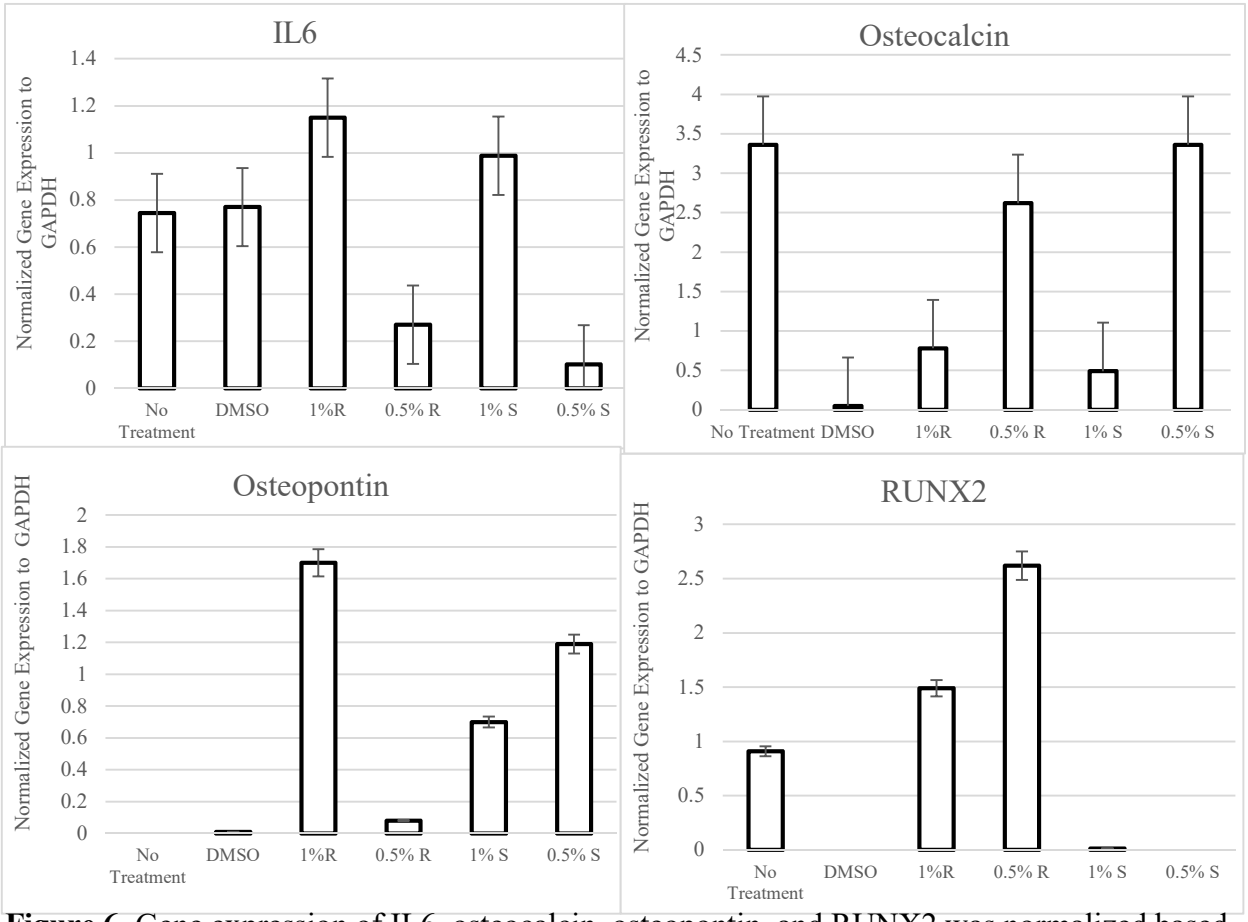

Figure 6. Gene expression of IL6, osteocalcin, osteopontin, and RUNX2 was normalized based on GAPDH gene expression after 6 hours. Analysis was done in triplets. R represents Marlboro Red or full flavor cigarettes while S represents Marlboro Silver or light Cigarettes. For all cases, n=2.

