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Cannabis sativa commonly known as Marijuana, is a well-known psychoactive drug that is frequently used in the United States (USA). Over 20 states have legalized marijuana for recreational or medical use, as such, understanding the impact of its use on human health becomes critical. Inhalation is a major route of exposure allowing substances to enter the body via the respiratory tract. Though smoke is typically exhaled right after being inhaled, its components are still interacting with the lining of the bronchus, specifically the bronchial epithelial cells. The effects of marijuana constituents on epithelial lining of the lungs are not fully understood. The objective of this study was to determine if Δ-9-THC (Δ-9-tetrahydrocannabinol) exposure leads to adverse outcomes in the respiratory epithelium using normal human bronchial epithelial cell line (BEAS-2B) at the cellular and transcriptional levels. The results showed that Δ-9-THC significantly decreased cell viability in 24 HRs 96 HRs, as well as in 9 days of culture. Transcriptome analysis of the Δ-9-THC-exposed BEAS-2B cells revealed genes with distinctly altered patterns of expression, particularly those specific to ferroptosis, unfolded protein response, and tumor microenvironment pathways, suggesting effects of marijuana smoking on various biochemical pathways in the lung. The Δ-9-THC-induced cell death was ameliorated by the inhibition of the ferroptosis pathway, whereas the agonist of ferroptosis exacerbated the process of cell death, suggesting that Δ-9-THC utilizes ferroptosis pathway to induce cell death in bronchial epithelial cells. Future studies are necessary to delineate other mechanisms predicted by transcriptome network analysis to understand the comprehensive picture of Δ-9-THC-induced cell death and other adverse respiratory health outcomes *in vitro* and *in vivo*.

RESPONSES OF HUMAN BRONCHIAL EPITHELIAL CELLS TO Δ-9-TETRAHYDROCANNABINOL EXPOSURE *IN VITRO*

by

Megan Doldron

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DEDICATION

I dedicate this dissertation to my mother, my grandmother, and my father. My grandmother came to America with the equivalent of a middle school education, a dream of a better life and less than $500 to her name. Her perseverance and relentlessness afforded her the opportunity to become a successful woman and that is how my mother, and I were able to come to the United States, because of her. My mother is also one of the hardest working women I know, she has made many sacrifices for me to be where I am today. My mother is brave, smart, consistent, diligent, caring, understanding, just incredible and I get to call her my mother, wow. My father, he was pure love, kind, cool, hardworking, giving, just an amazing man. When I started this journey, he was sitting on the sofa with me while I completed my application for my Doctoral studies. It breaks my heart that he is not here to see me finish, but I will continue to make him proud.

APPROVAL PAGE

This dissertation written by Megan Doldron has been approved by the following committee of the Faculty of The Graduate School at The University of North Carolina at Greensboro.

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CHAPTER I: INTRODUCTION

Significance

Cannabis sativa, commonly known as Marijuana, is one of the most commonly used illicit drugs in the United States (USA) with a reported estimate of 6% of the population aged 26 and older having used marijuana in 2019, for a total of 22.2 million users overall, and an annual prevalence rate of 144 million people consuming marijuana (McCance-Katz, 2017).

Over 20 states have legalized marijuana for recreational or medical use. As such, understanding the impact of its use on human health is critical. The major route of marijuana exposure in humans is through inhalation. The effects of marijuana constituents on epithelial lining of the lungs are not fully understood. While the vast majority of marijuana research focuses on its neurological effects, there is a lack of research on its impact on the lungs. This research aims to address that gap by determining the effects of marijuana constituent, Δ-9-tetrahydrocannabinol (D9-THC or THC) on the bronchial epithelial cells at the cellular and transcriptional levels.

Background Information

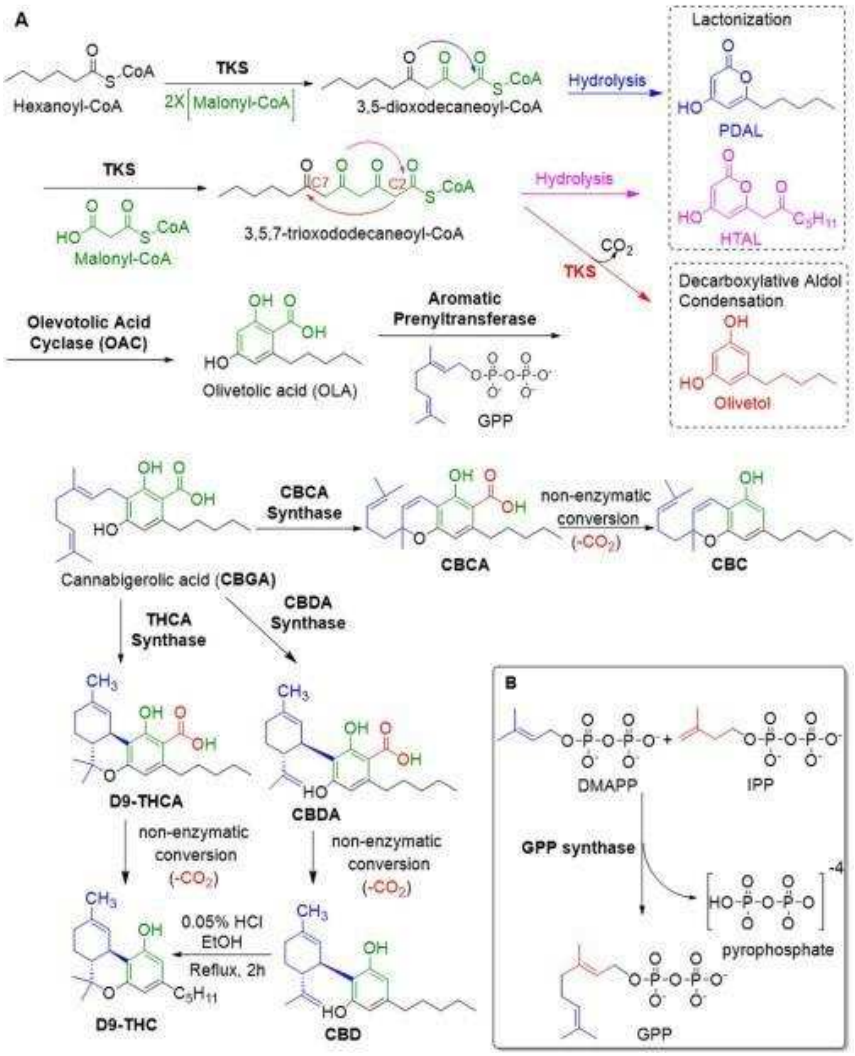
Cannabinoids - Synthesis and Activity

Marijuana is one of the oldest cultivated crops, with a history that extends to the Common Era (BCE). Historically, marijuana had many uses, and one of the primary ones was for medicine (Holland, 2010). This annual herb has recently gained increasing attention-as-many states are legalizing its use for medicinal or recreational purposes. Extensive research has also been conducted on the secondary components of marijuana (referred to as cannabinoids) regarding their associated health benefits (Lubek, 2018; Mücke et al., 2018; Sarafian et al.,

1999). Marijuana is composed of over 400 different chemical compounds with approximately 66 phytocannabinoids identified (Baron, 2015). These phytocannabinoids are naturally occurring oxygen-containing aromatic hydrocarbon compounds. Major constituents from the 66 phytocanabinoids are identified as the active ingredients of the plant.

Research has been conducted on the cannabinoid biosynthetic pathway, and progress has been made in determining how metabolites and active ingredients are synthesized. Cannabinoids are synthesized inside the head of the glandular trichomes on the female flower (Lubek, 2018; Baron, 2015). The trichome may also provide a defense mechanism for the plant, serving as a type of insecticide (Tahir, 2021). The active components of the plants are stored in and secreted from the trichome (DePetrocellis, 2017).

Figure 1. Biosynthetic Pathway of Cannabinoids



The biosynthetic pathway begins with condensation of olivetolate (OLA) and geranyl pyrophosphate via geranyltransferase (GPP), forming the central precursor of countless cannabinoids: cannabigerolic (CBG). Once CBG is formed, it is immediately converted to three oxidoreductases: tetrahydrocannabinolic acid (THCA), cannabidiol acid (CBDA, and cannabichromenic acid (CBCA). This is followed by decarboxylation via light or heat, forming the more common cannabinoids and active ingredients in marijuana (Goode, 2017; Olmo et al.,

2016; Preet et al., 2011). The active ingredients Δ-9- tetrahydrocannabinol (Δ-9-THC) and cannabidiol (CBD) shape how marijuana affects the body.

