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Investigation of the Effects of Curcumin on Woodsmoke-Induced Inflammation

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by

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Honors Thesis

Submitted to:

Biology Department

University of Richmond

Richmond, VA

April 30th, 2021

Advisor: Dr. Shannon Jones

This thesis has been accepted as part of the honors requirements in the Department of Biology.

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Abstract

Inflammatory diseases caused by biomass smoke exposure and indoor air pollution affect millions of people worldwide. These diseases can be caused by the burning of biomass (plastic, wood, rubber, tobacco, etc.) for light or heat. Acrolein is a common indoor and outdoor pollutant from tobacco smoke or organic combustion. Wood smoke is hypothesized to cause inflammation since it contains high concentrations of particulate matter and gaseous compounds and are similar in size to other well-known damaging particles. Natural remedies, like curcumin, are hypothesized to be a natural remedy for combating inflammation. The goal of this research was to investigate the ability of indoor air pollutants, like acrolein and wood smoke, to induce inflammatory responses. Many studies have found induced responses in animal models. This research also looked at the ability of natural remedies, like curcumin, to combat these inflammatory effects. Curcumin has been found to have therapeutic effects in many inflammatory diseases. This research was done on specific inflammatory cells and used cytokine secretion and gene expression to measure inflammatory responses. Cells were cultured for 24 hours to assess inflammatory gene expression (TNFα, MIP-2, and COX-2) via RT-PCR, gel electrophoresis, and ELISA. Woodsmoke was found to dose-dependently induce gene expression of COX-2 and MIP-2. Curcumin was found to dose-dependently decrease gene expression of COX-2 and MIP-2. However, neither woodsmoke or curcumin significantly affected secretion of TNFα and MIP-2 cytokines. Further research must be conducted to confirm these findings and determine whether curcumin has anti-inflammatory properties against biomass fuels like woodsmoke.

Background and Significance

Inflammatory diseases caused by biomass smoke exposure and indoor air pollution affect millions of people worldwide. Over 3 billion people rely on biomass fuels as a main source of heating for their homes, where the inflammatory agents in the smoke become concentrated in these poorly ventilated, indoor areas. Women and children in developing countries with fuel shortages are at the most significant risk for respiratory disease due to this indoor pollution. Prolonged, chronic exposure to air pollution has been linked to exacerbation of asthma, chronic upper respiratory infections, and an increased risk for heart disease and lung cancer (WHO). Current anti-inflammatory drugs used to manage lung disease such as inhaled corticosteroids are incredibly costly in many developing nations and can increase susceptibility to bacterial infections. Long-term treatments with corticosteroids can also have detrimental effects on human health. Alternative, less costly treatments are not currently profitable for pharmaceutical companies to pursue.

Compounds found in biomass fuel smoke, like woodsmoke, can activate macrophages and cause inflammation. Long term inflammation from chronic exposure to biomass smoke can lead to pulmonary and cardiovascular diseases such as high blood pressure, and serious infections such as pneumonia (Driscoll 1994). Woodsmoke and other combustion materials induce inflammation and oxidative stress in the airways (Swiston et. al. 2008). It is hypothesized that woodsmoke activates the nuclear factor-kappaB (NFκB) pathway and toll-like receptor (TLR) pathways (Schuller et al. 2020). These signaling cascades lead to oxidative stress in cells which can cause

mitochondrial and DNA damage, lipid peroxidation, and the signaling of pro-inflammatory genes (Silva et al. 2015) (Figure 1).
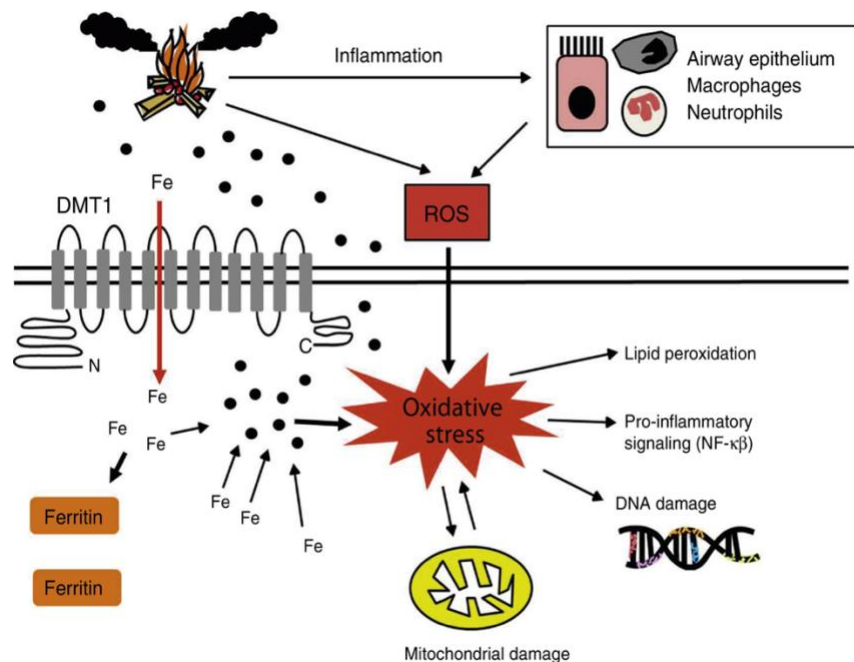

Figure 1: Woodsmoke oxidative mechanisms and genotoxicity. Oxidative stress generated by woodsmoke can cause direct damage to cell macromolecules, such as proteins or lipids, and can act as a genotoxic factor in producing DNA lesions. Mitochondrial membrane potential can also be affected by oxidative damage, resulting in increased reactive oxygen species (ROS)j production in these organelles (Figure from Silva et al. 2015).

