APIACEOUS VEGETABLES MITIGATE ACROLEIN-INDUCED LUNG INJURIES IN C57BL/6J MALE MICE

by

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ABSTRACT

Acrolein (Acr) is a ubiquitous, highly reactive aldehyde, abundant in polluted air and cigarette smoke. Acr causes oxidative stress and a cascade of catalytic events and has, thereby, been associated with increased risk of pulmonary disease and other inflammatory diseases. Apiaceous vegetables (carrot family vegetables) are noteworthy for dietary prevention of inflammation and various cancers. However, whether API could prevent Acr-induced pulmonary toxicity has not yet been explored. In this study, we investigated the effects of API on Acr-induced pulmonary damages in male C57BL/6J mice. A total of 20 mice were assigned to either negative control (NEG group; AIN-93G diet only), positive control (POS group; AIN-93G+Acr) or apiaceous vegetable intervention group (API group; AIN-93G+21% API+Acr). After one week of dietary intervention, the POS and API mice were exposed to Acr (10 μmol/kg body weight daily) for five days. During the treatment period, assigned diets remained the same. Prominent indicators of toxicity within the lungs of POS mice were found, including mucus accumulation, macrophage infiltration, and hemorrhage, which appeared to be ameliorated in API mice. Serum and lung inflammation markers, such as tumor necrosis factor alpha (TNF-α) and interleukin 1 beta (IL-1β), were also increased by Acr while reduced by API. Furthermore, poly (ADP-ribose) polymerase (PARP; a necrosis marker) was increased in the POS group while decreased in the lungs of API mice. Similarly, DNA repair genes [e.g., breast cancer type 1 susceptibility protein (*Brca1*) and proliferating cell nuclear antigen (*Pcna*)] within the

lungs were decreased in POS compared to NEG, but the genes were normalized in API mice. In the liver, API upregulated expression of glutathione *S*-transferases (GST), which enhanced the metabolism of Acr into water-soluble 3-hydroxypropyl mercapturic acid for excretion, which is consistent with observed reductions in serum Acr-protein adducts. In conclusion, apiaceous vegetables may provide protection against Acr-induced pulmonary damages via downregulation of inflammatory pathways and/or enhancement of the detoxification of Acr.

Chapter 1

INTRODUCTION

1.1 Acrolein

Acrolein (Acr) is a clear yellow or colorless flammable liquid at room temperature with a burnt, sweet, acrid odor. This aldehyde is produced from the incomplete combustion of organic matter, and is, thereby, ubiquitous in the environment, including in cigarette smoke.[1,2] The general population is primarily exposed to Acr by inhalation, and the most significant target of Acr toxicity is the first site of contact, which is commonly the respiratory tract.[1] Acr is highly water soluble compared to other common air pollutants hence is deposited mainly in the aqueous lining fluid of mucus membranes.[3] When Acr is inhaled by oral breathing (e.g., smoking cigarettes), lower respiratory tract retention is near complete at greater than 98%.[4] Although most Acr exposure is from inhalation of cigarette smoke or car exhaust, Acr may also be released into the atmosphere as fumes from burning of industrial incinerators or fireplaces or cooking foods and oils at high temperatures.[5,6] Furthermore, the consumption of specific foods (e.g., fish, cocoa beans, some cheeses, and fried foods) and endogenous production may also contribute to Acr accumulation; however, estimating the level of exposure from these sources is virtually impossible due to analytical difficulties and the lack of reliable content measurements.[1,7]

1.2 Acrolein toxicity

Many pathological effects of smoke-related lung diseases can be replicated by exposure to only Acr in animal models.[3] Inhalation of low levels of Acr causes nasal irritation, pulmonary resistance, bronchial hyperreactivity, and excessive mucus

production; therefore, even low concentrations produce histological features indicative of acute lung injury.[3] High levels of Acr elevate blood pressure and cause cessation of the heart.[3] Previously, rats, guinea pigs, dogs, and monkeys were continuously exposed to Acr 24 hours per day for 90 days, from which pulmonary inflammation, emphysema, and pulmonary hemorrhage were demonstrated (with varying degrees) across all species.[8] Similarly, squamous metaplasia, basal cell hyperplasia, hypersecretion, and necrosis were present in various parts of the respiratory tracts.[8] In addition to these studies, research experiments have demonstrated that Acr induces lung edema in dogs,[9] sheep,[10] and mice,[11] and it promotes lipopolysaccharide-induced acute lung injury in mice.[3,11]

Much of Acr toxicity may be explained by its high reactivity, which damages cellular structures and intracellular macromolecules (e.g., DNA) and results in production of reactive oxygen species (ROS).[12,13] In turn, ROS induce a condition referred to as oxidative stress that is characterized by increased lipid peroxidation, altered epithelial permeability, and depleted antioxidant glutathione (GSH), subsequently leading to inflammation and tissue injury.[12,13,14] Due to this cascade of oxidative stress and inflammation, chronic Acr exposure increases risk of inflammatory diseases[15] and is implicated with cancers.[2] In pursuit of stabilization, Acr reacts with and adheres to a variety of proteins in the cells and blood. For instance, Acr forms adducts with histone proteins and inhibits acetylation of cytosolic histones, thus compromising nuclear import of histones and chromatin assembly and increasing chromatin accessibility.[16] Moreover, transcriptional responses to Acr exposure correlate with changes in nucleosome occupancy at several genomic loci, meaning Acr interacts with the genome and influences genomic function.[16] Further, Acr binds to p53 protein and prevents p53-DNA binding, thus inhibiting tumor suppressor activity.[17] Coinciding with this evidence, Acr-induced mutations in p53 have been identified in the lung tumors of human smokers, suggesting Acr may contribute to the development of smoke-induced lung cancer.[2]

1.3 Acrolein metabolism

Hepatic biotransformation enzymes (BTE), such as glutathione-S-transferases (GST), cytochrome P450 (CYP), and N-acetyltransferases, mediate systemic elimination of various xenobiotics.[18] Acr is also metabolized by hepatic BTE. For instance, when exposed to Acr, GST conjugates Acr to form water-soluble 3-hydroxypropyl mercapturic acid (3-HPMA) for efficient urinary excretion.[19] 3-HPMA is therefore used as a biomarker of Acr exposure. Of note, an inverse relationship is present between levels of urinary 3-HPMA and Acr-DNA adducts (i.e., α-OH-Acr-dGuo) in the lungs of smokers.[19] If Acr is not conjugated to 3-HPMA and excreted from the system, cellular antioxidative defenses are disrupted by rapid depletion of GSH[20,21] and by decreased antioxidant enzyme activities (e.g., superoxide dismutase and glutathione peroxidase).[22] To restore GSH and decrease oxidative stress, nuclear factor erythroid 2-related factor 2 (Nrf2) signaling orchestrates the expression of key antioxidant enzymes and BTE, including GST.[23] Studies suggest Acr mediates the activation of Nrf2, thereby upregulating transcription of GST, which is essential in Acr detoxification.[24,23]

1.4 Whole-food approach: protective potential of apiaceous vegetables

Although smoking cessation is ideal for cigarette smoke-related disease prevention, smoking cessation is predominantly unsuccessful with 95-97% failure rates without professional assistance.[25] On the other hand, diet is a modifiable risk factor for various chronic diseases; current research suggests particular foods and nutrients (e.g., fruits, vegetables, and zinc) may offer protective effects against chronic diseases (e.g., lung cancer).[26] However, long-term use of high dose dietary supplements of retinol, β-carotene, B vitamins, or vitamin E has been linked to increased lung cancer risk in current and former smokers,[27] whereas consumption of these nutrients from whole food sources has not been associated with such risks. Therefore, research utilizing a ‘whole-

food approach' should be more emphasized to accurately evaluate dietary effects against diseases, and, in our case, Acr-induced toxicities.[26,27]