Δ-9-THC is a major constituent of *Cannabis sativa* and was originally isolated in 1964. It is chemically unstable, highly lipophilic, and is involved in the plant's defense mechanism Bouaboula et al., 1993; Lu & Mackie, 2016). Δ-9-THC has been found to be the primary cause of the psychoactive properties of in marijuana, and accounts for 40 percent of the plant's extract (Galiegue et al., 1995). Δ-9-THC impacts processes in the body by its interaction with cannabinoid receptors and by mimicking the endogenous cannabinoid (endocannabinoid) neurotransmitters. It has been shown to promote appetite in cancer and HIV patients (Lee et al., 2012; Turcotte et al., 2016). Studying the major active ingredients of marijuana is necessary to develop a comprehensive understanding of marijuana. Certain cells in the human body have receptors that readily interact with these cannabinoids and produce a response. Therefore, it is essential that the ways in which cannabinoids work both individually and as a collective unit are understood, including how they affect endogenous cannabinoid receptors and signaling pathways.

Numerous human cell types have cannabinoid receptors that interact with components of marijuana. These receptors are a part of a larger unit called the *endocannabinoid system*, that is composed of cell surface G protein-coupled Cannabinoid CB1 and CB2 receptors. The system also includes enzymes specific to biosynthesis and metabolism as well as endogenous endocannabinoids (2AG and AEA) (DePetrocellis et al., 2017; Preet et al., 2011). G protein-coupled receptors are in the cell membrane and induce cellular responses via signal transduction pathways in the brain. The cellular responses include cell survival, cell proliferation and exocytosis regulation (Olmo et al., 2016). The CB1 receptors also play a critical role in

mediating the effects of Δ-9-THC exposure on the brain, just as they play a predominant role in most psychoactive effects (Goode, 2017). Previous studies have shown that the CB1 receptors are expressed in the brain and peripheral tissues while CB2 receptors are expressed in immune cells (Lu & Mackie, 2016). However, recent studies have found CB1 receptor proteins and their messenger RNA (mRNA) in the liver, kidneys, testis, heart, uterus, ovaries and lungs (Bouaboula et al., 1993; Galiegue et al., 1995; Turcotte et al., 2016) providing a route for THC to impact multiple organ systems in the body. Bronchi in the lungs are the primary organs that come into direct contact with marijuana smoke. Thus, the impact of marijuana impact on respiratory health is a critical question (Turcotte et al., 2016).

Inhalation: A Major Route of Exposure

Inhalation is a major route of exposure to the components in marijuana, allowing them to enter the body via the respiratory tract where they are deposited. Though smoke is typically exhaled right after being inhaled, its components remain behind and interact with the lining of the bronchus, specifically the bronchial epithelial cells. Marijuana smokers have the tendency to hold the marijuana smoke in the respiratory tract for a longer period of time, compared to cigarette smokers. Cigarette smoke alters the epithelial cells of the bronchus, inducing inflammatory responses and oxidative stress as well as decreasing cell viability (Lee & Vassallo, 2012; Hoffmann et al., 2013). The impact of marijuana on the respiratory system, however, needs further exploration to understand its cellular impact. Symptoms indicative of a wide variety of respiratory diseases and conditions associated with marijuana smoke inhalation have been noted (Aldington et al., 2007; Macleod et al., 2015; Megarbane & Chevillar, 2013; Owen et al., 2014; Tan et al., 2009). These include coughing, wheezing, shortness of breath, acute bronchodilation, sputum production and airway obstruction (Aldington et al., 2007; Macleod et

al., 2015; Hancox et al., 2015). At issue are the underlying mechanisms associated with these altered respiratory health outcomes.

Known Effects of Marijuana Smoking and Underlying Mechanisms

Marijuana has been known to impact pulmonary function leading to respiratory complications both *in vivo* and *in vitro* (Martinasek et al., 2016; Tan et al., 2019; Tashkin, 2013; Volkow et al., 2014). Impacts include large airway impairments, inflammation, an accumulation of fluids that induce swelling, and excessive secretion of mucus in the respiratory tracts (Aldington et al., 2007; Howden & Naughton, 2011). These impacts, in turn, can lead to increases in wheezing, coughing, and production of sputum (Aldington et al., 2007; Ghasemiesfe et al., 2018). Such specific respiratory symptoms have a direct correlation to chronic bronchitis (Aldington et al., 2007). Increased risk for bronchitis occurs when sputum production accumulates and causes infection in the lining of the airway, severely reducing lung function (Hancox et al., 2015).

Overall particulate matter exposure when smoking unfiltered cannabis is about four times greater than when smoking the same amount of tobacco (Holland, 2010). Moreover, regular-use marijuana smokers can develop emphysematous bullae in the lungs, often resulting in lung asymmetrical bullous lung disease (Golwala, 2012; Johnson et al., 2000). This correlation is due to the longer inhaling and holding periods with marijuana smoking as compared to smoking other substances such as tobacco (Tashkin, 2013). The literature provides a general understanding of how marijuana smoke affects the respiratory tract as a whole. However, there is still little to no research on what specific marijuana components do to bronchial epithelial cells at the cellular and molecular levels.

Cannabinoids Effects on Cell Viability

Cannabinoids induce cell apoptosis in immune cells, dendritic cells, macrophages and airway cells (Rieder et al., 2010; Sarafian et al., 2003). Macrophages and lymphocytes exposed to THC cause significant DNA fragmentation as a result of increased BCL-2 gene expression and caspase-1 release (Zhu et al., 1998). Additionally, cortical neural cells exposed to THC resulted in membrane blebbing and translocation of cytochrome c in addition to caspase 3 activation (Campbell, 2001). Apoptosis can be a possible therapeutic implication, particularly to cure cancer (Fiandalo & Kyprianou, 2012). Apoptosis has been found in colon cancer cells, glioma cells, and prostate cancer cells exposed to cannabinoids (Cianchi et al., 2008; Greenhough et al., 2007; Ruiz et al., 1999; Salazar et al., 2009). Cannabinoids-induced apoptosis occurs in a ceramide-dependent manner, activating the release of caspase and resulting in cell death (Campbell, 2001; Carracedo et al., 2006). In contrast, lung cancer cells exposed to marijuana smoke and THC exhibited signs of induction of necrosis but inhibition of apoptosis. Specifically, marijuana smoke and THC exposure led to inhibition of caspase-3 and an increase in reactive oxygen species (Sarafian et al., 2001).

Oxidative stress is an imbalance of oxidants/radicals and antioxidants that plays an important role in triggering cell death (Orrenius et al., 2007; Ott et al., 2007). These substances that can mitigate the deleterious effects of reactive molecules using a myriad of mechanisms, for example, neutralization of free radicals by the donation of one of their own electrons. One occurrence that leads to oxidative stress is the dramatic release of reactive oxygen species (ROS), causing significant damage to lipids, protein, and DNA. Generations of ROS in lipids caused by the free radical chain reaction can produce lipid ozonation products (LOPs) (Schieber

& Chandel, 2014). When bronchial epithelial cells are exposed to LOPs, this causes activation of phospholipases and induction of inflammatory mediators such as prostaglandins.

Reactive oxygen species are generated in cells as part of normal physiological processes such as neutralization of bacteria by phagocytes. There are enzymatic and nonenzymatic antioxidants which detoxify ROS [47]. Glutathione (GSH), for example, is a well-known antioxidant that is found in the epithelial cells of the lungs. GSH has many functions, including antioxidant defense, metabolism of prostaglandins, gene expression, and apoptosis as well as signal transduction (Wu et al., 2004). One of its most noteworthy functions is in activating reactive oxygen species (Sies, 1991). The specific components in marijuana that cause an increase in ROS and lead to decreased levels of antioxidant GSH in bronchial epithelial cells are yet to be determined.