The red or full flavor cigarettes showed the most upregulation due to the fact that there are more chemicals and nicotine than in the silver cigarettes per puff (20). The response of IL6 was much as expected due to the fact that oxi- LDLs are known to be increased in cells that are affected by cigarette smoke (18). LDLs are also accompanied by macrophages that lead to the production of osteopontin and RUNX2 (2,6). In contrast, osteocalcin was shown to have the greatest amount of upregulation in the well without any treatment. This suggested that osteocalcin was downregulated by smoking but does not seems to be plausible when one compares the fact that smoking elicits the release of the inflammatory cells, such as macrophages, that have been

known to cause the release of osteocalcin due to the TGF-β1 activation (2, 6). Further study is needed in this regard.

When RT-PCR was performed on 24 hour extracts, we observed that IL6 was the only gene that was highly upregulated for 1% R and 0.5% R treated cells (Fig. 7).

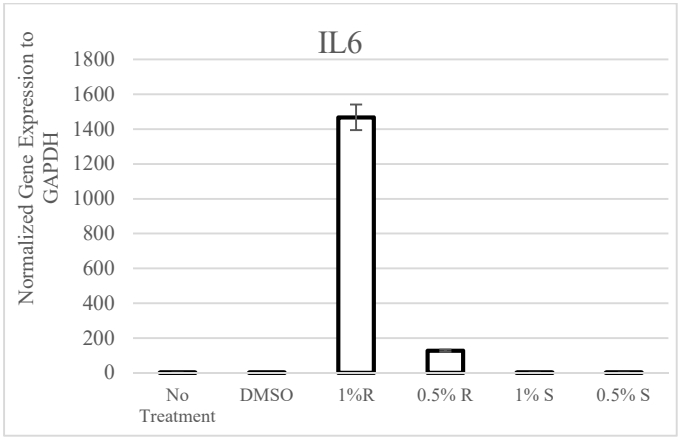

Figure 7. Gene expression of osteopontin, osteocalcin, RUNX2, and IL6 was normalized with GAPDH expression after 24 hours. IL6 was the only one that showed gene expression but experienced extreme abnormalities. Analysis was done in triplets. R represents Marlboro Red or full flavor cigarettes while S represents Marlboro Silver or light Cigarettes. For all cases, n=2.

There was no significant difference in osteopontin, osteocalcin or RUNX2 expression. They should have been present due to the fact that the T-lymphocytes that were activated by the inflammatory response to the smoke would have released both TGF-β1 and IL6. The TGF-β1 would have caused the formation of osteoblast that released all three of these molecules (6). Despite the evidence provided by the 6 hour and 24 hour RT-PCR analysis, there is some question as to what exactly happened during the 48 hour window. During this last trial, only osteopontin and RUNX2 were expressed and were upregulated more for DMSO and no treatment (Figure 8).

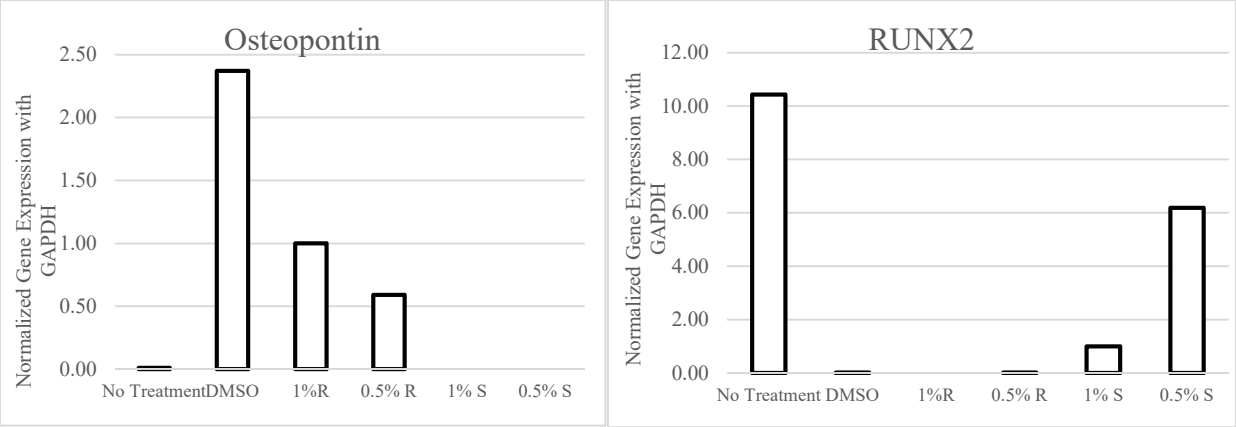

Figure 8. Gene Expression of osteopontin, osteocalcin, RUNX2, and IL6 was normalized with GAPDH expression after 48 hours. RUNX2 and osteopontin were the only genes expressed. R represents Marlboro Red or full flavor cigarettes while S represents Marlboro Silver or light Cigarettes. For all cases, n=2.