Acrolein is a simple aldehyde that is released during the combustion of organic material (include tobacco and wood smoke). It has been proposed that biomass smoke and acrolein can trigger inflammatory responses via TLR and NFκB pathways. When cell receptors, like TLR4, come in contact with acrolein a signal cascade activates the signals in the cell which control apoptosis and the synthesis of pro-inflammatory cytokines, including the NFκB pathway (Valacchi et. al. 2005). This causes an inflammatory response in the immune system and can lead to chronic illnesses, like emphysema.

Natural remedies, like curcumin, are hypothesized to combat inflammation (Aggarwal et al. 2009) This is also significantly more cost effective than current

treatments for inflammatory diseases. Curcumin is a nutritional compound located within the rootstalk of the turmeric plant (Tsuda 2018). Its active ingredient is diferuloylmethane, a hydrophobic polyphenol with a characteristic yellow-orange color. This natural extract possesses numerous physiological properties, including anti-inflammatory properties (Epstein et al. 2010). Its mechanisms include the inhibition of NFκB and TLR4 dependent signaling pathways through the inhibition of IκBα kinase and the AKT signaling pathway (Aggarwal et al., 2005). It also signals the activation of a peroxisome proliferator-activated receptor-gamma pathway which can lead to the production of anti-inflammatory cytokines (Shimizu et al. 2019) (Figure 2).
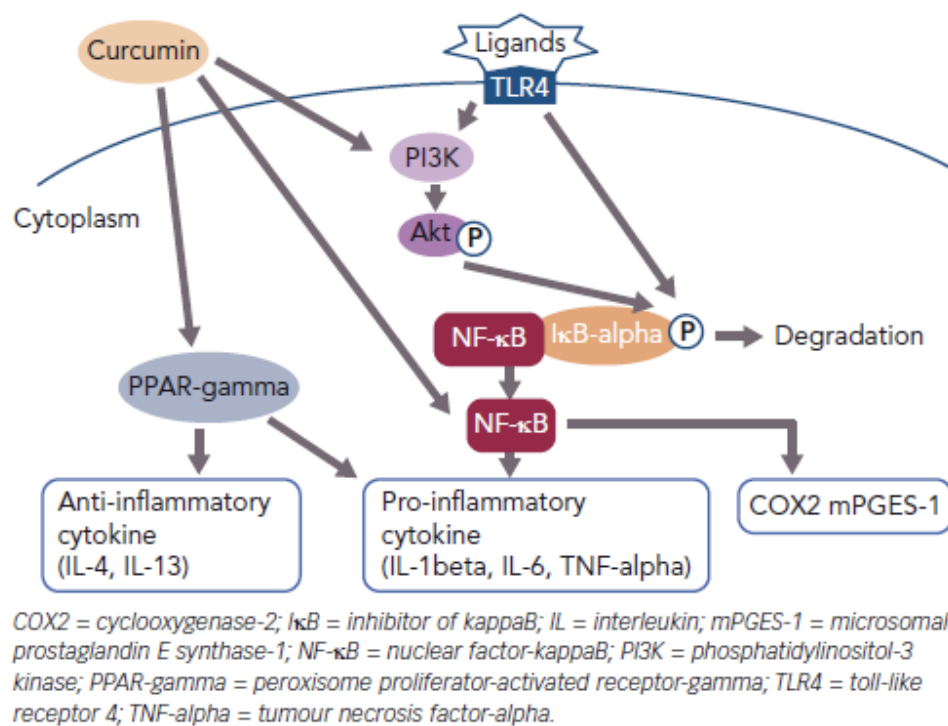

COX2 = cyclooxygenase-2; IκB = inhibitor of kappaB; IL = interleukin; mPGES-1 = microsomal prostaglandin E synthase-1; NF-κB = nuclear factor-kappaB; PI3K = phosphatidylinositol-3 kinase; PPAR-gamma = peroxisome proliferator-activated receptor-gamma; TLR4 = toll-like receptor 4; TNF-alpha = tumour necrosis factor-alpha.

Figure 2: Curcumin mechanism and anti-inflammatory effect. Curcumin inhibits the NFκB and TLR4 dependent signaling pathways. It also signals the activation of a peroxisome proliferator-activated receptor-gamma pathway which can lead to the production of anti-inflammatory cytokines (Figure from Shimizu et al. 2019).