An epidemiological study evaluated both oxidant and pro-inflammatory cytokine levels in patients who had a cannabis dependency. In comparison to the control group, the cannabis-dependent group had a significant increase in cytokine levels of IL-6, IL-8, and IL1-β, suggesting that exposure to cannabis can lead to oxidative stress and inflammation (Bayzit et al., 2017). Similar research showed that Δ-9- THC can induce brain mitochondrial respiratory chain dysfunction via dose-dependent inhibition of respiration. There was also a dose-dependent increase of H_2O_2 production due to THC exposure (Wolff et al., 2015), suggesting that Δ-9- THC exposure can result in oxidative stress. However, conflicting research found that Δ-9- THC was able to reduce N-Methyl-D-aspartate-initiated oxidative stress and apoptosis by decreasing reactive oxygen species and increasing cell proliferation in AF5 cells, suggesting it has neuroprotective properties (Chen et al., 2005). Δ-9- THC has also been associated with an inflammatory response. Chronically exposed adolescent rats entered a neuroinflammatory state,

with an increase in inflammatory mediators such as COX-2, TNF-α, and nitric oxide synthase (Chen et al., 2005). Similarly, topical Δ-9- THC reduced corneal injury inflammation resulting from ocular surface injury (Thapa et al., 2018).[53. Because research on the inflammatory response to Δ-9- THC exposure is conflicting, additional research is required to more fully understand the impact of Δ-9- THC on oxidative stress. Another open question is whether Δ-9-THC produces similar level of oxidative stress and inflammatory response in other cell types, including bronchial epithelium.

Major Research Question

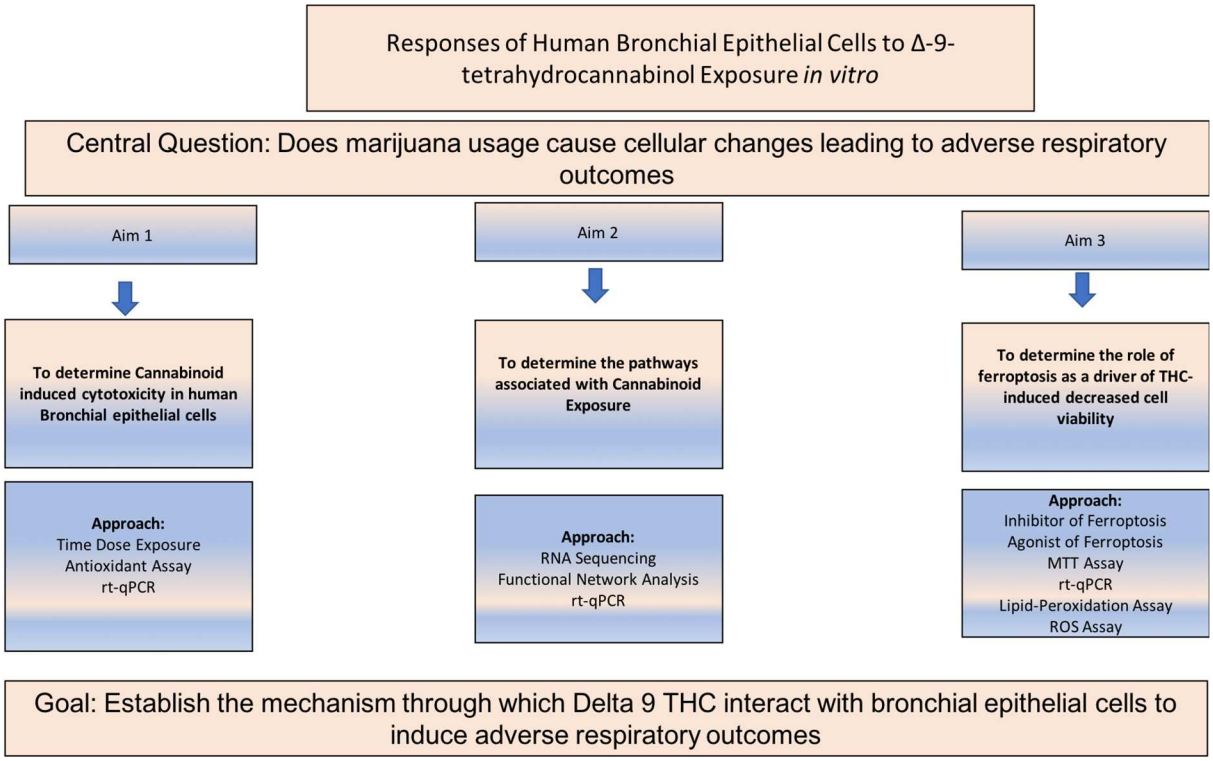
While Δ-9- THC has been shown to induce cytotoxicity, there is little understanding of how Δ-9-THC exposure causes cytotoxicity in bronchial epithelial cells. This dissertation explores three major questions to further our understanding. The major questions are:

i) Does Δ-9-THC induce cytotoxicity in human bronchial epithelial cells?

ii) What are the major molecular pathways associated with THC exposure-induced cytotoxicity in human bronchial epithelial cells?

iii) What role do the pathways play in Δ-9-THC-induced reduction in cell viability?

Figure 2. Research Approach



CHAPTER II: AIM 1: CELLULAR RESPONSES OF BRONCHIAL EPITHELIAL CELLS TO Δ-9-THC EXPOSURE IN VITRO

Introduction

Marijuana is a well-known substance with broad use in the North American population (Sarafina et al., 1999). Use of this drug has significantly increased during the COVID pandemic (McKay & Asmundson, 2020). Marijuana is composed of over 400 different chemical compounds with approximately 66 phytocannabinoids identified (Stout et al., 2012). Δ-9-tetrahydrocannabinol (Δ-9-THC), the primary psychoactive constituent, is also a partial agonist of the cannabinoid receptors. It mimics the endogenous cannabinoids of the body, producing a wide range of biological and behavioral responses (Jirschitzka et al., 2013).

Information on the impact of cannabinoid exposure on bronchial epithelial cells is limited. Cannabinoids induce cell apoptosis in immune cells, dendritic cells, and macrophages [34]. Δ-9-THC exposure can alter cell viability, induce cell death and produce oxidative stress *in vitro*. Macrophages and lymphocytes exposed to Δ-9-THC incur DNA fragmentation due to increased BCL-2 gene expression and caspase-1 release (Zhu et al., 1998). Additionally, cortical neural cells exposed to THC incur membrane blebbing and translocation of cytochrome c, in addition to caspase 3 activation (Campbell, 2001). Induction of apoptosis has possible therapeutic implications, particularly in the treatment of cancer (Fiandalo & Kyprianou, 2012). Induction of apoptosis has been found in colon cancer cells, glioma cells, and prostate cancer cells that were exposed to cannabinoids (Greenhough et al., 2007; Massi et al., 2006; Ruiz et al., 1999; Salazar et al., 2009). Cannabinoid-induced apoptosis occurs in a ceramide-dependent

manner, activating caspase and resulting in cell death (Campbell, 2001; Carracedo et al., 2006). Primary airway epithelial cells exposed to Δ-9-THC showed a decreased cell viability as well as reduced mitochondrial membrane potential, causing mitochondrial membrane damage (Abrams & Guzman, 2015; Pini et al., 2012). Similarly, neural studies showed that neural cells exposed to Δ-9-THC induce cell death via DNA damage, caspase activation, and cytochrome-c translocation. It is not known if bronchial epithelial cells respond in the equivalent way.

The first objective of this study is to determine cellular responses of bronchial epithelial cells exposed to Δ-9 THC. A range of concentrations of Δ-9 THC (0.06 to 6000 ng/ml) and time courses (24 hours to 9 days) was explored. From these results, one concentration and one-time course were selected, as they showed measurable effects. Following precedence in marijuana research, the Δ-9 THC was dissolved in both DMSO and methanol. Cannabinoid research requires a National Institute of Drug Abuse (NIDA) license that involves a lengthy application process. For efficiency, most research groups use Δ-9 THC that is commercially available from Sigma-Aldrich. Δ-9 THC from NIDA is dissolved in DMSO (Dimethyl Sulfoxide). Δ-9 THC from Sigma-Aldrich is dissolved in methanol (T4764). Δ-9 THC has different solubilities in DMSO and methanol, and that impacts Δ-9 THC uptake by bronchial epithelial cells.