Apiaceous vegetables (API) are one noteworthy example for potential dietary prevention of inflammation and various cancers, including prostate and colorectal cancer.[28–32] *Apiaceae* is the 16th-largest family of flowering plants, with more than 3,600 species in approximately 455 genera that are widely distributed all over the world.[33] Therefore, a variety of vegetables and herbs are classified as API, including carrots (*Daucus carota* L. subsp. *sativus),* celery (*Apium graveolens* L. var. *rapaceum*), fennel (*Foeniculum vulgare* subsp. *vulgare* var. *azoricum*), parsley (*Petroselinum crispum* subsp. *tuberosum*), and parsnip (*Pastinaca sativa* L. subsp. *sativa* var. *sativa*).[34,35] Furthermore, angelica (*Angelica archangelica*), anise (*Pimpinella anisum*), caraway (*Carum carvi*), coriander (cilantro; *Coriandrum sativum*), cumin (*Cuminum cyminum*), and dill (*Anethum graveolens*) are all classified as API.[36] Although these plants vary significantly, most *Apiaceae* are annual and perennial herbs with well-developed secretory systems that partake in deposition of essential oils with distinct odors and flavors.[35] Due to their distinct flavors, many plants from this family are consumed as vegetables, herbs, or spices.[35] The most used parts of API are fruits (21 species) followed by leaves (17 species), aerial parts (17 species), gum (13 species), root (12 species), stem (7 species), flowers (4 species), whole plant (4 species), seed (3 species) and rhizome (1 species).[37] It has been established that *Apiaceae* are rich in various vitamins and phytochemicals.[38,39] Therefore, recent research emphasizes the potential of these plants for developing functional foods.[38,40]

Using animal models, we have previously demonstrated that an achievable dose of API increased hepatic BTE activity in a dose-dependent manner and reduced carcinogen induced DNA adducts.[41] In line with these results, lipophilic fractions of carrot increased antioxidant enzyme activities,[42] and lipophilic polyacetylenes from API increased both antioxidant expression and activities.[43] In combination, these results

strongly suggest that API may enhance systemic clearance of Acr. Despite the proposed evidence, little is known regarding the specific effects of API against Acr-induced lung damages. Therefore, the aim of this study was to elucidate potential preventive mechanisms of API against Acr-induced pulmonary injury and inflammation and to examine API-induced modulation of the liver detoxification system involved in the metabolism and excretion of Acr.

Chapter 2

MANUSCRIPT

2.1 Introduction

Acrolein (Acr; 2-propenal) ubiquitously occurs from incomplete combustion of organic matter,[1] and it is one of the most abundant, reactive, and mutagenic aldehydes present in cigarette smoke.[2] Other sources of Acr include polluted air and fumes from high heat cooking processes, such as frying.[1,44] Due to its reactivity, the United States Environmental Protection Agency lists Acr as a high priority toxin.[45] A primary target of Acr is the tissue at site of contact, for instance, the respiratory tract in the case of inhalation.[4] As a reactive aldehyde, Acr forms reactive oxygen species (ROS) that result in a condition referred to as oxidative stress;[12,13] oxidative stress triggers a cascade of critical consequences, such as epithelial permeability, lipid peroxidation, and cellular depletion of the antioxidant glutathione (GSH) in alveoli, subsequently leading to inflammation and tissue injury.[14] As a result, evidence suggests that Acr exposure increases risk of cancers,[2] as well as inflammatory diseases.[15]

As a defensive mechanism, hepatic biotransformation enzymes (BTE) play a major role in systemic elimination of toxins, including Acr.[18] Acr is conjugated by the hepatic BTE, glutathione-S-transferases (GST), to form water soluble metabolites such as 3-hydroxypropyl mercapturic acid (3-HPMA), for which the reduced form of GSH is essential.[19] If Acr is not properly detoxified, intracellular GSH is rapidly depleted,[20,21] and antioxidative enzyme activities are disrupted (e.g., superoxide dismutase and GSH peroxidase)[22]. In Acr detoxification, Kelch-like ECH-associated protein 1 (Keap1)-nuclear factor erythroid 2-related factor 2 (Nrf2) signaling is critical, as it orchestrates expression of a range of GST and enzymes related to GSH recycling.[23] Therefore, effective protection against Acr toxicity may be achieved by quenching Acr-induced

ROS and/or by transactivating the Keap1-Nrf2 pathway, thereby supporting Acr detoxification.

Smokers are regularly exposed to high levels Acr; however, epidemiological studies have shown that vegetable consumption correlates with reduced lung cancer risk in this population.[46] Similarly, in humans, consumption of fruits and vegetables is associated with reduced serum levels of C-reactive protein, tumor necrosis factor alpha (TNF-α), and interleukin 6 (IL-6), all of which are closely related to chronic inflammation.[47] In a recent randomized controlled trial, apiaceous and cruciferous vegetables reduced circulating IL-6 in healthy young adults, suggesting anti-inflammatory potential of apiaceous vegetables.[48] In addition, in a rat model, we have demonstrated that achievable servings of apiaceous vegetables increase hepatic BTE activity in a dose-dependent manner and reduce carcinogen induced DNA adducts in the colon.[41] In line with these results, it was also reported that lipophilic fractions of carrot increased hepatic antioxidative enzyme activity (e.g., superoxide dismutase, catalase, and GST),[42] and lipophilic polyacetylenes (which are present in apiaceous vegetables) potently increased both GST expression and activities through induction of the Nrf2 pathway.[43] Collectively, the evidence in the literature suggests that apiaceous vegetables may be protective against Acr toxicity by alleviating inflammation and/or facilitating systemic clearance of Acr via transactivation of the Nrf2 pathway.

Despite the proposed evidence, little is known regarding the specific effects of apiaceous vegetables against Acr-induced lung damages. Therefore, the aim of this study was to elucidate potential preventive mechanisms of apiaceous vegetables against Acr-induced pulmonary injury. Considering that 1) in vitro models cannot address the systemic interplay of the lungs and the liver, and 2) the typical route of exposure of Acr in smokers is inhalation, we exposed Acr to mice intranasally. For further translational purposes, apiaceous vegetables (i.e., 21% wet wt:wt) were supplemented into mice diets

at an equivalent of approximately 1 1/3 cup vegetable per day for humans; this is certainly an achievable level in daily life.

2.2 Materials and Methods

2.2.1 Animal study design and diet preparation

For the animal intervention study, a total of 20 male C57BL/6J mice were assigned to either a negative control [NEG group; American Institute of Nutrition (AIN)-93G], positive control (POS group; AIN-93G+Acr) or apiaceous vegetable intervention group (API group; 21% apiaceous vegetables supplemented in AIN-93G+Acr). The AIN-93G diet was used as a background diet for all groups.[49] The API diet was balanced for calories by matching macronutrient and fiber contents, as justified elsewhere.[50] Detailed nutrient composition is provided in Table 1. As stated, supplementation of API was equivalent to approximately 1 1/3 cup of vegetable per day in humans.

For the API diet, organic fresh celery and parsnips were purchased from a local market and supplemented (21%, wet wt:wt; 10.5% per each). In brief, the fresh vegetables were first washed, trimmed, and manually chopped. The vegetables were then ground by a food processor (Model DFP-11; Cuisinart, Stamford, CT) and mixed into the background diet (i.e., AIN-93G) for 15 min with a mechanical mixer (Model A-200-D; Hobart, Troy, OH). This duration of diet mixing was previously determined to avoid heating and minimize risk of oxidation, while achieving a homogenous mixture of powdered diet.[41] The API diet was then visually inspected for homogeneity. Diets were divided into plastic bags designated for each day of the feeding period, stored at -80°C, and thawed before feeding.

After one week of dietary intervention, the POS and API group mice were exposed to Acr (10 μmol/kg body weight daily) for five days. During the treatment period, assigned diets remained the same. For Acr treatment, following isofluorane

exposure for 5-10 seconds until unconscious, Acr was administered intranasally, as described elsewhere.[51] Acr was dissolved in saline, and administration volume did not exceed 15 μL. The NEG group mice were exposed to the same isofluorane condition followed by Acr-free saline solution. After, all mice were euthanized by exsanguination, and harvested tissues were stored in RNALater (Thermo Fisher Scientific, Waltham, MA) at -80°C or fixed in 10% neutral buffered formalin solution before further analyses.

2.2.2 Assessment of pulmonary damages

Harvested lung tissues were cryopreserved and embedded in an optimal cutting temperature compound with isopentane cooled in liquid nitrogen prior to sectioning at 10 μm with a cryostat set to -25°C and transferring to slides. Subsequently, slides were stored at -80°C until stained with hematoxylin and eosin Y or trichrome stain. Inflammatory lesions, mucus accumulation, macrophage infiltration, hemorrhaging, alveolar size, collagen degradation, and elastin fibrosis were assessed as indicators of lung tissue damage by a double-blinded veterinary pathologist.

2.2.3 Assessment of systemic inflammation

Proteome Profiler Mouse Cytokine Array Panel A (ARY006; R&D Systems, Minneapolis, MN) was used to measure serum inflammatory cytokine profiles; procedures were performed according to the manufacturer's instructions. The intensities of the dot blots were quantified using ImageJ software (National Institute of Health, Bethesda, MD).