This effect could be due to the fact that at this time point, the cells were beginning to reach the maximum cell density that the wells can handle and this could have elicited enough of a stress response for CAVD mechanisms to begin to occur (14). That the wells with smoking extracts did not have as much expression could possibly be due to the fact that the smoking extract slowed the cell growth cycle. Further study is needed to verify this particular hypothesis. From the RT-PCR, it can be concluded that there is some connection between smoking and CAVD but the extent to which that connection occurs cannot be determined at this time.

The cells were then analyzed for the 24 and 48 hour treatments through western blot to examine the changes in protein expression when the cells were exposed to the smoking extracts (Fig. 9-10).

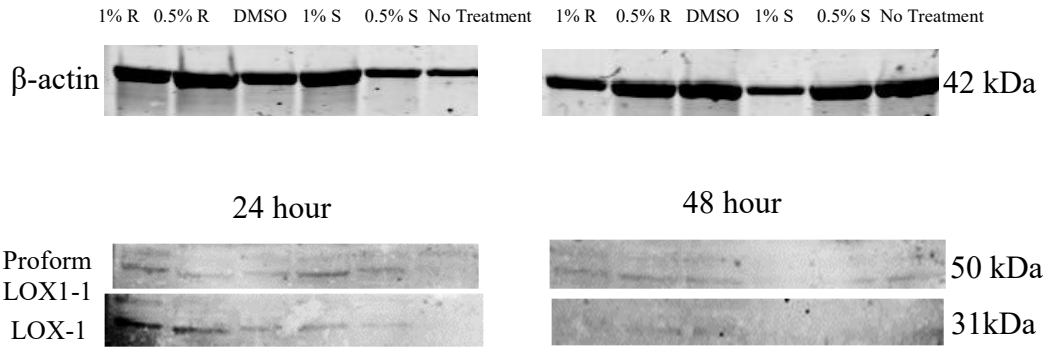

Figure 9. The intensity of LOX 1 was compared to that of β-actin from 30 ug protein samples. The samples are in the following order; 1% R, 0.5% R, DMSO, 1% S, 0.5% S and no treatment for each time. TGF-β1, osteocalcin, osteopontin, and RUNX2 did not display any bands in the western blot.