Materials and Methods

Cell Culture and THC Treatment and Sample Collection

Cell culture used immortalized human bronchial epithelial cells (BEAS-2B) obtained from American Type Culture Collection (ATCC) in Manassas, Virginia. Cells were grown in LHC-9 (1x) media and maintained at 37° C in a humidified incubator with 5% CO2 and 95% air in vented T-75 flasks (430641U, New York). Δ-9-THC was purchased from Sigma Aldrich (T4764, Missouri). When cells were 95-100% confluent they were seeded in 24 well plates and

exposed to a range of concentration between 0.06 ng/ml and 6000 ng/ml of Δ-9-THC and vehicle (MEOH) for 24 hours. The cells were harvested after being exposed for 24 HRs, 96 HRs, and 9 days respectively, after which they were collected and frozen down.

Cell Viability Analysis

Once exposed, the cells were assessed using an inhouse 3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide (MTT) cell proliferation according to [28]. Briefly, the MTT assay measures the conversion of MTT (3-(4,5-Dimethylthiazol-2-Yl)-2,5-10 Diphenyltetrazolium Bromide) to an insoluble formazan. The MTT stock solution (2 mg/ml) in 1 X phosphate buffered saline (PBS) was diluted to a 1:9 ratio with media and added to the cells. The cells were incubated at 37°C for two hours. After incubation, the MTT solution was decanted, and cells were rinsed with 1 X PBS. DMSO was added and the plate scanned in a Biotek microplate reader at an absorbance of 570 nm and values were adjusted as percent control, graphed using Graphpad prism and analyzed using One-way ANOVA followed by Dunnett's post-hoc test for multiple comparisons.

RNA/DNA Extraction and cDNA Synthesis

Total RNA and DNA was scraped and extracted in bronchial epithelial cells using the ZR Duet kit (Zymo Research, D7003) per the user's guide. cDNA was synthesized from 2µg of total RNA per sample using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, 43668814). Oligo(dT) and random primer mix were used per the user's guide.

Real-Time Quantitative PCR (RT-qPCR)

Realtime qPCR was conducted using PowerUp SYBR ™ Green PCR Master Mix (Applied Biosystems) with a QuantStudio 3 (Applied Biosystems). Primers specific to Oxidative stress (*SENS2, IL8),* DNA Methylation *(DNMT and DNMT3a)* and Beta Actin (*BAC*) were

examined. Total RNA was reversed transcribed to generate cDNA, and transcript levels for these genes were determined by qPCR (SYBR Green method). Expression of the housekeeping gene Beta-actin was used to normalize the expression of target genes. Transcripts have been presented as fold change difference against control.

Table 1. Primer Sequences

Name	Primer Forward	Primer Reverse
BAC	5' GAA GAT CAA GAT CAT TGC TCC T 3'	5' TAC TCC TGC TTG CTG ATC CA 3'
hDNMT1	5' AGA TCT TGC CAA GA 3'	5' TGC GTC TCT CTT TT
HDNMT3A	5' CTG GAA GGA GGC TGA GA 3'	5' CTC CAC CTT CTG AGA CTC CC 3'
SENS2	5' GGC AGA AAC TTT GGG ATT GT 3'	5' GAC GCC TCT TCC CT 3'
IL8	5' CTC TGT GTG AAG GTG CAG 3'	5' AAA CTT CTC CAC AAC CCT CTG 3'

Protein Isolation and Protein Quantification

After the 9-day exposure, cells were collected and re-suspended in ice-cold 50 mM potassium phosphate buffer. The cell suspension was sonicated on setting 2 at 30 second intervals for 4 pulses, followed by centrifugation at 13,000 x g for 10 minutes at 4°C to remove cell debris. The supernatants were collected, and the protein concentration quantified using the Bio-Rad protein assay (23236, Bradford, Hercules, CA) using bovine serum albumin (BSA) as the standard.

Glutathione Assay

The measurement of total cellular glutathione (GSH) to measure the protein lysate from treated cells per accepted procedures (Jia et al., 2008). Fluorescence intensity was measured at an excitation wavelength of 350 nm and an emission wavelength of 420 nm. The sample GSH

content was calculated using a GSH (in-house protocol) standard curve and expressed as nanomoles of GSH per milligram of sample protein.

5-mC Assay

Global DNA methylation levels were measured in the DNA isolated from both the exposed and control cells using a Zymo research 5-mC DNA ELISA kit according to manufacturer's instruction (D5325, California). The wells were coated with DNA, Samples (100 ng) and Standards (100 ng) were added to PCR tubes and mixed with 5-mC Coating Buffer. DNA was denatured at 98°C for 5 minutes and then transferred to wells of a plate and incubated at 37°C for 1 hour. Following incubation, the plate was blocked, the buffer was discarded, and wells were washed 3 times with 200 µl of 5-mC ELISA Buffer. These steps were followed by 200 µl of 5-mC ELISA Buffer incubation at 37°C for 30 minutes. Antibody was then added according to the protocol and incubated at 37°C for 1 hour. This step was followed by Color development with HRP developer and absorbance measured at 405-450 nm using a plate reader.

Statistical Analysis

Results are expressed as mean ± SEM (standard error of the mean). Differences between the two groups were analyzed using a t-test. Dose response and time courses were analyzed by One-way ANOVA followed by Dunnetts post-hoc test for multiple comparisons. Statistical significance was considered at $p < .05$, unless otherwise stated.

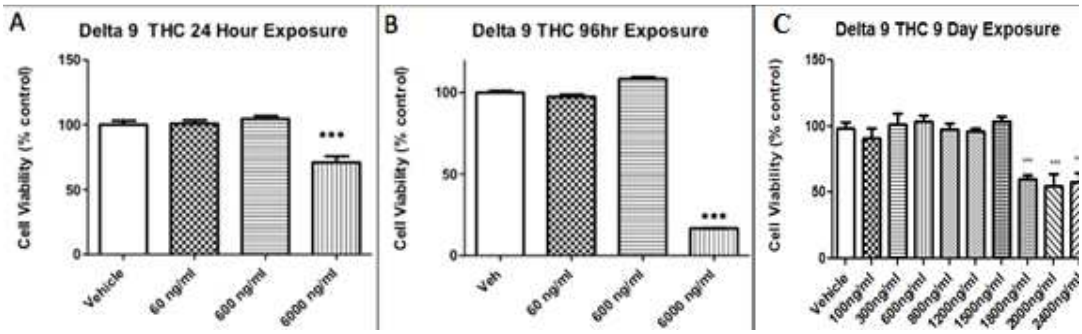
Results

Cell Viability

Research shows that in a marijuana cigarette Δ-9-THC ranges from 30 mg/ml to 60 mg/ml (Matthias et al., 1997). However, the amount of Δ-9 THC that is reportedly inhaled from the cigarette is roughly 82 µg/ml [62, 63]. Thus, the concentrations used for this study ranged

from 0.06 ng/ml to 1800 ng/ml. Though not as high as the actual concentrations, this is the highest amount that did not cause cell death *in vitro*.

Figure 3. Viability Levels of BEAS-2B Cells Treated with Δ-9-THC for 24 Hrs, 96 Hrs and 9 Days



When cells were 95-100% confluent they were seeded in 24 well plates and exposed to 60 ng/ml – 6000 ng/ml of Δ-9-THC and vehicle for 1-9 days. Early time points were sampled to gauge intermediate results. The later time points were selected to see the response after cells were given time to proliferate. After exposure, a 3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide (MTT) cell proliferation assay was performed. The results showed a significant decrease in cell viability at 6000 ng/ml in both 24 and 48 HR exposures as seen in Figs. 3A and 3B. When BEAS-2B cells were chronically exposed to Δ-9-THC for 9 days, there is a significant decrease in cell viability at concentrations of 1800 ng/ml-6000 ng/ml as seen in Figure 3C. These results show that the viability of airway cells exposed to Δ-9-THC is altered. The concentrations selected for the remainder of the study were 1500 ng/ml and 1800 ng/ml. The lower dose stimulated a response without affecting the cell viability, and the higher induced cell death. Based on these observations, it is evident that chronic exposure to Δ-9-THC induces cell death in bronchial epithelial cells. However, the question of which mechanism is causing cell

death is still not clear. The next step was to determine if the mechanism behind cell death was oxidative stress or epigenetic changes.