2.2.4 Measurement of serum Acr-protein adduct

To monitor systemic Acr levels, serum samples were pooled within groups, serially diluted in phosphate buffered saline, and applied onto nitrocellulose membranes. After blocking the membranes with 5% bovine serum albumin in Tris-buffered saline (0.5 M Tris base, 9% NaCl, and 2% Tween 20; pH 7.8), the membranes were incubated

with an anti-Acr primary antibody (Invitrogen, Carlsbad, CA). Subsequently, the membranes were washed and incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. After washing, membranes were visualized using a chemiluminometer (FluorChem R; Bio-Techne, San Jose, CA), and the intensity was quantified using ImageJ software (National Institutes of Health). To account for serum matrices effects, dot blots were analyzed as relative quantities based on signal intensity slopes as concentration declined. A greater absolute slope (more significant decline in signal) signified a lower concentration.

2.2.5 GSH/GSSG ratio detection assay

A GSH/oxidized glutathione disulfide (GSSG) Ratio Detection Assay Kit (ab138881; Abcam, Cambridge, UK) was used to assess hepatic redox status, as indicated by the ratio of reduced GSH to GSSG. Liver tissues were homogenized in NP-40 lysis buffer (Pierce™ IP Lysis Buffer; Thermo Fisher Scientific) for extraction. The liver lysate was deproteinized using the Deproteinizing Sample Preparation Kit-TCA (ab204708; Abcam) prior to the assay, and deproteination was validated via BCA protein assay. Resulting deproteinized samples were diluted with equal volume of Assay Buffer. All procedures followed the Product Protocols associated with their respective kit. Fluorescence was monitored at Ex/Em = 490/520 nm using a microplate reader (SpectraMax i3x; Molecular Devices, Sunnyvale, CA) following 15, 30, and 45 min of incubation while protected from light at room temperature.

2.2.6 Measurement of protein expression

Expression levels of proteins relating to lung damage were measured by conventional western blot (WB) analysis. Total tissue protein was prepared at 1 mg/mL in Laemmli buffer. β-actin and cyclophilin B were used as housekeeping proteins in WB analyses, and final results were calculated as a ratio of protein/housekeeping protein to

factor in intraassay variation. The membranes were visualized using a chemiluminometer (FluorChem R; ProteinSimple Bio-Techne), and the intensity was quantified using ImageJ software (National Institutes of Health).

2.2.7 Measurement of gene expression

To measure changes in mRNA, tissue samples were homogenized and lysed in QIAzol Lysis Reagent (Qiagen, Hilden, Germany). Total RNA was isolated using the RNeasy Plus Universal Mini Kit (Qiagen), and quality of RNA was assessed using conventional A260/280 ratio and A260/230 ratios (SpectraMax i3x; Molecular Devices). Then 2 μg of total RNA were reverse transcribed using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA) following the manufacturer's protocol. The cDNA samples were stored at -80°C until analyzed. Expression of mRNA was measured by quantitative reverse transcription polymerase chain reaction (RT-qPCR) analysis using the StepOnePlus system (Applied Biosystems) in a reaction mixture containing TaqMan Gene Expression Mastermix, primers tagged with TaqMan probe, and cDNA. Amplification was conducted under the instructed conditions: one cycle at 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of denaturation (95°C for 15 s) and annealing (60°C for 1 min). Genes of interest were normalized with reference genes to account for intraassay variation. Data was analyzed with StepOne Software using the 2-ΔΔCT method.

2.2.8 Measurement of serum 3-HPMA (Results pending)

The Acr metabolite 3-HPMA was measured in serum samples using LC-MS to assess GSH mediated detoxification of Acr. Solid phase extraction with Isolute ENV+ cartridges (Biotage, Charlotte, NC) was conducted to prepare each sample before LC-MS analysis. In brief, cartridges were conditioned with 1 mL of methanol, 1 mL of water, and 1 mL of 0.1% formic acid in water in succession. Fifty microliters of each serum sample

were combined with: 200 ng of deuterated 3-HPMA (d3-3-HPMA) (Toronto Research Chemicals, Toronto, Canada), 500 µL of 50mM ammonium formate, and 10 µL of undiluted formic acid and then applied into the prepared solid phase cartridges. The cartridges were subsequently washed twice with 1 mL of 0.1% formic acid and 1 mL of 10% methanol/90% 0.1% formic acid in water in succession. The cartridges were dried with nitrogen. 3-HPMA was eluted with three volumes of 600 µL 2% formic acid in methanol, which were combined and dried with a nitrogen evaporator (Organomation, Berlin, MA). Samples were reconstituted in 25 µL of 0.1% formic acid prior to LC-MS analysis.

The LC-MS system used was a Q Exactive Orbitrap interfaced with a Dionex ultimate 3000 UHPLC system (Thermo Fisher Scientific). In brief, samples were subjected in an ACQUITY UPLC BEH C18 1.7 µm reverse-phase column (50 mm × 2.1 mm) and then analyzed using a 5-min gradient going from 0% B to 95% B at a flow rate of 0.5 mL/min. Solvent A was water containing 0.1% formic acid and solvent B was acetonitrile containing 0.1% formic acid. MS data were acquired in negative mode using a data-dependent top 5 method dynamically choosing the most abundant precursor ions from the survey scan for HCD fragmentation using a stepped normalized collision energy of 30, 30, and 40 eV. Survey scans were acquired at a resolution of 70,000 at m/z 200 on the Q Exactive.

2.2.9 Measurement of Nrf2 transcription factor activity (Results pending)

Cytoplasmic and nuclear fractions were extracted from liver tissues and separated using a Nuclear Extraction Kit (Cayman Chemical Company, Ann Arbor, MI). After, the protein concentration was quantified using spectrophotometry at 280 nm (SpectraMax i3x; Molecular Devices). To confirm enrichment of the fraction, relative nuclear protein concentration was estimated by nuclear housekeeping protein LaminB1 by direct ELISA methods using a precoated microplate (ab206978; Abcam) and the associated Abcam

direct ELISA protocol. Nrf2 activity will soon be measured using a Nrf2 Transcription Factor Assay Kit (600590; Cayman Chemical Company) according to the manufacturer's instruction.

2.2.10 Statistical Analysis

For phenotypic histological damages, 2 × 2 contingency table chi-square analyses were used to compare NEG vs POS and POS vs API. Categories included experimental group (NEG, POS, or API) and whether each damage marker was present or not present. Alternatively, hemorrhage severity was scored 0-3 and analyzed by chi-square methods. For serum dot blot analyses, samples were pooled by experimental group, and data was expressed as a slope of signal intensity at varied concentrations. All other data were expressed as means ± standard error of means across measures, and the POS and NEG groups, as well as POS and API groups, were compared by t-tests. A p-value of 0.05 or less was considered statistically significant (GraphPad Prism 9.0.2; GraphPad Software, San Diego, CA).

2.3 Results

2.3.1 Body weight, food intake, and tissue weight

Body weight gain and food intake were monitored throughout the study period. Harvested liver, colon, and lung were weighed as well. The POS group mice gained less weight than the NEG and API groups, but average body weight at the end of the study did not significantly differ between groups (Table 2). There were no differences in food intake between the NEG and POS groups; however, the API group had a higher food intake per day by weight compared to the POS group. When tissues were harvested, average liver weight and the average ratio of liver weight to body weight of API group mice were lower than those of POS mice, and average colon weight and the average ratio

of colon weight to body weight in the POS group were lower than the NEG group (Table 2).

2.3.2 Histopathological assessment of pulmonary damages

Histopathological assessment of Acr-induced pulmonary damages was completed by a double-blinded pathologist. A greater percentage of POS group mice presented with lung damages for each type of pulmonary lesion observed, and a lower percentage of POS group mice presented no significant histological lesions in the lung tissues compared to NEG group mice, suggesting Acr-exposure resulted in fewer mice in good pulmonary condition. In contrast, as shown in Figure 1, API group mice had lower percentages of tissues with histological lesions across all damage markers. To be specific, mucus accumulation within the alveoli was present in 0.0% of NEG group mice, 62.5% of POS group mice, and 33.3% of API group mice. Likewise, pulmonary alveolar infiltration with macrophages was identified in 0.0% NEG group mice, 50.0% POS group mice, and 33.3% API group mice. Hemorrhaging, scored in severity from 0-3, was most severe in the POS group mice compared to both NEG and POS groups. Due to small sample size, analyses of histological lesions only revealed statistical significance for an Acr-induced increase in the percentage of mice with mucus accumulation ($p<0.05$) and a marginal increase in the percentage of mice with macrophage infiltration ($p=0.08$) between NEG and POS groups. Statistical significance was not met for differences between POS and API groups (Figure 1).