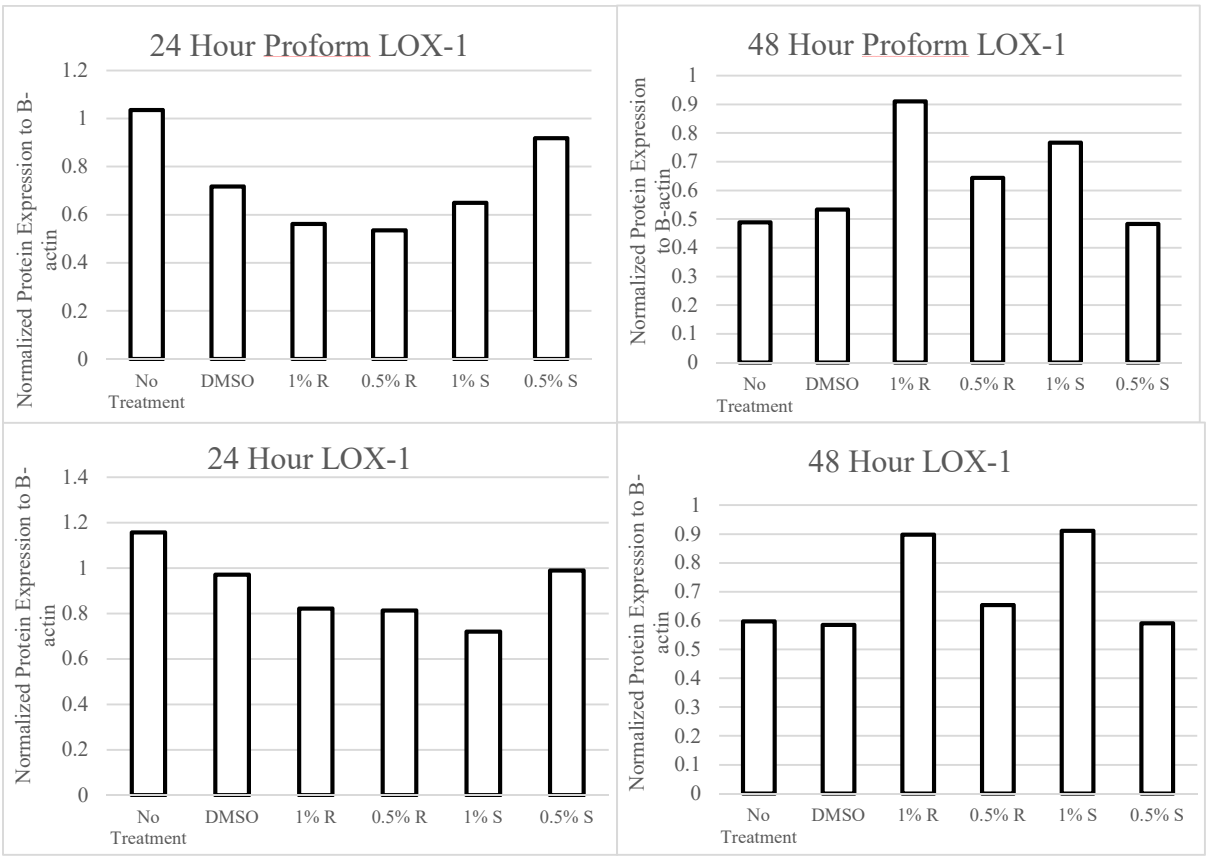

Figure 10. The amount of preform and mature LOX 1 was normalized based on the amount of β-actin in the gel and compared by both time and type of smoking extract added. R represents Marlboro Red or full flavor cigarettes while S represents Marlboro Silver or light Cigarettes. For all cases, n=2.

Protein expression of β-actin was used as a way to normalize the data between the wells. During both the 24 hour and 48 hour treatments, the only protein that was expressed was LOX-1 in both its proform at 50 kDa and its mature form at 31 kDa. LOX-1 was produced more in the wells with 1% R, 0.5% R and 1% S compared to those treated with DMSO and no treatment at all. This is expected and follows the hypothesis due to the fact that LDLs are increased in during smoking (9). It should be noted that the 24 hour treatment does the reverse in that it shows lower amounts of LOX-1 than are seen in the wells with no treatment and DMSO. LOX-1 and it's preform showed the same trend. This difference between the time points was unexpected but the presence of both forms of LOX-1 combined with the fact that IL6 was also present in the 24 hour treatment suggest that smoking does increase the presence of oxidized LDLs (6). This increase could possibly lead to the formation of plaques if the cells were grown in the smoking extracts long enough. The end result of this study is that smoking elicited an increase in the LDL concentration in the VICs due to the upregulation of IL6 genes and the presence of LOX-1 proteins.

Conclusion and Future Directions

This study has provided results demonstrating that smoking causes a pathological response much like that of cellular oxidative stress by inhibiting nitric oxide. The upregulation of IL6 and the presence of LOX-1 points to an increase of oxidized LDLs that are characteristic of oxidative stress. A possible treatment for this that should be tested is whether the use of nitric oxide donors inhibit this effect since they have been shown to inhibit calcifying nodules in porcine VICs (17). Also, inhibitors of IL6 and LOX-1 should be examined to determine if this will alleviate the effects of the disease. These results, in combination with the fact that osteopontin, osteocalcin, and RUNX2 gene expression were upregulated at the 6 hour and 48 hour time points, also suggest that there is still a possibility that smoking could induce an osteogenic response. If IL6 was present, then TGF-β1 should have been present for the release of osteopontin, osteocalcin and RUNX2. Further studies will have to be done to prove this due to the lack of consistency in the results.