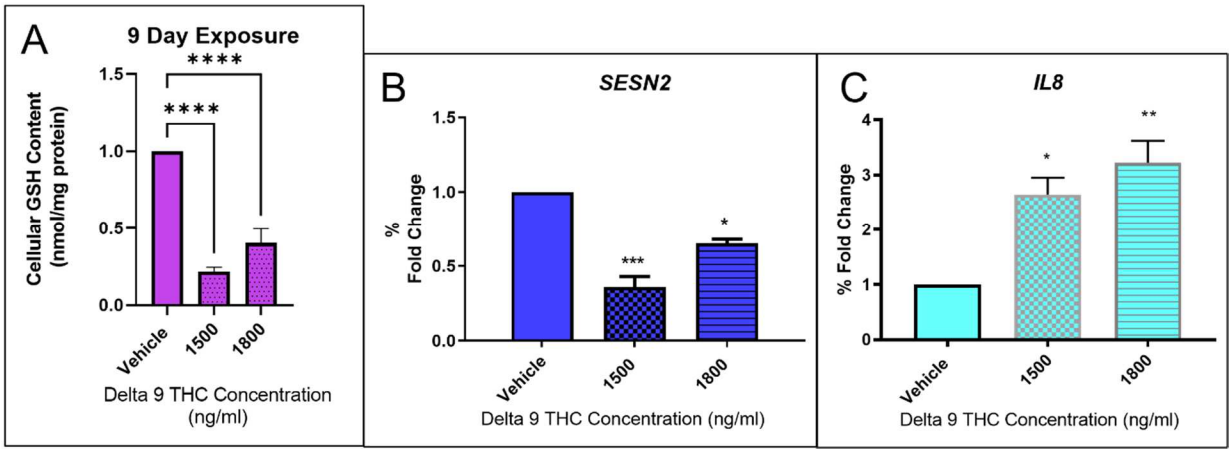
Oxidative Stress and Inflammation

Next, Δ-9-THC cells exposed to 1500 ng/ml and 1800 ng/ml were examined for changes in GSH levels as an indicator of oxidative stress. Results indicate that bronchial epithelial cells that are chronically exposed to Δ-9-THC show a decrease in cellular GSH levels (see Figure 4A). These data suggest that Δ-9-THC may be causing an increase in ROS by decreasing antioxidant levels. Detailed molecular and cellular processes that are involved were not identified.

Two other indicators of oxidative stress were also analyzed. Sestrins are a family of proteins that are known to populate in cells due to stress (Saveljeva et al., 2016). Sestrin 2 is specifically known to mediate intracellular reactive oxygen species accumulation (Hagenbuchner et al., 2012; Kopnin et al., 2007). Interleukin 8 (IL8) is a mediator of inflammatory response as it recruits and activate inflammatory cells (Remick, 2005).

To examine if sestrin 2 and IL8 were altered, gene expression was determined in bronchial cells chronically exposed to 1500 ng/ml and 1800 ng/ml of Δ-9-THC for 9 days. *SESN2* gene expression was significantly decreased and *IL8* expression was significantly increased in bronchial cells exposed to Δ-9-THC at 1500 ng/ml and 1800 ng/ml compared to the control (see Figure 4). This further suggests that oxidative stress and possible inflammation are both caused by Δ-9-THC exposure.

Figure 4. Oxidative Stress and Inflammatory Indicators in BEAS-2B Cells Treated with Δ-9-THC for 9 Days

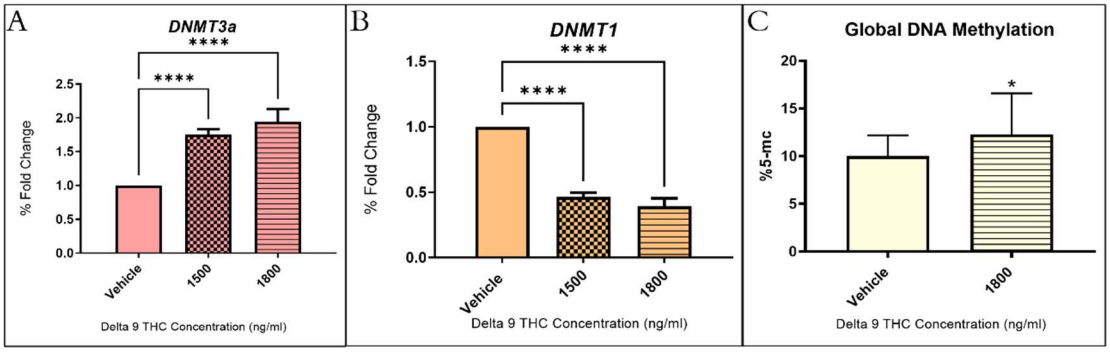


DNA Methylation

Two kinds of evidence can indicate changes in epigenetic parameters: alterations in expression patterns of genes involved in DNA methylation, and global level of 5mC methylation on DNA To examine if Δ-9-THC alters epigenetic parameters, bronchial cells were exposed with two concentrations of Δ-9 THC for 9 days, and all the parameters mentioned in the previous section were measured. Transcripts levels are presented as a fold change difference relative to the control (beta-actin). Results show that Δ-9-THC exposure caused a decrease in expression of DNMT3a and an increase in expression of DNMT1 genes, suggesting that Δ-9-THC induces epigenetic changes in bronchial cells *in vitro*.

Next, a 5-mC DNA ELISA was used to determine if a chronic 9-day exposure to Δ-9-THC alters global DNA methylation patterns in bronchial cells. Results found that Δ-9-THC exposure causes an increase in DNA methylation levels in bronchial cells *in vitro* (see Figure 5).

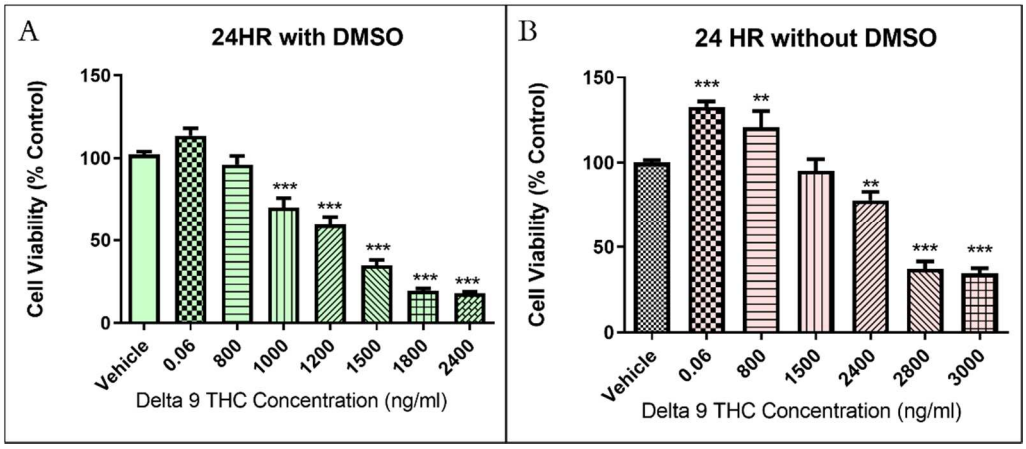
Figure 5. Expression of DNA Methyltransferase Genes (DNMT1 and DNMT3a) and Global DNA Methylation in BEAS-2B Cells Treated with Δ-9-9 THC



Cytotoxicity

To examine the potentiating effects of methanol and DMSO on Δ-9-THC, BEAS-2B cells were incubated with different concentrations (0.06 ng/ml-30000 ng/ml) of Δ-9-THC dissolved in methanol or methanol/DMSO mixed together. Viability was determined 24 hours after exposure. As shown in Figure 6, cell viability was significantly decreased by Δ-9-THC with the DMSO + methanol solvent at the concentration 1000 ng/mL. Cells exposed to Δ-9-THC solely in solvent methanol had a decrease at 2400 ng/mL. Δ-9-THC decreased cell viability at concentrations of 1800ng/mL-2400ng/mL during a 9-day exposure.