Macrophages play a key role in the immune response in all tissues of the body. They survey the bloodstream and tissues for pathogens, and when specialized

receptors on their cell membranes bind with stimuli such as bacterial products, the inflammatory response within the macrophage is activated. This allows the macrophages greater phagocytic activity and causes it to secrete inflammatory mediators to recruit more macrophages or other immune cells, like T-cells. (Driscoll, et al. 1997). Some of these inflammatory mediators are tumor necrosis factor-α (TNFα), macrophage inflammatory protein 2 (MIP-2), and cyclooxygenase-2 (COX-2). TNFα is an inflammatory cytokine produced by macrophages during acute inflammation and is responsible for a diverse range of signaling events within cells, ultimately leading to necrosis or apoptosis (Idriss et al. 2000). MIP-2 is produced mainly by macrophages, monocytes and epithelial cells and is responsible for chemotaxis to the source of inflammation and activation of neutrophils (Driscoll 1994). COX-2 is an enzyme expressed in response to inflammatory and other physiologic stimuli and growth factors and is involved in the production of prostaglandins that mediate pain and support the inflammatory process (Simon et al. 1999).

The goal of this research was to investigate the ability of indoor air pollutants, like acrolein and wood smoke, to induce inflammatory responses and the ability of natural remedies, like curcumin, to combat these inflammatory effects. This research was done on macrophages and used cytokine secretion and gene expression to measure inflammatory responses. Genes which were measured include TNFα, MIP-2, and COX-2 as these are commonly expressed in inflammatory responses.

Materials and Methods

The mouse macrophage cell line (RAW 264.7) was cultured in RPMI media at 37°C and with constant 5% CO_2. Approximately 6×10^6 cells were harvested to perform

each experiment. Approximately 5×10^5 cells/well were divided into 12 well plates. When assessing both cytokine release and gene expression, cells were incubated for 24 hours.

When assessing the inflammatory response of RAW264.7 macrophages after exposure to wood smoke and acrolein, two wells were not exposed to any inflammatory stressors to act as a control for background inflammation. Two wells per experiment were exposed to 5µg/mL of zymosan to act as a positive control to compare inflammation induced by wood smoke and acrolein. Zymosan is a fungal cell wall component and a potent TLR agonist. In preliminary studies it was determined concentrations of woodsmoke up to 400 µg/mL were non-toxic to cells. Lactate dehydrogenase (LDH) Cytotoxicity Assays were used to measure the cytotoxicity of each concentration. In preliminary studies, up to 5µM of acrolein was used. Data from these studies indicated that this concentration proved to be highly toxic to the cells. It was therefore determined that concentrations of acrolein up to 2.5 µM was non-toxic to cells. Based on this data, a range of wood smoke concentrations (100-400 µg/mL) and a range of acrolein (0.3125-2.5 µM) were tested.

When assessing the anti-inflammatory effects of curcumin in cells treated with wood smoke and acrolein, one well was not exposed to any inflammatory stressors to act as a control. The number of wells was reduced in order to properly fit the number of samples needed into one 12 well plate. One well per experiment was exposed to 5µL/mL of zymosan to act as a positive control. Initially, concentrations of curcumin up to 20µM were used, however these proved to be toxic to cells (over 70% cell death). After preliminary studies, concentrations of curcumin up to 15 µM were used. It was

determined that concentrations of woodsmoke up to 400 μg/mL were non-toxic to the macrophage cells, even when cells were exposed to both wood smoke and curcumin. Preliminary studies showed concentrations of acrolein up to 2.5μM were toxic to cells when exposed to acrolein and curcumin (over 70% cell death). After this, concentrations of acrolein up to 1.25uM were used. Based on this data, a range of curcumin (2.5-15μM) were tested. A concentration of 400mg/mL wood smoke was used, and a concentration of 1.25uM acrolein were tested.

Two main methods of study were used to assess the inflammatory response in RAW 264.7 cells. The secretion of pro-inflammatory cytokines was assessed via enzyme-linked immunosorbent assays (ELISA). All ELISA kits were purchased from R&D Systems. To identify the levels of inflammatory cytokines released by macrophages, supernatants were collected from 24-hour cultured cells. TNF-α and MIP-2 cytokines were chosen because they are common cytokines released in an inflammatory response and therefore can be used to measure the total inflammatory response.

The expression of pro-inflammatory genes was assessed via reverse transcription-polymerase chain reaction (RT-PCR) and gel electrophoresis. Homogenates from 24-hour cultured cells were collected for RT-PCR and gel electrophoresis to measure the expression levels of pro-inflammatory genes. RNA was isolated using a Purelink RNA isolation kit (Invitrogen). Following purification, RNA concentration and purity of each sample was found using a UV-Vis spectrophotometer in preparation for RT-PCR. (Nanodrop 2000). cDNA was subsequently synthesized using M-MLV reverse transcriptase. TNF-α, MIP-2, and COX-2 proinflammatory genes

were assessed. All primers were tested to determine optimal concentrations for use during PCR. Gel electrophoresis was performed using agarose gels and ethidium bromide (EtBr), a fluorescence dye that is capable of DNA intercalation.