2.3.3 Systemic inflammation

An unbiased serum cytokine array showed, in general, apiaceous vegetables reversed inflammatory cytokine responses induced by Acr (Figure 2A). Among 16 cytokines that were significantly different between POS and API groups, the only exceptions were cytokines B-lymphocyte chemoattractant (BLC) and IL-3. In contrast,

these two cytokines were minorly increased in POS and significantly increased in API rather than decreased. Also, of importance, relative levels of IL-1 receptor antagonist [IL-1RA; an inhibitor of proinflammatory IL-1 beta (IL-1β)] were dramatically reduced in mice exposed to Acr only ($p<0.001$), whereas the API group showed significantly increased IL-1RA compared to the POS group ($p<0.05$). Furthermore, although TNF-α was not dramatically affected by Acr in the POS group, the API group had reduced serum TNF-α compared to POS group mice ($p<0.05$).

2.3.4 Pulmonary inflammation

Overall, expression levels of gene and protein markers of inflammation were significantly upregulated by Acr-exposure, whereas apiaceous vegetables downregulated most inflammatory markers, including the key players in the NF-κB pathway [i.e. REL-associated protein (*Rela;* p65 gene), p65, phosphorylated p65 (p-p65)]. Acr exposure significantly elevated protein expression of both p65 and p-p65, as well as inducible nitric oxide synthase (iNOS; Figure 2B). Furthermore, Acr marginally increased both precursor and mature forms of TNF-α ($p=0.06$ and $p=0.09$, respectively). Protein expressions of IL-6 and IL-1β were increased due to Acr (64% increase and 22% increase in the POS group vs NEG group, respectively), but these findings were not statistically significant due large variation within groups. Interestingly, compared to the POS group, apiaceous vegetables resulted in reductions in expression of most inflammatory protein markers in the lungs, including p65, p-p65, IL-1β, precursor and mature forms of TNF-α, and iNOS, but apiaceous vegetables did not reduce expression of IL-6 compared to the POS group.

In support of the protein expression results, gene expression of *Rela* and *Tnf* in the lung tissues was increased in the POS mice compared to the NEG group. In contrast, *Rela* and NF-kappa-B inhibitor alpha (*Nfkbia*) were decreased in the API group compared to the POS group (Figure 2C). However, gene expression data did not coincide with protein

expression results in respect to expression levels of IL-1β in the POS group; gene expression of *IL-1β* was reduced in the POS group compared to the NEG group. Nevertheless, in compliment to the protein expression, *IL-1β* was lower in the API group compared to the POS group.

2.3.5 Pulmonary DNA damage, cell cycle regulation, and apoptosis

Protein expression of p85α and poly (ADP-ribose) polymerase (PARP) were increased by Acr exposure; however, cleaved PARP (c-PARP) and cleaved caspase 3 (c-Casp3) were not affected. In contrast to mice exposed to Acr only, the API group mice had significantly lower levels of p85α and both PARP and c-PARP compared to the POS group (Figure 3A).

At the transcriptional level, in the POS group, DNA repair genes [e.g., breast cancer type 1 susceptibility protein (*Brca1*) and proliferating cell nuclear antigen (*Pcna*)] were decreased compared to the NEG group mice. In addition, apoptotic phosphoinositide-3-kinase regulatory subunit 1 (*Pik3r1;* p85 gene) and anti-apoptotic B-cell lymphoma 2 (*Bcl2*) were upregulated in the lungs of POS mice (Figure 3B). Conversely, when compared to the POS group, API group mice displayed increased mRNA levels of *Brca1* and *Pcna*, and decreased levels of *Pik3r1* and *Bcl2*. The overall observation was that apiaceous vegetables normalized most Acr-induced changes in mRNAs and proteins that were assessed related to DNA damage and cell cycle regulation.

2.3.6 Systemic clearance of Acr

In the serum dot blot analysis, the POS group had the most persistent signal for Acr-protein adducts, suggesting higher serum Acr concentrations (Figure 4A). The API group showed a less persistent signal when diluted, suggesting a lower Acr concentration in the serum of API group mice compared to the POS mice. Each slope, quantified by

signal intensity reduction rate with dilution, was adjusted relative to the POS, and the inverse relative slopes appear in Figure 4A with their associated R^2 values.

To further investigate the effects of API on hepatic detoxification of Acr, depletion of GSH was assessed by measuring GSH/GSSG ratios within the liver. As expected, Acr depleted GSH ($p<0.05$; Figure 4B), but there was no significant difference between POS and API. However, compared to the POS group, the API group had higher average levels of *Gst* mRNA in the liver for all *Gst* measured (a3, m1, p1, k1, and z1) with statistically significant increases in *Gstk1* and *Gstp1* (Figure 4E).

The metabolite of Acr detoxification 3-HPMA will be measured to assess if reduced serum Acr levels could be accounted for by enhanced detoxification efficiency. LC-MS analyses are currently being conducted and will appear in Figure 4 prior to publication. Also, to elucidate a potential underlying pathway impacting *Gst* mRNA expression, Nrf2 transcription factor binding activity will be measured, and the data will appear in Figure 4.

2.4 Discussion

2.4.1 Acr-exposure resulted in reduced body weight gain

During the study, food intake was calculated on the basis of wet weight, and, similar to previous studies supplementing 21% apiaceous vegetables, greater food intake in the API group may be accounted for by the higher water content and lower energy density of the added fresh vegetables.[41] Lower energy density of the supplemented diet indicates more food consumption by weight in the API group may have actually resulted in isocaloric intake compared to other groups. Water intake was not measured due to the variability of unavoidable bottle leakage, but this may be modified in future studies to account for water intake and food intake correlations.

At tissue harvest, few anthropometric measurements significantly differed between groups. However, one difference of significance was mice in the POS group experienced lower body weight gain during the study. This is explained by the fact that reduced weight gain is a well-established clinical indicator of toxicity relating to generally stressful stimuli.[52] Of note, a general inverse association has been established in humans between cigarette smoking and body weight or body mass index, but weight is certainly multifactorial in human populations.[53–55] Ratios of organ weight to body weight are better indicators of toxicological effects due to the tendency of organ weights to vary with body weight.[56] However, both significant organ weight differences (lower colon weight in the POS group compared to NEG group and lower liver weight in the API group compared to the POS group) maintained statistical significance when expressed as ratios to their respective body weights. There is no clear indication that Acr would affect colon weight, but experimental variation in tissue harvesting and weighing may be a plausible cause of skewed data. Similarly, given the small sample size and our previous studies showing no difference in liver weight after feeding apiaceous vegetables (same dose),[41] the apparent reduction in liver weight may have been caused by the same technical variations in tissue harvesting and weighing.

2.4.2 API improved Acr-induced pulmonary damages

Histological pulmonary findings were consistent with previous studies. In a study of intranasal Acr exposure over four weeks, similar results with more profound disease phenotypes were revealed, including extensive hemorrhaging in the lung tissue beginning at week 1 and continuing to week 4 and increased immune cell infiltration of the lung.[57] Addressing mucus accumulation, both *in vitro* and *in vivo* studies have determined that Acr and inflammatory mediators, including TNF-α, increase expression of the predominant mucin produced by airway epithelial cells (i.e., mucin5AC).[58,59] At both the mRNA and protein levels, mucin5AC is upregulated by Acr in human and animal lung

cells and, as a result, induces mucus hypersecretion in animal models.[58,59] In addition, activation of NF-κB (upregulated under our conditions), may partake in induced mucin5AC expression; evidence has demonstrated there are NF-κB binding sites within the functional promoter region of mucin5AC.[60] Based on our study, the proposed apiaceous vegetable enhancement of Acr detoxification and anti-inflammatory protections (e.g., downregulation of NF-κB and reduced TNF-α) could explain the trend of decreased lung mucus accumulation in the API mice (Figure 1A).

Due to the acute nature of our study, advanced phenotypic damages, such as fibrosis, could not have occurred. A study of particulate and soluble chemical fibrogenic agents, which induce pulmonary fibrosis, demonstrated that the climax of acute lesions and the junction of transition from an acute response to chronic fibrosis is seven days post-exposure.[61] Likely due to the early onset of morphological changes at only five days and small sample size, some damages by Acr and the protective effects of apiaceous vegetables were not found under our conditions. However, as shown in the Figure 1, there was a trend of reduction in pulmonary lesions across all measures in the API group compared to the POS group mice. In future studies with larger sample sizes and longer durations of chronic exposure, disease phenotype may be more progressive, allowing for stronger differentiation between pulmonary morphological changes.