Figure 6. Visibility Levels of BEAS-2B Cells Treated with Δ-9-THC



Discussion

The present study investigated the cellular effects of Δ-9-THC exposure on bronchial epithelial cells. Δ-9-THC exposure resulted in decreased cell viability in a dose-dependent manner. The results showed that 24 HRs BEAS-2B cells exposed to Δ-9-THC in the vehicle mixture, MEOH/DMSO, resulted in a decrease in cell viability at 2400 ng/mL. At 96 hours Δ-9-THC resulted in a decrease in cell viability at 6000 ng/mL. And at 9 days, Δ-9-THC resulted in a decrease in cell viability at 1800 ng/mL. Bronchial cells exposed to marijuana smoke condensate showed a similar pattern of decreased cell viability (Turcotte et al., 2016). In human lung cancer cells, Δ-9-THC caused a dose-dependent decrease in mitochondrial membrane potential (Hoffman et al., 2013). This decrease was tied directly to cell death and a decreased production of ATP, and thus plays an essential role in apoptotic cell death (Adlington et al., 2007). Glutathione (GSH) is the major free radical scavenger in humans. Diminished GSH levels elevate cellular vulnerability to oxidative stress that is characterized by accumulating reactive oxygen species. In the current studies, exposure to Δ-9-THC to BEAS-2B cells for 9 days resulted in a decrease in GSH level. This outcome differs from other studies showing that smoke concentrate exposure leads to a decrease in ROS generation in bronchial cells, together with an increase in SOD and catalase. In mice, Δ-9-THC exposure resulted in a dose-dependent increase in mitochondrial H_20_2 production and exhibited oxidative stress as a result of exposure (Macleod et al., 2015).

Proinflammatory biomarker IL8 was significantly increased in our studies. Similar to an epidemiological study which evaluated both oxidant and pro-inflammatory cytokine levels in patients who had a cannabis dependence had an increase in proinflammatory biomarkers. Specifically, the cannabis dependent group had a significant increase in cytokine levels of IL-6,

IL-8, and IL1-β, in comparison to the control group. This suggests that exposure to cannabis can lead to oxidative stress and inflammation (Bayatzi, et al., 2017). Similar research (Wolff et al., 2015) showed that Δ-9-THC could induce brain mitochondrial respiratory chain dysfunction via dose-dependent inhibition of respiration. There was also a dose-dependent increase of H_2O_2 production as a result of Δ-9-THC exposure, suggesting that Δ-9-THC exposure can result in oxidative stress and inflammation.

In the present study, global DNA methylation levels and *DNMT3a* expression were significantly increased as a result of chronic, 9-day Δ-9-THC exposure in BEAS-2B cells. These results support a study that found hypermethylation in the sperm of the rats exposed to Δ-9 THC, compared to the control (Acarya et al., 2020). However, when Murphy et al. (2018) compared human sperm of marijuana to non-marijuana smokers, a decrease in DNA methylation was detected in the marijuana users. Δ-9-THC has also been found to cross-generationally alter DNA methylation in the nucleus accumbens of rats that were exposed (Watson et al., 2015). Taken altogether, there are numerous studies that show the effects of exposure to marijuana and its components on both sperm and neural cells (Boggs et al., 2018; Downer & Campbell, 2010; Ronan et al., 2016). DNA hypomethylation is known to be mediated by DNMT3a, and dysregulation of DNMT3a has been associated with ovarian cancer, testicular cancer, lung cancer and lung tumor progression (Chen & Chan, 2014; Chen et al., 2014; Gao, et al., 2011; Liu et al., 2018; Mostowska et al., 2013; Spencer et al., 2017; Yang et al., 2016). In our studies there was a decrease in *DNMT1* expression and an increase in *DNMT3a* expression as well as an increase in global methylation levels, suggesting that hypermethylation might be occurring as a result of exposure. However, the effect may not be maintained through DNA replication. There is a plethora of research that connects oxidative stress to DNA methylation and suggesting that

oxidative stress can alter DNA methylation (Donkena et al., 2010; Niu et al., 2015). Marijuana can elicit epigenetic modification, cytotoxicity and cell death in a variety of cell types.

Respiratory symptoms can be associated with marijuana exposure in humans (Aldington et al., 2007; Hancox et al., 2015; Macleod et al., 2015). The mechanisms underlying cannabinoid-induced respiratory effects are not well understood. The present study to addresses the effects of Δ-9-THC exposure on bronchial cells using the concentration below the circulating levels of Δ-9-THC in marijuana smokers after 24 hours. In this study Δ-9-THC in BEAS-2B exposed cells showed a similar decrease in viability in both the 24 HR and 9-day exposure. Because this study replicates cytotoxicity results from other studies, and because many other related studies use a 24 HR format, the remaining studies used just the 24 HR exposure (Chang et al., 2018; Sarafin et al., 2005). The current experiment found that the 1200 ng/mL concentration of Δ-9-THC is effective but not very toxic to BEAS-2B cells. Therefore, the same concentration will be used to characterize the mechanisms and pathways associated with the cell death phenotype observed.

Responses of BEAS-2B cells to Δ-9-THC exposure were extremely variable. An extensive literature search suggested that the intake of Δ-9-THC is not homogeneous due to the presence of solvent methanol. The addition of DMSO to the dissolved Δ-9-THC was tested to see if it would change the responses of BEAS-2B cells. Results found that bronchial cells exposed to Δ-9-THC dissolved in both methanol and DMSO decreased cell viability and had a dose dependent potentiation effect on cells. However, the cells that were exposed to Δ-9-THC dissolved in DMSO had substantial decrease in viability in comparison to the Δ-9-THC dissolved in methanol. DMSO is a highly polar organic solvent that is commonly used due to its having exceptionally low toxicity at low concentrations. DMSO is commonly used to solubilize poorly

soluble chemicals and is a well-known vehicle. DMSO at a 0.24% concentration is not toxic to cells and allows for the uptake of Δ-9 THC (45). This is consistent with the current literature as studies find that DMSO has little to no cytotoxic effect on colon tumor cells in concentrations up to 10% (Ott et al., 2007). A similar study showed that at 5% DMSO was not toxic and did not affect membrane integrity in isolated rat intestinal tissue. Although MEOH was previously used in this study as the primary solvent, most studies use Δ-9 THC provided by NIDA that is dissolved in DMSO. For the remaining studies 0.24% DMSO was used as a solvent.

CHAPTER III: AIM 2: TRANSCRIPTIONAL ALTERATIONS ASSOCIATED WITH Δ-9-THC-INDUCED CYTOTOXICITY IN BRONCHIAL EPITHELIAL CELLS

Introduction

Marijuana, derived from *Cannabis sativa,* is one of the most commonly used illicit drugs in the United States for both medicinal and recreational purposes (Mental Health Services Administration, 2017). Although marijuana is one of the oldest multi-usage cultivated crops, the primary use has always been medicinal (Holland, 2010). Delta 9 tetrahydrocannabinol (Δ-9-THC), the primary psychoactive constituent, is also a partial agonist of the cannabinoid receptors and mimics the endogenous cannabinoids of the body producing a wide range of biological and behavioral responses (Cooper & Hanery, 2009).

The endogenous cannabinoid receptors are part of a larger unit called the endocannabinoid system, which is composed of cell surface G protein-coupled cannabinoid CB1 and CB2 receptors, which are known to regulate cellular processes such as cell survival, cell death and cell proliferation (Goode, 2017; Lu & Mackie, 2016). The CB1 receptors are located throughout the body and can be found in the brain, liver, kidneys, ovaries and the lung (Bouaboula et al., 1993, Galiegue et al., 1995; Turcotte et al., 2016). The lungs are a major route of exposure as inhalation allows for substances to enter the body and interact with the components of the respiratory tract. Inhalation of marijuana smoke has been associated with respiratory symptoms such as cough, shortness of breath, acute bronchodilation, wheezing, and airway obstruction (Aldington et al., 2007; Hancox et al., 2015; Macleod et al., 2015). Epidemiological studies have found an increase in the prevalence of symptoms indicative of a

wide variety of respiratory diseases and conditions (Adlington et al., 2007; Megarbaine & Chevillard, 2013; Owen et al., 2014; Tan et al., 2009). Cough, wheezing, shortness of breath, acute bronchodilation, sputum production and airway obstruction were among symptoms (Adlington et al., 2007; Hancox et al., 2015). Taken together, marijuana exposure to airway cells can have adverse effects. However, the underlying mechanism of these adverse effects is not fully understood.