Results

The LDH assay was used to assess toxicity of woodsmoke and acrolein (Figure 3). It was found that neither compound induced more than 50% toxicity in the macrophage cells. Woodsmoke and acrolein do induce TNF-α cytokine secretion, however there was no significant dose-dependent effect. (Figure 4). There was no significant effect of woodsmoke and acrolein on MIP-2 cytokine levels at any dose, indicating that woodsmoke and acrolein do not induce the MIP-2 pathway (Figure 5). When looking at gene expression via gel electrophoresis, GAPDH was used as a control gene (Figure 6). No substantial dose-dependent TNF-α expression was observed in gel electrophoresis of samples treated with woodsmoke and acrolein, expression remained constant (Figure 7). Substantial dose-dependent MIP-2 expression was observed in gel electrophoresis of samples treated with woodsmoke and acrolein (Figure 8). As the concentration of woodsmoke and acrolein increased, the expression of MIP-2 substantially increased compared to the negative control. COX-2 showed slight dose-dependent expression in gel electrophoresis (Figure 9). At higher concentrations of woodsmoke and acrolein, there was slightly more expression compared to the negative control.

The LDH assay was also used to assess the toxicity of woodsmoke, acrolein, and curcumin (Figure 10). There was no significant increase or decrease of TNF-α cytokine secretion, indicating that curcumin did not induce or block the TNF-α pathway

(Figure 11). There was no significant effect of woodsmoke and acrolein on MIP-2 cytokine levels at any dose, indicating that curcumin did not induce or block the MIP-2 pathway (Figure 12). GAPDH was again used as a control gene (Figure 13). There was no significant increase or decrease of TNF-α gene expression in gel electrophoresis of samples treated with curcumin, supporting the idea that curcumin did not induce or block the TNF-α pathway (Figure 14). Curcumin had a dose-dependently decreasing effect on the gene expression of MIP-2 in samples treated with woodsmoke and acrolein. (Figure 15). COX-2 had a slight dose-dependent decrease in expression as the concentration of curcumin increased in the woodsmoke and acrolein treated samples (Figure 16).
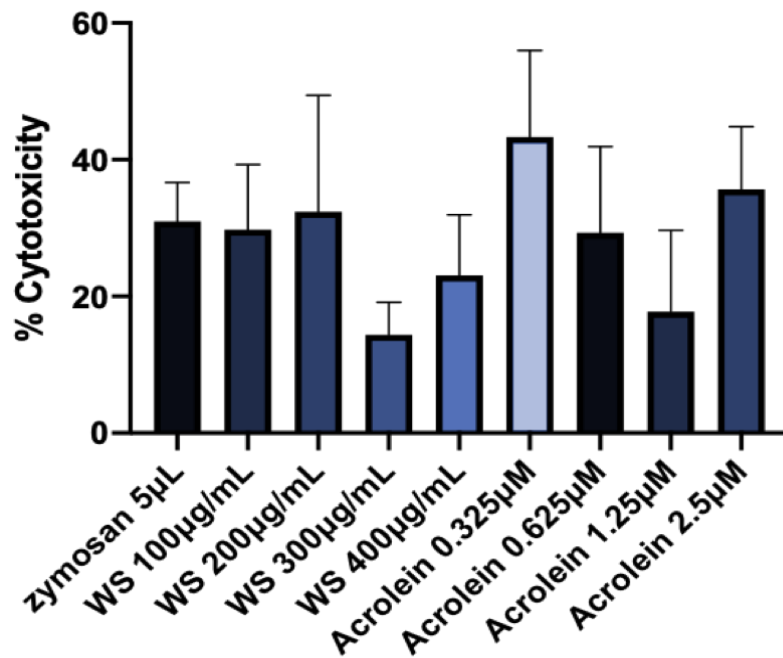