2.4.3 API ameliorated Acr-induced systemic inflammation responses

Although Acr is most volatile at the first site of contact, the lungs, a systemic response is a hallmark of both acute and chronic lung inflammatory conditions. Inflammatory mediators produced in the lung "spill over" into circulation, activating release of acute-phase proteins from the liver and release of leukocytes and platelets from the bone marrow.[62,63] Therefore, repeated exposure to toxicants, such as Acr, causes systemic chronic inflammation.[64] Systemic chronic inflammation maintains high levels of proinflammatory cytokines, which have a causal role in the development of not just a few

select disorders but several diseases that represent the leading causes of disability and mortality worldwide.[64]

Based on the serum cytokine array results, there was a clear pattern of Acr-induced changes in inflammatory cytokines within the serum that was reversed or normalized in the API group (Figure 2). Important inflammatory markers to emphasize included IL-1RA and TNF-α. TNF-α is one of the most significant proinflammatory cytokines, which has been associated with oxidative stress, systemic inflammatory pathways, and acute phase reactions.[65] In contrast, IL-1RA is an anti-inflammatory antagonist of the receptor for IL-1 proinflammatory cytokines, including IL-1β.[66] Acr exposure reduced serum IL-1RA, potentially upregulating IL-1β activity, but, when supplemented with apiaceous vegetable, serum IL-1RA was increased while serum TNF-α was reduced. Across the 16 cytokines and in the scope of IL-1RA and TNF-α particularly, these findings suggest apiaceous vegetables potentially produced an anti-inflammatory effect against Acr-induced inflammatory responses. Systemic inflammation, as seen in the POS group mice, may be correlated with other organ systems. In relation to lung damages, studies have reported significant associations between systemic inflammation and impaired lung function that may not be unidirectional;[67,68] elevated serum proinflammatory cytokines and macrophage activation result in lung function decrement in a strong dose-dependent manner.[68] Thereby, there may be systemic "crosstalk" between the lungs and distal organs,[69,70] and evidence suggests the complex network of cytokines and chemokines in the bloodstream can initiate and amplify lung injury.[71,72] Many inflammatory mediators involved in the systemic response can both damage lungs directly and stimulate the bone marrow to release leukocytes into circulation.[73] Altogether, the systemic response induced by pulmonary damage may feed back to the lungs and perpetuate the localized inflammatory response,[74] but the causality of this relationship and how apiaceous vegetables intervened in Acr-induced inflammatory responses are to be elucidated.

2.4.4 API protected against Acr-induced pulmonary inflammation

In order to confirm our histopathological observations, specifically Acr-induced macrophage infiltration of alveoli, we measured inflammatory markers within the lung tissue. Similar to the systemic inflammatory responses, overall, Acr-induced inflammatory markers in the lung while apiaceous vegetables ameliorated. To be specific, protein expression levels of p65 and p-p65, IL-1β, TNF-α, and iNOS were all significantly reduced in the API group compared to the POS group (Figure 2B). Further, at mRNA level, *Rela* (p65), *Nfkbia* (p50), and *IL-1β* were all decreased in the API group mice lungs, further suggesting anti-inflammatory potential of apiaceous vegetables. Although Acr only marginally affected protein expression of proinflammatory TNF-α in the POS mice lung (precursor TNF-α, $p=0.06$; mature TNF-α, $p=0.09$), previous studies have shown Acr upregulates TNF-α, *in vitro* and *in vivo*.[75,76] It should also be noted that gene expression of *Tnf* was increased in the POS group compared to NEG, and API did reduce expression of TNF-α protein. Overall, our data collectively suggests that apiaceous vegetables reversed the inflammatory responses in the lung.

We assessed a few key upstream (e.g., p65) and downstream players (e.g., IL-1β) of the NF-κB pathway, which orchestrates cellular responses (e.g., inflammation) against external stimuli such Acr[57] and oxidative stress.[75] Similarly, under our treatment conditions, Acr transactivates NF-κB signaling (increased both p65 and p-p65) while API reduced the responses (e.g., p65, p-p65, TNF-α, and iNOS). In contrast, however, some research has suggested that Acr directly inhibits the binding of p50 (i.e., one of transcription factors of the signaling pathway) to the IL-2 promoter binding domain, thereby suppressing overall inflammatory cytokine production in T lymphocytes.[77] On the other hand, bronchial biopsies from smokers revealed increased p65 predominantly in the epithelium, and, in those with COPD, disease severity was associated with increased epithelial expression of NF-κB.[78] COPD patients are particularly relevant because both lung and plasma Acr levels are significantly higher in COPD patients than non-COPD

smokers ($p<0.001$), and Acr concentrations directly correlate with oxidant status in patients with COPD (R=0.69, $p<0.05$).[79] The mechanism by which apiaceous vegetables transactivate this pathway is not clear at this moment; it was demonstrated that falcarinol and falcarindiol (profoundly present in apiaceous vegetables) downregulate NF-κB and its downstream inflammatory cytokines (e.g., TNF-α, IL-6),[28] thus additional mechanistic studies using the phytonutrients are warranted.

2.4.5 Acr induced variable effects on cell cycle regulation

Acr produces ROS and DNA damages both of which can induce apoptotic and necrotic cell deaths.[80] In general, moderate oxidative stress induces apoptosis, whereas higher exposure to ROS triggers necrosis.[81–83] When it comes to apoptotic responses, ROS induce apoptosis through p53 activation and induction of the regulatory protein of phosphatidylinositol-3-kinases, p85.[84–86] However, effects of Acr on apoptosis are unclear in this study. Although p85α protein expression and *Pik3r1* gene expression (encodes p85α) were increased in Acr treated lungs, changes in c-PARP and c-Casp3 (apoptotic marker proteins) did not occur in the POS group mice. Further PARP, when not cleaved, is a marker of necrosis and, in agreement with anti-apoptotic gene *Bcl2*, was increased in POS group mice (Figure 3). This may indicate that cells were more likely to undergo necrosis rather than apoptosis. In fact, past studies have demonstrated that high Acr concentrations actually decrease c-Casp3 and apoptosis and result in necrosis.[87] It has, thereby, been proposed that because persistent neutrophilic inflammation is common in smoking-related lung diseases, it is plausible that Acr stimulates production of proinflammatory cytokines and interferes with normal apoptotic processes, which contributes to amplified and more chronic inflammatory processes via necrosis.[87] In contrast to the POS mice, reductions in p85α, PARP, and c-PARP may suggest that fewer DNA damages occurred in the API group mice. Therefore, DNA repair genes (e.g., *Brca1* and *Pcna*) were measured to further investigate DNA damage pathways. Although a

causal relationship is yet to be elucidated, Acr decreased both *Brca1* and *Pcna*, potentially inhibiting DNA repair, whereas normalized levels of DNA repair genes in API group mice could explain less pronounced DNA damage cascades (Figure 3). To elucidate the Acr-induced necrosis theory, necrotic markers including receptor interacting protein 1 and 3 (RIP1 and RIP3) will be measured. When caspases are inhibited, which may be the case in our model, kinase RIP1 interacts with RIP3, and initiates the necrotic pathway.[88] Obviously, especially given the complexity of cell cycle regulation and cell death signaling, additional studies (e.g., unbiased omics or mechanistic in vitro studies) are needed to fully comprehend the outcomes of API group in relation to Acr-induced cell deaths.

2.4.6 API modulated detoxification of Acr within the liver

Evidence suggests that apiaceous vegetables increase hepatic BTE activity in a dose-dependent manner and reduce DNA damages.[41] In the context of our study, we assessed all subtypes of GST, which conjugate GSH for Acr detoxification to form an excretable metabolite, 3-HPMA. First, in our condition, Acr lowered GSH in POS mice, but no difference was shown between POS and API groups. However, gene expression of hepatic GST (*Gstk1* and *Gstp1*) was higher in the API group, suggesting that the vegetable supplementation may have transactivated the Nrf2 signaling pathway which can be validated by a Nrf2 transcription factor binding activity assay (Result pending). These results agree with systemic Acr-protein adduct results (Figure 4A); lower levels of serum Acr in the API might have been facilitated by efficient excretion of Acr via GST-mediated detoxification. Currently, serum 3-HPMA levels are being analyzed, which will provide corroborating evidence as to the impacts of apiaceous vegetables on Acr metabolism.