Human cells or cell lines exposed to Δ-9 THC result in cell death, inflammation and oxidative stress (Blanco et al., 1998; Mohanraj et al., 2011; Zaha et al., 2020). Oxidative stress induced by marijuana exposure may also mediate the release of pro-inflammatory cytokines. In an epidemiological study, patients who had a cannabis dependency exhibited elevated levels of IL-6 and IL-8, indicating that exposure can induce inflammation in humans (Mohanraj et al., 2011). Primary airway epithelial cells that were exposed to Δ-9-THC showed a decreased cell viability as well as mitochondrial membrane potential, causing mitochondrial membrane damage. These findings are consistent with studies which show that neural cells exposed to Δ-9-THC undergo cell death via DNA damage, caspase activation and cytochrome-c translocation (Rigoglou & Papavassiliou, 2013). Previous studies have also demonstrated that exposure to cannabinoids induces oxidative stress (Blanco et al., 1998). Oxidative stress occurs as a result of an accumulation of reactive oxygen species (ROS). The human body has many defense mechanisms to counteract oxidative stress. The antioxidant glutathione (GSH) and the protein senstrin2 (SENS2) are known to repress and detoxify ROS. Δ-9-THC has been found to increase H_2O_2 production, which can also lead to oxidative stress, whereas CBD has been found to decrease ROS by increasing GSH levels [89, 93]. Literature clearly suggests that the Δ-9-THC

exposure can result in cell death, which may be the consequence of increased ROS; however, the mechanism behind cell death is not clearly understood.

The present study seeks to determine the mechanism underlying Δ-9-THC-induced bronchial cell death that was demonstrated in the previous chapter. At issue is if Δ-9-THC produces the same transcriptional responses in bronchial epithelial cells as it does in the bronchi of cannabis smokers. To understand the effects of Δ-9 THC at the molecular level, RNAseq was performed using BEAS-2B cells exposed to Δ-9-THC at various concentrations, ranging from low to high (Lc50). The dose-responses of Δ-9-THC exposure using human relative concentrations, on bronchial epithelial cells *in vitro* were determined. The transcriptional responses of bronchial epithelial cells were determined by RNA sequencing followed by gene ontology enrichment (GO) and pathway analyses. Results reveal a novel cell death pathway caused by Δ-9-THC exposure in bronchial epithelial cells.

Materials and Methods

Cell Culture, Δ-9 THC Exposure, and Sample Collection

Cell culture was conducted using immortalized human bronchial epithelial cells (BEAS-2B), which were obtained from American Type Culture Collection (ATCC) in Manassas, Virginia. Cells were grown in LHC-9 (1x) media and maintained at 37° C in a humidified incubator with 5% CO2 and 95% air in vented T-75 flasks (430641U, New York). Δ-9-THC was purchased from Sigma Aldrich (T4764, Missouri). When cells were 95-100% confluent they were seeded in 24 well plates and exposed to 800 ng/ml, 1000 ng/ml 1200 ng/ml and 1500 ng/ml of Δ-9-THC and vehicle (.24% DMSO and 0.015 MEOH) for 24 hours. The cells were harvested after the 24-hour exposure, collected and frozen down.

Cell Viability Analysis

After exposure was completed, cells were assessed using an inhouse 3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide (MTT) cell proliferation according to [28]. Briefly, the MTT assay measures the conversion of MTT (3-(4,5-Dimethylthiazol-2-Yl)-2,5-10 Diphenyltetrazolium Bromide) to an insoluble formazan. The MTT stock solution (2 mg/ml in 1 X phosphate buffered saline (PBS) was diluted to 1:9 ratio with media and added to the cells. The cells were incubated at 37°C for two hours. After incubation, the MTT solution was decanted, and cells were rinsed with 1 X PBS, DMSO was added and the plate scanned in a Biotek microplate reader at an absorbance of 570 nm and values were adjusted as percent control, graphed using Graphpad prism and analyzed using One-way ANOVA followed by the Dunnett's post-hoc test for multiple comparisons.

RNA Extraction and cDNA Synthesis

Total RNA was extracted from the cells using the ZR Duet kit (Zymo Research, D7003) according to the user's guide. The concentration and purity of the RNA was determined by using a nanodrop. Following extraction, cDNA was synthesized from 2µg of total RNA per sample using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, 43668814). Oligo(dT) and random primer mix were used following the guidelines provided by the user's guide.

Library Preparation and RNA Sequencing

The RNA-sequencing library was prepared in accordance to the NEBNext® Ultra II RNA Library Prep Kit for Illumina (E7775, New England BioLabs) according to manufacturer's instruction. RNA was then purified, fragmented, isolated and primed for double stranded cDNA synthesis. Sequence adaptors and indexes were then ligated to the end of the dscDNA sample.

Polymerase chain reaction was then used to amplify the sample through 13 cycles. Quality control was then completed by cleaning up, assessing and quantifying the amplified cDNA on a bioanlyzer. RNA Sequencing was performed on a HiSeq X Ten (Illumina) platform (150 bp PE) at Novogene Corporation (Davis, California) at a target depth of 40 million reads per sample. Raw sequence data was analyzed using DAVID 6.8 gene set enrichment webtool.

Pathway Analysis

Enrichment analysis of significant genes were performed using Gene Ontology and Ingenuity Pathway Analysis (IPA). IPA software was also used to reveal the pathways that were most altered as a result of exposure to different concentrations of Δ-9-THC.

Gene Validation by Real-Time Quantitative PCR (qPCR)

Realtime qPCR was conducted using PowerUp SYBR ™ Green PCR Master Mix (4385610, Applied Biosystems) with a QuantStudio 3 (Applied Biosystems). Primers specific to Ferroptosis (*HO1, FTH1, FTL,* and *GCLC*), unfolded protein response (*PERK*) and the NRF2 pathway (*NRF2 and SQSTM1*) were examined.

Table 2. Seq Primer Sequences

FTH1	5' GCA CGA GCA AGT CAA GAC CAT 3'	5' CTT GTC GAA CAG GTA CTC AGC 3'
SQSTM1	5' GCC ATA CCC TCT TCG ACT ACG 3'	5' GAT TCT GGC ATC TGT AGG 3'
PERK	5' CTT ATG CCA GAC ACA CAG AA 3'	5' TCC ATC GTG CTG AAT GGA ATA C 3'
HO1	5' CTC TGA AGT TTA GGC CAT TG 3'	5' AGT TGC TGT AGG GCT TTA TG 3'
FTL	5' CAG CCT GGT CAA TTT GTA CCT 3'	5' GCC AAT TCG CGG AAG AAG TG 3'

Statistical Analysis

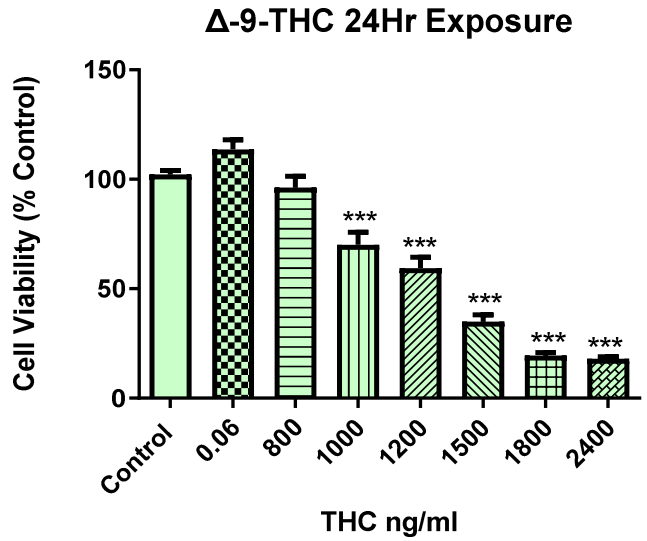
The viability and qPCR gene expression data are expressed as mean ±SEM. Differences between two groups were analyzed using a T-test. Following One-way ANOVA, Dunnetts post-hoc test was used for comparison between the three groups. Statistical significance was considered at $p < 0.05$, unless otherwise stated.

Results

Cell Viability

To examine the toxic effects of Δ-9-THC, BEAS-2B cells were incubated with different concentrations (0.06 ng/ml-2400 ng/ml) of Δ-9-THC and viability was determined 24 hours after exposure. As shown on Figure 7, cell viability was significantly decreased by Δ-9-THC at the concentration 1000 ng/mL and above.