Figure 3: LDH release in woodsmoke and acrolein challenged RAW264.7 macrophages; 5×10^5 cells/mL media were cultured in a 12 well dish. Cells were incubated with increasing concentrations of solubilized woodsmoke or acrolein for 24 hours. Supernatants were collected and used for the LDH assay. No significant cell death was observed compared to unexposed cells.
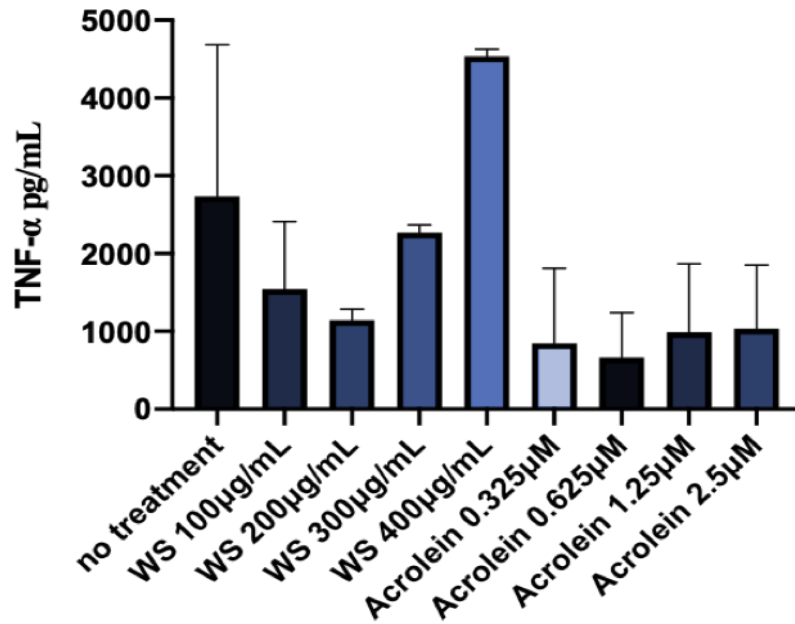
Figure 4: TNF-alpha secretion woodsmoke and acrolein challenged RAW264.7 macrophages. 5 X 10^5 cells/mL media were cultured in a 12 well dish. Cells were incubated with increasing concentrations of solubilized woodsmoke or acrolein for 24 hours. Supernatants were collected and used for an ELISA. No significant changes in TNA-alpha secretion were observed.
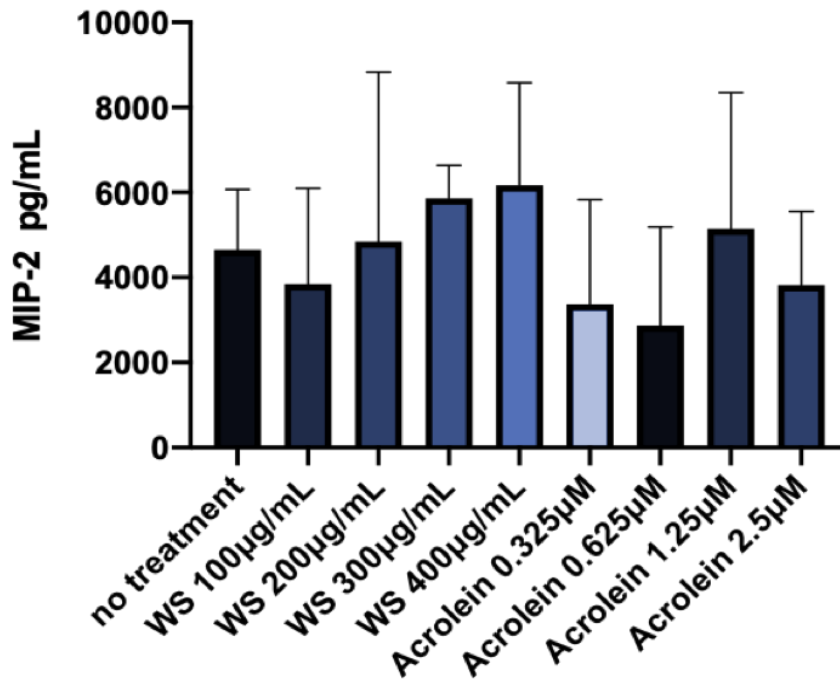

Figure 5: MIP-2 secretion woodsmoke and acrolein challenged RAW264.7 macrophages. 5 X 10^5 cells/mL media were cultured in a 12 well dish. Cells were incubated with increasing concentrations of solubilized woodsmoke or acrolein for 24 hours. Supernatants were collected and used for an ELISA. No significant changes in MIP-2 secretion were observed.
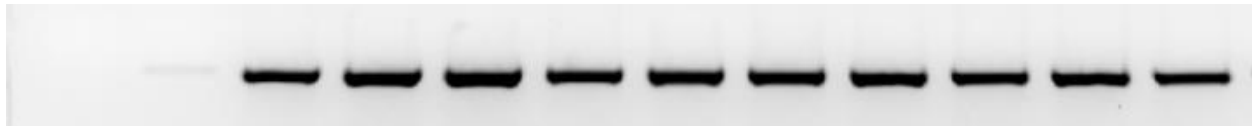
Figure 6: GAPDH gel electrophoresis of RAW 264.7 macrophage cells (500,000cells/mL) treated from left to right as follows:(1) None (2) None (3) zymosan 5µL/mL (4) zymosan 5µL/mL(5) WS 100µg/mL (6) WS 200µg/mL (7) WS 300µg/mL (8) WS 400µg/mL (9) acrolein 0.325µM (10) acrolein 0.625µM (11) acrolein 1.25µM (12) acrolein 2.5µM. No significant treatments were observed.

Figure 7: TNF-α gel electrophoresis of RAW 264.7 macrophage cells (500,000cells/mL) treated from left to right as follows:(1) None (2) None (3) zymosan 5µL/mL (4) zymosan 5µL/mL(5) WS 100µg/mL (6) WS 200µg/mL (7) WS 300µg/mL (8) WS 400µg/mL (9) acrolein 0.325µM (10) acrolein 0.625µM (11) acrolein 1.25µM (12) acrolein 2.5µM. No significant treatments were observed.
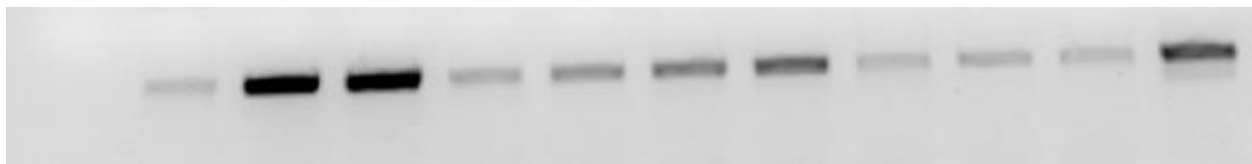