Possible future studies would include a gene microarray that would be performed on the mRNA of the VICs to examine all of the genes that are manipulated by the smoking extracts. Once the microarray has identified more of the key factors then possible inhibitors can be tested. RT-PCR and western blots should be performed again but only after the cells have grown in the smoking extracts for a much longer times. Many other studies that examined the pathological response of VICs in osteogenic media did not analyze the cells until at least four days after culture. For example, in a study by Kennedy et al, VICs grown in the presence of TGF-β1 showed peak calcifying nodule formation between 4 and 8 days (8). Another study by Clark-Greuel et al did not show a confirmed presence of calcification of sheep aortic VICs until 7 days and no evidence to suggest calcification was shown until 72 hours (5). Yip et al showed that

calcified aggregates did not occur until 5 days (19). None of these studies have examined the response of VICs to smoking. What all of these studies did suggest, though, was that CAVD is a much slower process than originally thought, possibly due to all of the mechanisms involved, and leads to an explanation as to why the results with the other candidate genes/ proteins were inconclusive. That being said, these experiments should be repeated for longer time periods with RT-PCR and Western Blots being re-analyzed. To better test for an osteogenic response, the cells should be subjected to Alizarin Red staining to test for the presence of calcium in the cells. In addition to that, there has been some evidence that autophagy is related to CAVD and live-dead assays could be performed to analyze this response. The final hope of this study is that someday, this knowledge can be used to develop a better strategy to halt CAVD progression.

References

1. Ambrose, John A, Barua, Rajat S, The pathophysiology of cigarette smoking and cardiovascular disease: An update, Journal of the American College of Cardiology, Volume 43, Issue 10, 19 May 2004, Pages 1731-1737, ISSN 0735-1097, http://dx.doi.org/10.1016/j.jacc.2003.12.047. (http://www.sciencedirect.com/science/article/pii/S0735109704004346)

2. Benowitz, Neal L, Cigarette smoking and cardiovascular disease: pathophysiology and implications for treatment, Progress in Cardiovascular Diseases, Volume 46, Issue 1, July–August 2003, Pages 91-1, ISSN 0033-0620, http://dx.doi.org/10.1016/S0033-0620(03)00087-2. (http://www.sciencedirect.com/science/article/pii/S0033062003000872)

3. Benowitz, Neal L, The Role of Nicotine in Smoking-Related Cardiovascular Disease, Preventive Medicine, Volume 26, Issue 4, July 1997, Pages 412-417, ISSN 0091-7435, http://dx.doi.org/10.1006/pmed.1997.0175. (http://www.sciencedirect.com/science/article/pii/S0091743597901754)

4. Butcher, J.T. "Porcine Aortic Valve Interstitial Cells in Three-dimensional Culture: Comparison of Phenotype with Aortic Smooth Muscle Cells." J Heart Valve Dis 13.3 (2004): 478-85. Web. 6 Apr. 2016.

5. Clark-Greuel, J. N., Connolly, J. M., Sorichillo, E., Narula, N. R., Rapoport, H. S., Mohler, E. R., . . . Levy, R. J. (2007). Transforming Growth Factor-β1 Mechanisms in Aortic Valve Calcification: Increased Alkaline Phosphatase and Related Events. The Annals of Thoracic Surgery, 83(3), 946-953. Retrieved February 1, 2015.

6. Freeman, R. V., and Catherine M. Otto. "Spectrum Of Calcific Aortic Valve Disease: Pathogenesis, Disease Progression, And Treatment Strategies." Circulation 11.24 (2004): 3316-326. Circulation. American Heart Association. Web. 4 Mar. 2015. (http://circ.ahajournals.org/content/111/24/3316).

7. Gould, Russell A., and Jonathan T. Butcher. "Isolation of Valvular Endothelial Cells." Journal of Visualized Experiments JoVE 46 (2010). Web.

8. Kennedy, J. A., Hua, X., Mishra, K., Murphy, G. A., Rosenkranz, A. C., & Horowitz, J. D. (2009). Inhibition of calcifying nodule formation in cultured porcine aortic valve cells by nitric oxide donors. European Journal of Pharmacology, 602(1), 28-35. Retrieved September 10, 2015.

9. Lee, Seung Hyun, and Jae-Hoon Choi. "Involvement of Immune Cell Network in Aortic Valve Stenosis: Communication between Valvular Interstitial Cells and Immune Cells." Immune Network 16.1 (2016): 26. Web. 4 Apr. 2016.