Apiaceous vegetables contain a wide array of phytonutrients, so the particular chemicals and mechanisms underlying this effect require further investigation. For

example, lipophilic polyacetylenes in apiaceous vegetables increase both expression and activities of antioxidant enzymes.[42,43] Another candidate phytochemical in apiaceous vegetables is chlorogenic acid (CGA), which increases the expression of nuclear Nrf2 and Nrf2-regulated antioxidant genes.[89] Additionally, CGA decreases malondialdehyde levels and increases GSH, superoxide dismutase, and catalase levels in liver tissues,[89] which could affect both oxidative defenses and metabolism of Acr by increasing GSH availability; however, increased GSH availability was not produced by API in our model. Further evidence enforces that a CGA isoform, isochlorogenic acid B, upregulates Nrf2, thereby suppressing fibrogenic factors.[90] Aside from transactivation of hepatic Nrf2 signaling, apiaceous vegetable constituents may have a general Acr scavenging ability; research has demonstrated that there is a possible protective role of polyphenols in neutralizing reactive aldehydes, including Acr.[44] For instance, ferulic acid, which is abundant in apiaceous vegetables, was found to have the highest efficiency in scavenging Acr under physiological conditions,[91] presenting a possible mechanism of apiaceous vegetables.

There are a few limitations to be noted. Acr was instilled nasally, which is the natural site of exposure, but Acr was delivered via liquid saline rather than vapor. However, nasal instillation of small volumes of liquid offers complete control of dosage and delivery which 1) introduces less experimental variation and 2) still results in effective respiratory exposure as if Acr was inhaled.[51,57] Second, the dosage of Acr was very high, 10 μM/kg bodyweight per day, translating to approximately 25 times the average daily Acr exposure per kilogram body weight of a smoker in the United States.[92] However, the level of Acr introduced in the study was necessary to induce disease phenotypes and is still lower than other previous studies.[57] Another limitation includes the small sample size of only male mice, which was due to the nature of our work, a preliminary study to optimize appropriate experimental conditions.

The current study presents a few strengths as well. To the best of our knowledge, this pilot study was the first to examine the impacts of apiaceous vegetables supplementation on pulmonary and systemic effects of Acr exposure. Second, in contrast to our high Acr dosage, the diet formulation for apiaceous vegetable supplementation was very translatable and achievable in the human diet, amounting to only approximately 1 1/3 cup of vegetable per day in humans.[41] Considering the short duration and low dosage, the effects of apiaceous vegetables are noteworthy. Furthermore, despite the popularity of studying pure nutrients and extracted phytochemicals, we adopted the 'whole-food approach', which is more translatable and realistic. Last, our reference molecule, Acr, is significant given its wide presence in many environments (e.g., cigarette smoke and polluted air). Avoiding polluted air is virtually impossible in some geographic locations, and smoking cessation without professional help is only successful in 3-5% of cases.[25] Each year, less than 1 in 10 United States adult cigarette smokers successfully quit smoking (defined as 6 months or longer).[93]

2.5 Conclusion

Using an mice model, we demonstrated that Acr caused both systemic and pulmonary inflammatory responses, potentially induced inhibition of DNA repair and apoptosis, and resulted in phenotypic damages to the lung tissues. In contrast, apiaceous vegetables, in most cases, reversed these responses in Acr-exposed mice. Potential mechanisms of apiaceous vegetables include anti-inflammatory potential and enhancement of the hepatic detoxification of Acr. Apiaceous vegetables may offer a protective dietary intervention against Acr toxicity.

Chapter 3

CONCLUSION

In male C57BL/6J mice, we demonstrated that Acr caused both systemic and pulmonary inflammatory responses, potentially inhibited DNA repair and apoptosis, and resulted in phenotypic pulmonary damages. In contrast, dietary supplementation of apiaceous vegetables, in most cases, normalized or reversed these responses in Acr-exposed mice. Potential mechanisms include anti-inflammatory actions and enhancement of the hepatic detoxification of Acr. Future studies, including our current ongoing projects, will incorporate both males and females, varied Acr dosages, additional exposure routes and durations, varied dietary contribution of apiaceous vegetables, and analyses of pathways that influence additional metabolic pathways and body systems. *In vitro* studies may be necessary to test specific causative interactions of apiaceous vegetable bioactives and Acr within molecular pathways and to pinpoint protective mechanisms. On another note, Acr exposure has been identified as an instigator of cardiovascular disease. Therefore, future studies should examine the additional roles apiaceous vegetables may play in maintaining cardiovascular health under Acr-exposure conditions.

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Appendix A

TABLES

Table 1. Composition of AIN-93G and apiaceous vegetable (API)[1] diets

Diet ingredients	AIN-93G	21% API
Cornstarch, g	398	382 (15.2)
Dextrinized cornstarch, g	132	132
Casein, g	200	198 (2.00)
Sucrose, g	100	100
Cellulose, g	50.0	43.2 (6.80)
Mineral mix[2], g	35.0	35.0
Vitamin mix[3], g	10.0	10.0
L-Cystine, g	3.00	3.00
Choline bitartrate, g	2.50	2.50
Soybean oil[4], g	70.0	69.5 (0.500)
API, g	Not applicable	210.0
Total, kg	1.00	1.20

[1]Values in parentheses represent the contribution of the respective macronutrients from the apiaceous vegetables (API; celery and parsnip). All diets were balanced for macronutrients on a dry weight basis using the USDA National Nutrient Database.
[2]AIN-93G mineral mix.[49]
[3]AIN-93G vitamin mix.[49]
[4]The antioxidant, t-butylhydroquinone (0.02% wt:v) was included in soybean oil.

Table 2. Phytochemical constituents of celery and parsnip[1]

Phytochemical Group	Active Compound	Concentration in Celery and Parsnip		References
Phenolic compounds	Apigenin	Celery	• 17.4-46.1 mg/kg FW • 338.5 mg/kg DW	94,95,96,97,98
		Parsnip	• Undetermined	
	Luteolin	Celery	• 8.4-10.5 mg/kg FW, • 80.5-316 mg/kg DW	94,97,98
		Parsnip	• Undetermined	
	Kaempferol	Celery	• 2.2-7.7 mg/kg FW	94,99,100,98
		Parsnip	• 66.4 mg/kg DW	
	Quercetin	Celery	• 3.9 mg/kg FW, • 250.8 mg/kg DW	99,100
		Parsnip	• 9.9 mg/kg DW	
	Caffeic acid	Celery	• 676.5 mg/kg DW	101,98,102
		Parsnip	• 18 mg/kg FW	
	P-coumaric acid	Celery	• 1143 mg/kg DW	101,98,102
		Parsnip	• 3.4 mg/kg FW	
	Ferulic acid	Celery	• 216.7 mg/kg DW	101,98,103
		Parsnip	• 22 mg/kg FW	
	Chlorogenic acid	Celery	• 102.3 mg/kg DW	101,104,102
		Parsnip	• 11 mg/kg FW	
	Gallic acid	Celery	• 243.6 mg/kg DW	101,105
		Parsnip	• 82.1 mg/kg FW	

Furanocoumarins	Psoralen	Celery	• 8.0-11.9 mg/kg FW	[106,107]
		Parsnip	• 4.3 mg/kg FW	
	8-methoxypsoralen	Celery	• 4.7 mg/kg FW	[106]
		Parsnip	• 17.9 mg/kg FW	
	Angelicin	Celery	• Undetermined	[106]
		Parsnip	• 21.6 mg/kg FW	
	5-methoxypsoralen	Celery	• 0.9 mg/kg FW	[106]
		Parsnip	• 16.3 mg/kg FW	
	Isopimpinellin	Celery	• 0.4 mg/kg FW	[106]
		Parsnip	• 3.0 mg/kg FW	
	Sphondin	Celery	• Undetermined	[106]
		Parsnip	• 2.3 mg/kg FW	
	Oxypeucidanin	Celery	• 0.2 mg/kg FW	[106]
		Parsnip	• 0.1 mg/kg FW	
	Imperatorin	Celery	• Undetermined	[106]
		Parsnip	• 1.3 mg/kg FW	
	Dihydrobergamottin	Celery	• Undetermined	[106]
		Parsnip	• 5.8 mg/kg FW	
	Xanthotoxin	Celery	• 1.3 mg/kg FW	[108]
		Parsnip	• 8.5 mg/kg FW	

Polyacetylenes	Falcarindiol	Celery	• 2010-4580 mg/kg DW	109,34
		Parsnip	• 5770 mg/kg DW	
	Falcarinol	Celery	• 230-1620 mg/kg DW	109,34
		Parsnip	• 1600 mg/kg DW	
	Falcarinone	Celery	• Undetermined	110
		Parsnip	• Present	
	Falcarinolone	Celery	• Undetermined	110
		Parsnip	• Present	
	Papaxydiol	Celery	• 20-60 mg/kg DW	34
		Parsnip	• Undetermined	
	8-*O*-methylfalcarindiol	Celery	• 40-170 mg/kg DW	34
		Parsnip	• Undetermined	

[1]All values are expressed in mg/kg, but some sources analyzed samples based on fresh weight (FW) whereas others analyzed samples based on dry weight (DW). "Present" indicates that literature suggests the phytochemical is present in the respective vegetable. "Undetermined" indicates that concentrations were too low to detect, were not clearly published, or have not yet been measured.