Figure 8: MIP-2 gel electrophoresis of RAW 264.7 macrophage cells (500,000cells/mL) treated from left to right as follows:(1) None (2) None (3) zymosan 5µL/mL (4) zymosan 5µL/mL(5) WS 100µg/mL (6) WS 200µg/mL (7) WS 300µg/mL (8) WS 400µg/mL (9) acrolein 0.325µM (10) acrolein 0.625µM (11) acrolein 1.25µM (12) acrolein 2.5µM. MIP-2 gene expression increased as the concentration of woodsmoke and acrolein increased.
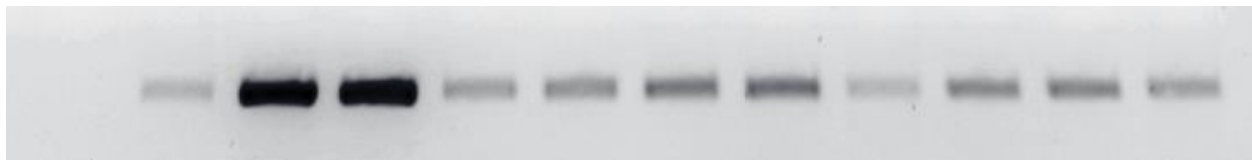

Figure 9: COX-2 gel electrophoresis of RAW 264.7 macrophage cells (500,000cells/mL) treated from left to right as follows:(1) None (2) None (3) zymosan 5µL/mL (4) zymosan 5µL/mL(5) WS 100µg/mL (6) WS 200µg/mL (7) WS 300µg/mL (8) WS 400µg/mL (9) acrolein 0.325µM (10) acrolein 0.625µM (11) acrolein 1.25µM (12) acrolein 2.5µM. COX-2 gene expression slightly increased as the concentration of woodsmoke and acrolein increased.
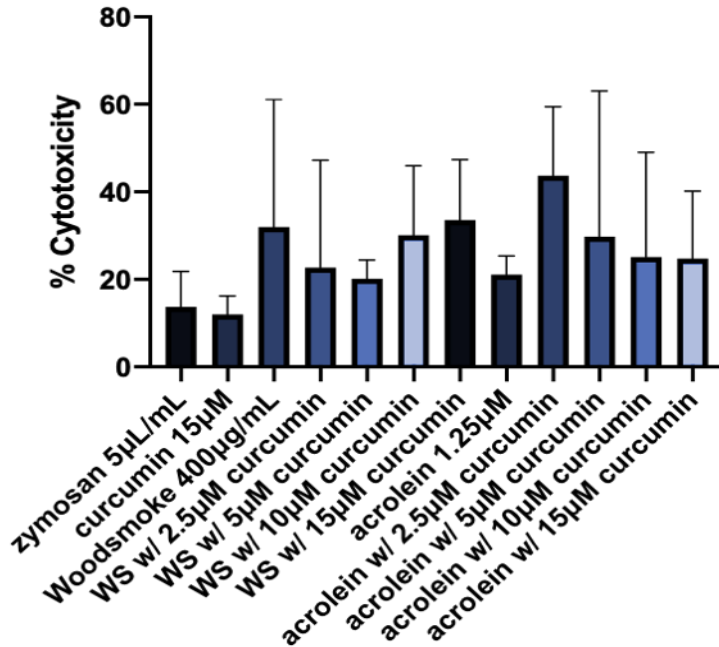
Figure 10: LDH release in woodsmoke and acrolein challenged RAW264.7 macrophages, following pretreatment with curcumin; 5 X 10^5 cells/mL media were cultured in a 12 well dish. Cells were incubated with solubilized woodsmoke or acrolein and increasing concentrations of curcumin for 24 hours. Supernatants were collected and used for the LDH assay. No significant cell death was observed, compared to unexposed cells.
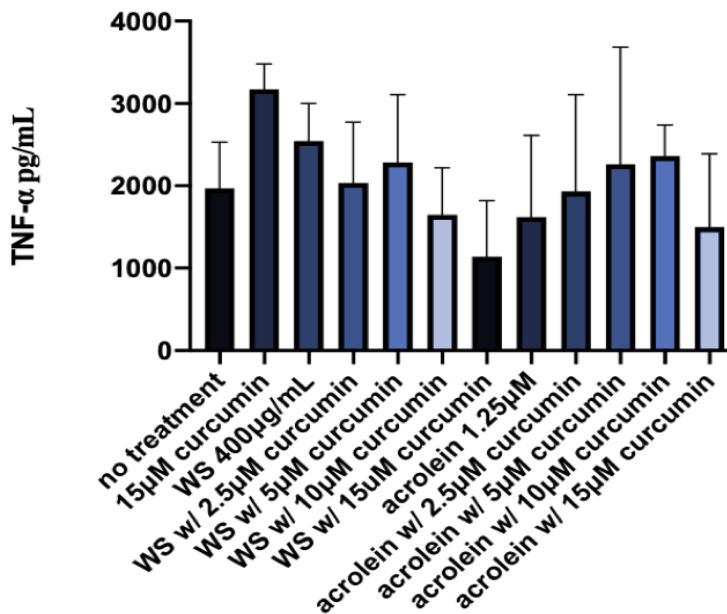