10. Leopold, J. A. "Cellular Mechanisms of Aortic Valve Calcification." Circulation: Cardiovascular Interventions 5.4 (2012): 605-14. National Institute of Health. PubMed Central. Web. 9 Mar. 2015. (http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3427002/)

11. Martinet, Wim, Michiel W.m. Knaapen, Mark M. Kockx, and Guido R.y. De Meyer. "Autophagy in Cardiovascular Disease." Trends in Molecular Medicine 13.11 (2007): 482-91. Science Direct. Web. 6 Apr. 2016.

12. Mistiaen, Wilhelm P., Pamela Somers, Michiel W.m. Knaapen, and Mark M. Kockx. "Autophagy as Mechanism for Cell Death in Degenerative Aortic Valve Disease: An Underestimated Phenomenon in Cardiovascular Diseases." Autophagy 2.3 (2006): 221-23. Science Direct. Web. 6 Apr. 2016.

13. Pyrgakis, Vlassis N. "Smoking and Cardiovascular Disease." Hellenic Journal of CArdiology 50 (2009): 231-34. Hellenic Journal of Cardiolgoy. Department of Cardiology. Web. 4 Mar. 2015. (http://hellenicjcardiol.com/archive/full_text/2009/3/2009_3_231)

14. Rajamannan, N. M., F. J. Evans, E. Aikawa, K. J. Grande-Allen, L. L. Demer, D. D. Heistad, C. A. Simmons, K. S. Masters, P. Mathieu, K. D. O'brien, F. J. Schoen, D. A. Towler, A. P. Yoganathan, and C. M. Otto. "Calcific Aortic Valve Disease: Not Simply a Degenerative Process: A Review and Agenda for Research From the National Heart and Lung and Blood Institute Aortic Stenosis Working Group * Executive Summary: Calcific Aortic Valve Disease - 2011 Update." Circulation 124 (2011): 1783-791. Circulation. Web. 4 Mar. 2015. (http://circ.ahajournals.org).

15. Richards, Jennifer, Ismail El-Hamamsy, Si Chen, Zubair Sarang, Padmini Sarathchandra, Magdi H. Yacoub, Adrian H. Chester, and Jonathan T. Butcher. "Side-Specific Endothelial-Dependent Regulation of Aortic Valve Calcification." The American Journal of Pathology 182.5 (2013): 1922-931. Elsevier. Web. 10 Sept. 2015. (http://ajp.amjpathol.org).

16. Tseng, Hubert, Liezl R. Balaoing, Bagrat Grigoryan, Robert M. Raphael, T.c. Killian, Glauco R. Souza, and K. Jane Grande-Allen. "A Three-dimensional Co-culture Model of the Aortic Valve Using Magnetic Levitation." Acta Biomaterialia 10.1 (2014): 173-82. Elsevier. Web. 1 Feb. 2015.

17. Warnock, James N., Shane C. Burgess, Allen Shack, Ajit P. Yoganathan. “Differential Immediate-Early Gene Responses to Elevated Pressure in Porcine Aortic Valve Interstitial Cells. The Journal of Heart Valve Disease 15 (2006): 34-42. Research Gate. Web. 10 September 2015. (http://www.researchgate.net/publication/7297014).

18. Warnock, James N., Bindu Nanduri, Carol A. Pregonero Gamez, Juliet Tang, Daniel Koback, William M. Muir, and Shane C. Burgess. "Gene Profiling of Aortic Valve Interstitial Cells under Elevated Pressure Conditions: Modulation of Inflammatory Gene Networks." International Journal of Inflammation 2011 (2011): 1-10. SAGE-Hindawi. Web. 1 Feb. 2015.

19. Yip, C. Y. Y., J.-H. Chen, R. Zhao, and C. A. Simmons. "Calcification by Valve Interstitial Cells Is Regulated by the Stiffness of the Extracellular Matrix." Arteriosclerosis, Thrombosis, and Vascular Biology 29.6 (2009): 936-42. Web. 1 Feb. 2015.

20. “"Light" Cigarettes and Cancer Risk." National Cancer Institute. 28 Oct. 2010. Web. 06 Apr. 2016. (http://www.cancer.gov/about-cancer/causes-prevention/risk/tobacco/light-cigarettes-fact-sheet)