Table 3. Food intake, tissue, and body weight after intervention of API[1]

	Food intake, g·d^{-1}	Weight gain, g	Weight, mg				Weight/body weight, ratio		
			Body	Liver	Colon	Lung	Liver	Colon	Lung
NEG	3.88 ± 0.43	3.83 ± 0.31	26.5 ± 1.04	1.37 ± 0.10	0.16 ± 0.01	0.15 ± 0.00	0.0511 ± 0.002	0.0061 ± 0.0003	0.0056 ± 0.0002
POS	3.42 ± 0.37	2.53 ± 0.28*	25.7 ± 0.51	1.46 ± 0.06	0.13 ± 0.01*	0.19 ± 0.02	0.0569 ± 0.002	0.0051 ± 0.0002*	0.0075 ± 0.0008
API	4.43 ± 0.39	3.48 ± 0.35#	24.0 ± 0.52	1.13 ± 0.09#	0.13 ± 0.01	0.17 ± 0.01	0.0468 ± 0.003#	0.0053 ± 0.0001	0.0069 ± 0.0007

[1]All results are expressed as least squares mean ± SEM. Means within the positive control (POS) column with * indicate a difference between the negative control (NEG) and POS groups, and # indicates a statistical difference between POS and apiaceous vegetable intervention (API) groups, $p<0.05$.

Appendix B

FIGURES

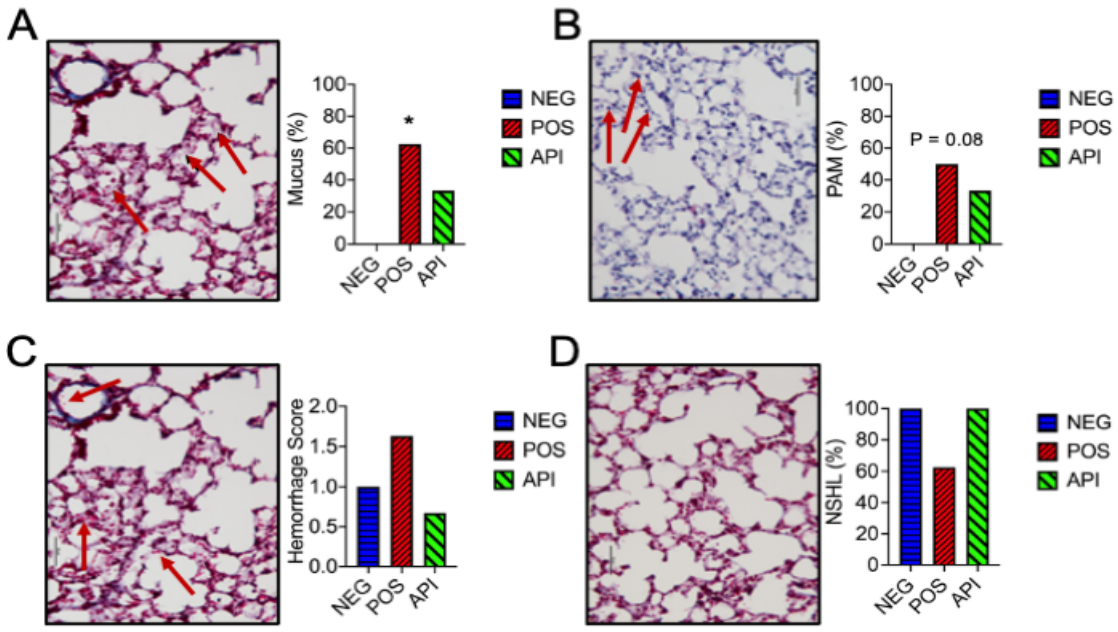

Figure 1. API alleviates Acr-induced lung tissue damages in mice.

Based on a blinded veterinary pathology report of lung tissue slides from the negative control group (NEG), positive control group (POS), and apiaceous vegetable intervention group (API) stained with H&E and Trichrome stain, significant phenotypic pulmonary changes were presence of **(A)** collapsed alveoli coated or filled with wispy mucinous or mucoid material (arrows), suspected to be mucus (Trichrome, 20× magnification), and **(B)** small numbers of pulmonary alveolar macrophages (arrows) infiltrating affected alveoli in an apparent attempt to clear the mucoid material within alveolar lumens (Hematoxylin and Eosin Y, 20× magnification), which were both

quantified as present or not present and expressed as percentage of lung tissue slides with the respective markers present. **(C)** Hemorrhage severity, identified by free red blood cells (arrows; Trichrome, 20× magnification) was scored on a scale from 0 = no hemorrhaging to 3 = severe hemorrhaging, and data was expressed as average hemorrhage score. **(D)** The record also included identification of slides, such as depicted in D (Trichrome, 20× magnification), with no significant histological lesions (NSHL), which is expressed as the percentage of tissue slides within each group with NSHL. Statistical differences were calculated using chi-square analyses. * indicates statistical significance between NEG and POS groups, $p<0.05$.

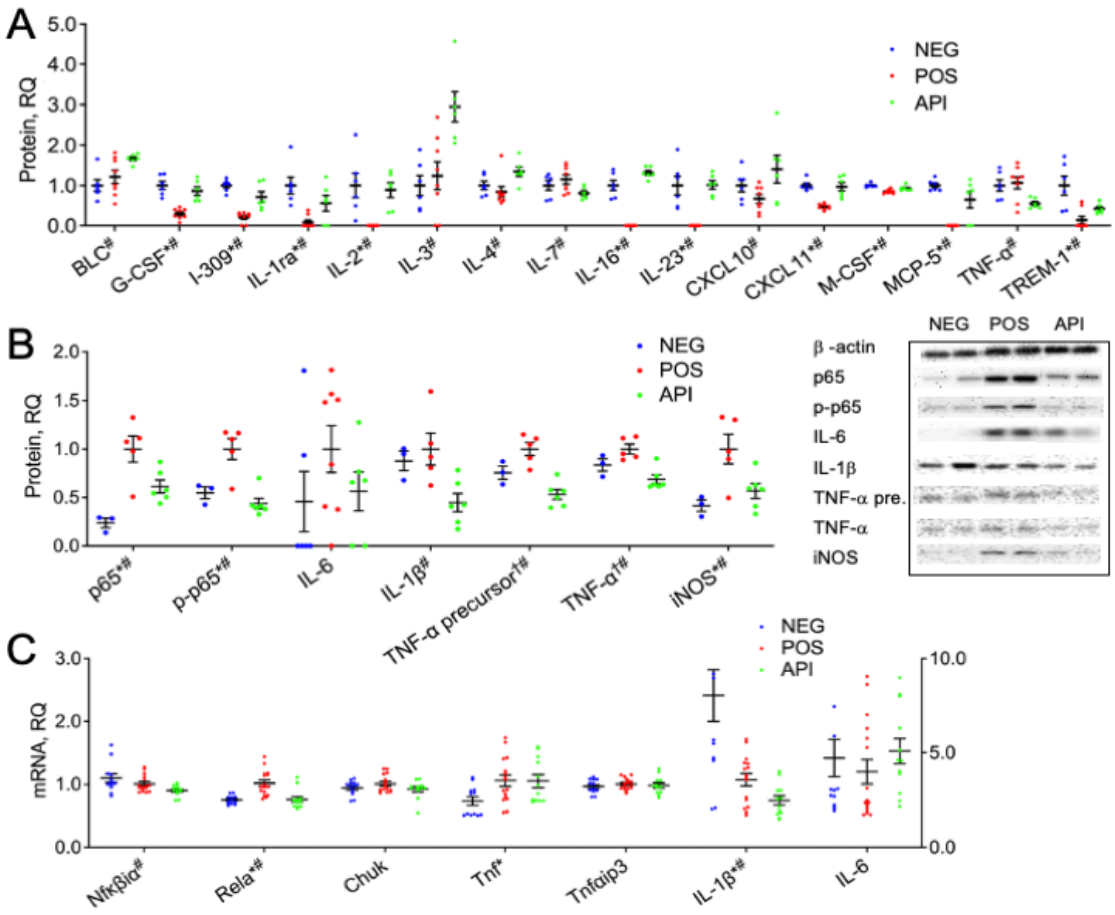

Figure 2. API supplementation reduced both systemic and pulmonary inflammatory responses induced by Acr

(A) Immunoblot serum cytokine array analyses, expressed as relative protein quantity based on mean pixel intensity, revealed API supplementation generally reversed inflammatory responses induced by Acr. **(B)** Western blot techniques were used to measure inflammatory markers within the lung tissue, and data was expressed as relative quantity of protein. **(C)** RT-qPCR analyses were conducted to investigate inflammatory pathways within the lungs, and data was expressed as relative quantity of mRNA. Error bars depict means ± standard error of means (SEM) across measures. Negative control (NEG) groups and positive control (POS) groups, as well as POS and apiaceous vegetable intervention (API) groups, were compared by t-tests. * alongside a target name

indicates statistically significant differences between NEG and POS groups, and [#] indicates significant differences between POS and API groups, $p<0.05$. [†] indicates a marginal difference between NEG and POS groups, $p<0.10$.