Figure 11: TNF-alpha secretion woodsmoke and acrolein challenged RAW264.7 macrophages following pretreatment with curcumin. 5 X 10^5 cells/mL media were cultured in a 12 well dish. Cells were incubated with increasing concentrations of solubilized woodsmoke or acrolein and increasing concentrations of curcumin for 24 hours. Supernatants were collected and used for an ELISA. No significant changes in TNA-alpha secretion were observed.
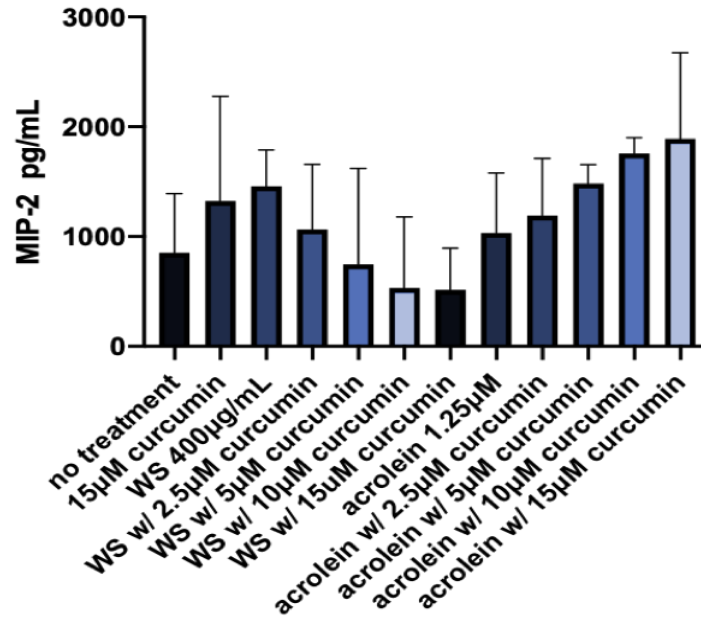
Figure 12: MIP-2 secretion woodsmoke and acrolein challenged RAW264.7 macrophages following pretreatment with curcumin. 5 X 10^5 cells/mL media were cultured in a 12 well dish. Cells were incubated with increasing concentrations of solubilized woodsmoke or acrolein and increasing concentrations of curcumin for 24 hours. Supernatants were collected and used for an ELISA. No significant changes in MIP-2 secretion were observed.

Figure 13: GAPDH gel electrophoresis of RAW 264.7 macrophage cells (500,000cells/mL) treated from left to right as follows:(1) None (2) zymosan 5µL/mL (3) curcumin 15µM (4) WS 400µg/mL (5) WS 400µg/mL +2.5µM curcumin (6) WS 400µg/mL +5µM curcumin (7) WS 400µg/mL + 10µM curcumin (8) WS 400µg/mL +15µM curcumin (9) acrolein 1.25µM (10) acrolein 1.25µM + 2.5µM curcumin (11) acrolein 1.25µM + 5µM curcumin (12) acrolein 1.25µM + 10µM curcumin (13) acrolein 1.25µM + 15µM curcumin. No significant treatments were observed.

Figure 14: TNF-α gel electrophoresis of RAW 264.7 macrophage cells (500,000cells/mL) treated from left to right as follows: (1) None (2) zymosan 5µL/mL (3) curcumin 15µM (4) WS 400µg/mL (5) WS 400µg/mL +2.5µM curcumin (6) WS 400µg/mL +5µM curcumin (7) WS 400µg/mL + 10µM curcumin (8) WS 400µg/mL +15µM curcumin (9) acrolein 1.25µM (10) acrolein 1.25µM + 2.5µM curcumin (11) acrolein 1.25µM + 5µM curcumin (12) acrolein 1.25µM + 10µM curcumin (13) acrolein 1.25µM + 15µM curcumin. No significant treatments were observed.
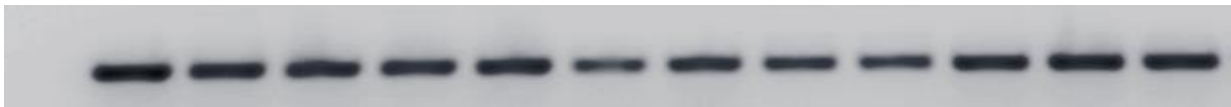
Figure 15: MIP-2 gel electrophoresis of RAW 264.7 macrophage cells (500,000cells/mL) treated from left to right as follows: (1) None (2) zymosan 5μL/mL (3) curcumin 15μM (4) WS 400μg/mL (5) WS 400μg/mL +2.5μM curcumin (6) WS 400μg/mL +5μM curcumin (7) WS 400μg/mL + 10μM curcumin (8) WS 400μg/mL +15μM curcumin (9) acrolein 1.25μM (10) acrolein 1.25μM + 2.5μM curcumin (11) acrolein 1.25μM + 5μM curcumin (12) acrolein 1.25μM + 10μM curcumin (13) acrolein 1.25μM + 15μM curcumin. Curcumin decreased gene expression of MIP-2 as concentration increased in both woodsmoke and acrolein samples.