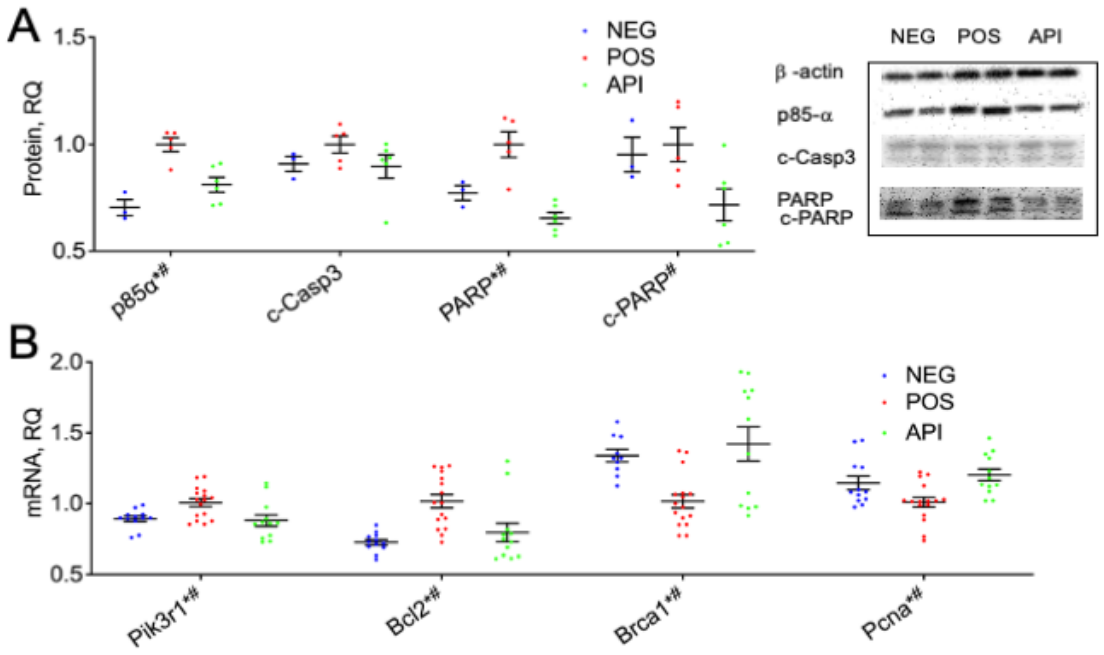

Figure 3. API supplementation prevents modulation of various DNA damage, cell cycle, apoptotic pathways induced by Acr in the lungs

(A) Western blot techniques were used to measure DNA damage, cell cycle, and apoptotic markers within the lung tissue, and data was expressed as relative protein quantities. **(B)** RT-qPCR analyses were conducted to further investigate these pathways within the lungs, and data was expressed as relative quantities of mRNA. Error bars depict means ± standard error of means (SEM) across measures. Negative control (NEG) groups and positive control (POS) groups, as well as POS and apiaceous vegetable intervention (API) groups, were compared by t-tests. * alongside a target name indicates statistically significant differences between NEG and POS groups, and # indicates significant differences between POS and API groups, $p<0.05$.

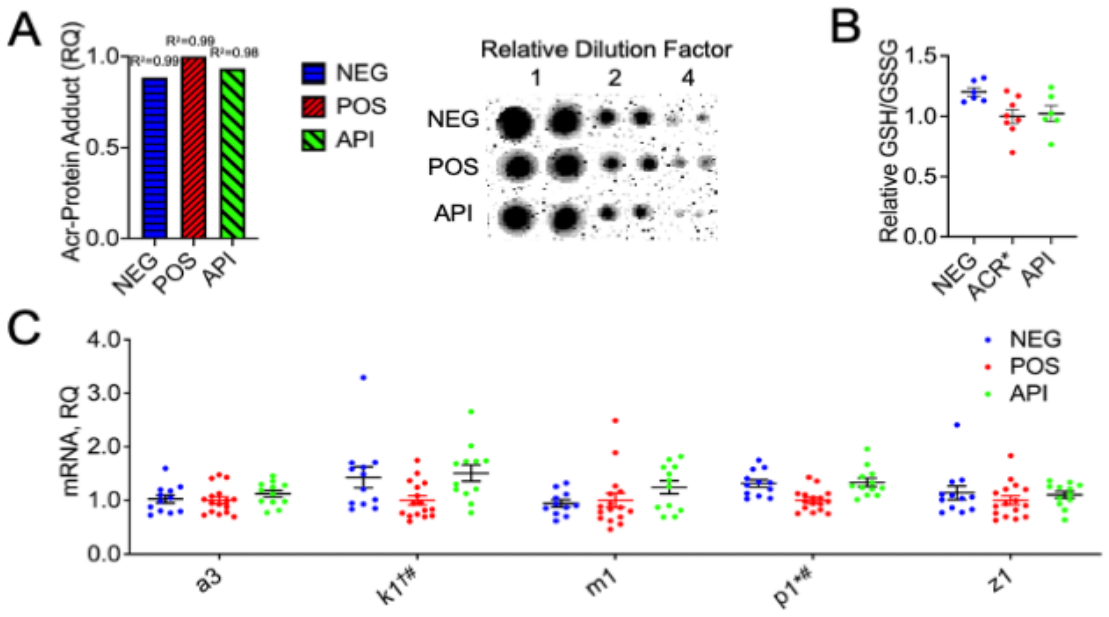

Figure 4. API upregulate GST expression and enhance Acr detoxification in liver

(A) Dot blotting methods were utilized to assess Acr-protein adduct levels within the serum. Serum samples were pooled by experimental group to account for intergroup variation, and, to account for serum matrices effects, dot blots were analyzed as relative quantities based on signal intensity slopes as concentration declined. A greater absolute slope (more significant decline in signal) signified a lower concentration. Data was expressed as relative quantities of Acr-protein adduct concentrations with associated R^2 values. **(B)** The ratios of reduced to oxidized glutathione (GSH/GSSG) were measured using a GSH/GSSG Ratio Detection Assay kit (ab138881; Abcam, Cambridge, UK) to assess GSH depletion in the liver, and data was expressed as relative quantities of GSH/GSSG. **(C)** RT-qPCR analyses were conducted to measure various isoforms of liver *Gst*, and data was expressed as relative quantities of mRNA. Error bars depict means ± standard error of means (SEM) across measures. For (B) and (C), negative control (NEG) groups and positive control (POS) groups, as well as POS and apiaceous vegetable intervention (API) groups, were compared by t-tests. * alongside a target name indicates

statistically significant differences between NEG and POS groups, and [#] indicates significant differences between POS and API groups, $p<0.05$.[†] indicates a marginal difference between NEG and POS groups, $p<0.1$.

Appendix C

IACUC/ANIMAL SUBJECTS APPROVAL

UNIVERSITY OF ARKANSAS
Office of Research Compliance

To:	Jae Kyeom Kim
Fr:	Craig Coon
Date:	December 17th, 2017
Subject:	IACUC Approval
Expiration Date:	December 15th, 2018

The Institutional Animal Care and Use Committee (IACUC) has APPROVED your protocol # **18001:** ***MicroRNA-mediated chemopreventive mechanisms of apiaceous vegetables against acrolein-induced lung injury***.

In granting its approval, the IACUC has approved only the information provided. Should there be any further changes to the protocol during the research, please notify the IACUC in writing (via the Modification form) prior to initiating the changes. If the study period is expected to extend beyond December 15th, 2018 you can submit a modification to extend project up to 3 years, or submit a new protocol. By policy the IACUC cannot approve a study for more than 3 years at a time.

The following individuals are approved to work on this study: Jae Kim and Jeong Pan. Please submit personnel additions to this protocol via the modification form prior to their start of work.

The IACUC appreciates your cooperation in complying with University and Federal guidelines involving animal subjects.

CNC/tmp

18001