Figure 16: COX-2 gel electrophoresis of RAW 264.7 macrophage cells (500,000cells/mL) treated from left to right as follows:(1) None (2) zymosan 5μL/mL (3) curcumin 15μM (4) WS 400μg/mL (5) WS 400μg/mL +2.5μM curcumin (6) WS 400μg/mL +5μM curcumin (7) WS 400μg/mL + 10μM curcumin (8) WS 400μg/mL +15μM curcumin (9) acrolein 1.25μM (10) acrolein 1.25μM + 2.5μM curcumin (11) acrolein 1.25μM + 5μM curcumin (12) acrolein 1.25μM + 10μM curcumin (13) acrolein 1.25μM + 15μM curcumin. Curcumin decreased gene expression of COX-2 as concentration increased in both woodsmoke and acrolein samples.

Discussion and Conclusions

Overall, we found that woodsmoke and acrolein induce an inflammatory response in murine macrophage cells compared to the unstimulated controls. Additionally, our data suggest that curcumin may mitigate some of the pro-inflammatory effects of woodsmoke and acrolein. Further studies are needed to confirm these results.

When assessing the inflammatory effects of woodsmoke and acrolein, we found that neither woodsmoke or acrolein significantly affected TNF-α cytokine secretion or gene expression. There is a slight increase of MIP-2 cytokine expression as concentrations of woodsmoke and acrolein, however there was no significant trend. However, our PCR data indicates expression of MIP-2 increased dose-dependently This indicates that woodsmoke and acrolein do induce the MIP-2 pathway and therefore cause expression of this inflammatory gene. Woodsmoke and acrolein had a slight dose-dependent effect on COX-2 gene expression as well, indicating they do induce

expression of the COX-2 enzyme. This supports the hypothesis that woodsmoke and acrolein act on NFκB and TLR-dependent signaling pathways (Schuller et al. 2020). This signal cascade is causing oxidative stress in the cell, which causes signaling of pro-inflammatory genes like MIP-2 and COX-2 (Silva et al. 2015; Valacchi et. al. 2005).

When assessing the anti-inflammatory effects of curcumin, it was determined that curcumin may be an effective, natural alternative anti-inflammatory compound, however more studies are needed to confirm these results. Curcumin had no effect on TNF-α cytokine secretion or gene expression. Curcumin did seem to dose-dependently decrease MIP-2 gene expression as the concentration increased. There also seems to be a slight decrease in MIP-2 cytokine secretion in samples treated with woodsmoke and increasing concentrations of curcumin, however this research needs to be replicated in order to confirm these results as there was no significant difference. The expression of COX-2 was also dose-dependently affected by curcumin. This supports the hypothesis that curcumin is inhibiting the of NFκB and TLR4 dependent signaling pathways (Shimizu et al. 2019). The inhibition of these pathways would lead to the inhibition of MIP-2 and COX-2 gene expression. The dose-dependent decrease of MIP-2 and COX-2 indicates that increasing concentrations of curcumin have a greater ability to block the of NFκB and TLR4 dependent signaling pathways and therefore inhibit inflammation. Current research shows that curcumins ability to inhibit these pathways could help decrease inflammation in many chronic diseases, including Crohn's disease, ulcerative colitis, and many forms of cancer (Epstein et al. 2010). Gaining a better understanding of the mechanisms of curcumin could lead to finding more of its health benefits.

Unfortunately, due to the COVID-19 crisis, we were unable to perform further replicates of these experiments. Specifically, curcumin's anti-inflammatory and anti-oxidant properties need to be assessed further. Current research hypothesizes that woodsmoke does induce cytokine secretion and oxidative stress (Kocbach et al. 2008; Migliaccio et al. 2010; Rao et al. 2018). Going forward, it should be assessed whether woodsmoke does dose-dependently induce cytokine secretion and oxidative stress. It is also important to assess other inflammatory mediators in order to discover the full effect of woodsmoke and acrolein in the cell, as it is hypothesized woodsmoke and acrolein do signal other inflammatory mediators (Kocbach et al. 2008; Migliaccio et al. 2010) It should also be assessed whether curcumin can properly combat these inflammatory effects. This could also be assessed by looking at the levels of anti-inflammatory cytokines linked to the peroxisome proliferator-activated receptor-gamma pathway, like IL-4 and IL-13 (Shimizu et al. 2019). Current research also suggests curcumin inhibits IκBα kinase (Yuan et al. 2018). Gene expression of IκBα could be assessed to see whether this is the part of the pathway curcumin inhibits. Long term inflammation from chronic exposure to biomass smoke can lead to multiple pulmonary and cardiovascular diseases (Driscoll 1994; Rao et al. 2018). Therefore, it is crucial to continue identifying other natural remedies as inflammation caused by biomass smoke is an ongoing and serious issue in the world today.

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