Figure 7. BEAS-2B Cells Treated with Δ-9-THC for 24 Hours



Differentially Expressed Genes

To investigate the impact of Δ-9-THC exposure on bronchial epithelial cells, RNA-seq analysis was performed. RNA was extracted from BEAS-2B cells exposed to concentrations ranging from 800 ng/mL to 1500 ng/mL for 24 HRs as shown in Figure 7. The isolated RNA was sequenced as described in Materials and Methods. Differentially expressed genes (relative to the untreated control) were identified using DAVID 6.8 gene set enrichment webtool. The differentially expressed genes were then analyzed using Ingenuity Pathway Analysis ® (IPA ® v. 9.0) and R scripts were used to generate and determine the pathway of differentially expressed

genes in the different Δ-9-THC concentrations. There were more similarities in gene expression in concentrations 800 ng/mL and 1000 ng/mL and 1200 ng/mL and 1500 ng/mL. (See Figure 8A). There were 47 transcripts that were significantly changed in at all concentrations of Δ-9 THC exposed cells. (See Figure 8B). Hierarchical clustering of these transcripts reveals different mRNA signatures in the Δ-9-THC exposed bronchial epithelial cells (Figure 9). The dendrogram analysis of relative expression indicated that the 800 ng/mL concentration was more similar to the vehicle control whereas 1200 and 1500 ng/mL were more similar to one another but different from the controls and the other treatment groups. (See Figure 10). Even with the differences, there were similarities in all concentrations with both upregulated and down regulated genes.

Figure 8. Heatmap Showing High Upregulated and Downregulated and Venn Diagram of Differentially Expressed Genes

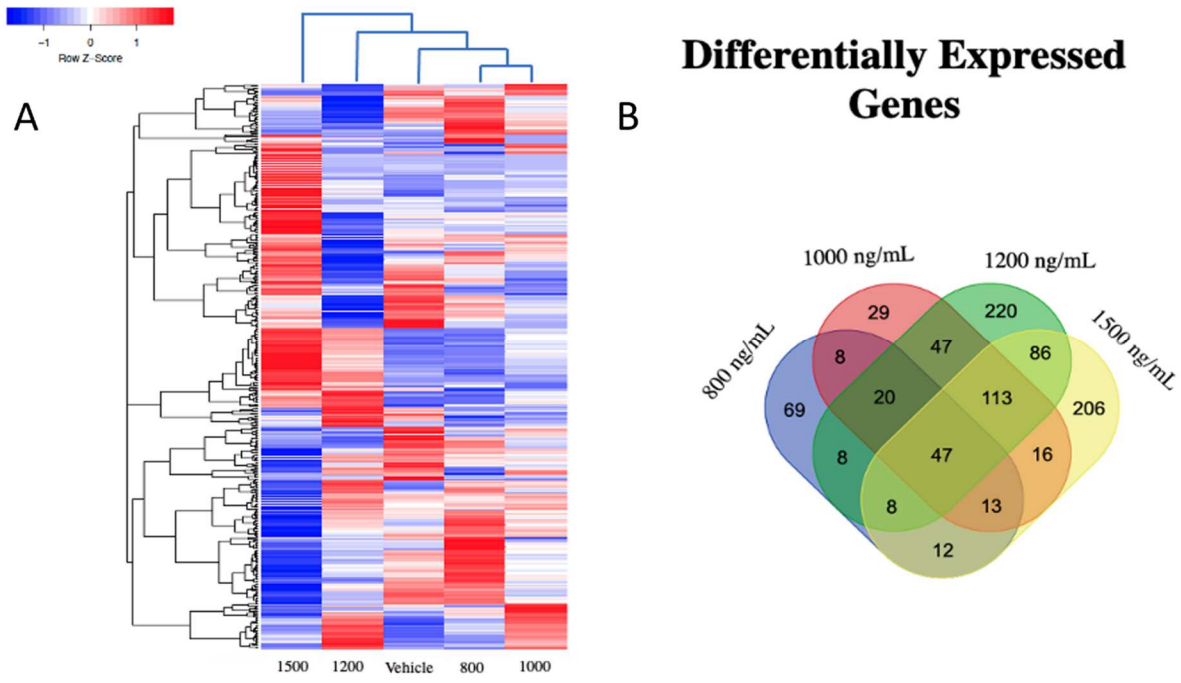


Figure 9. Hierarchical Clustering of Genes with the Most Significant Log 2-Fold Change

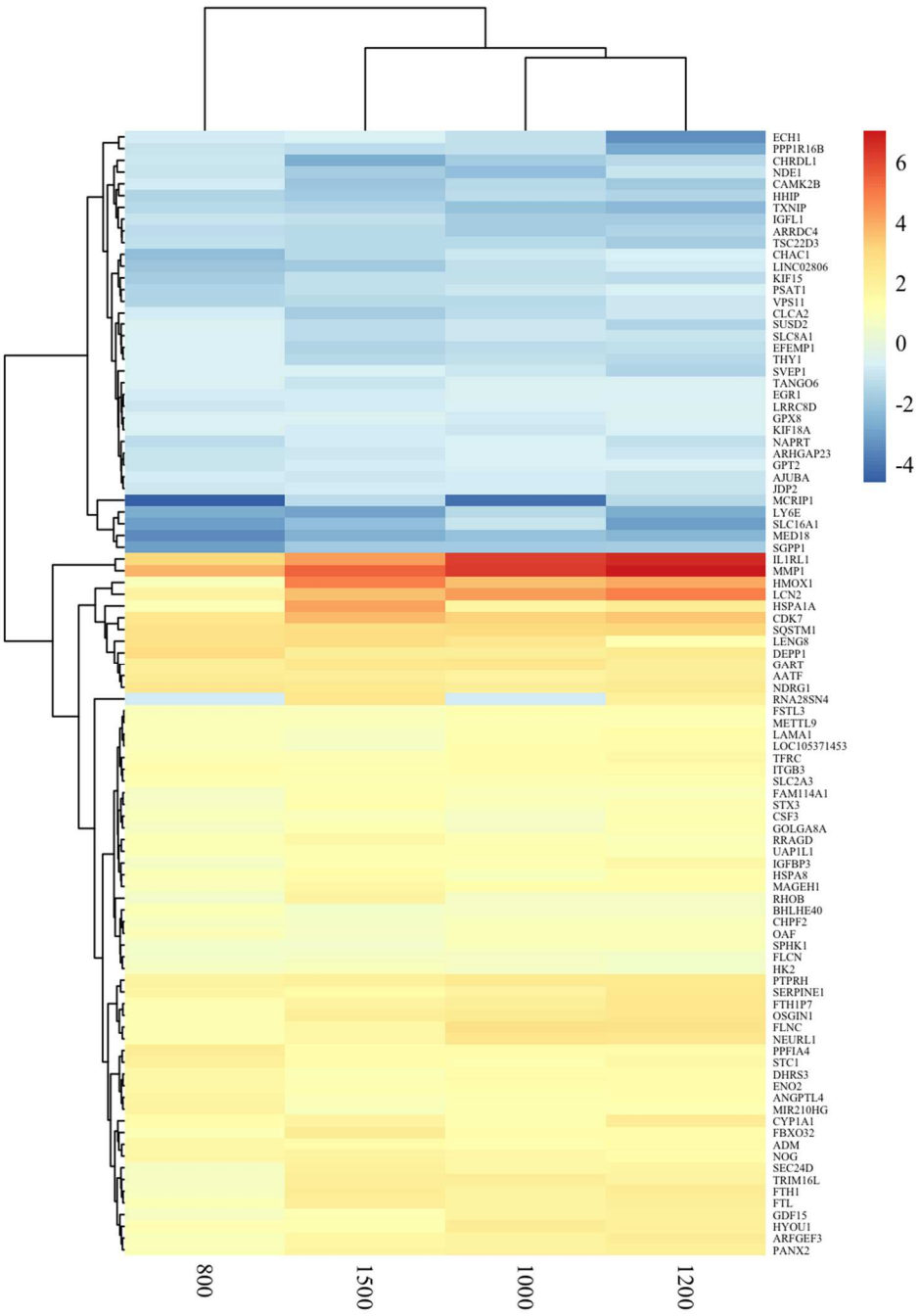


Figure 10. Dendrogram of Gene Relationship

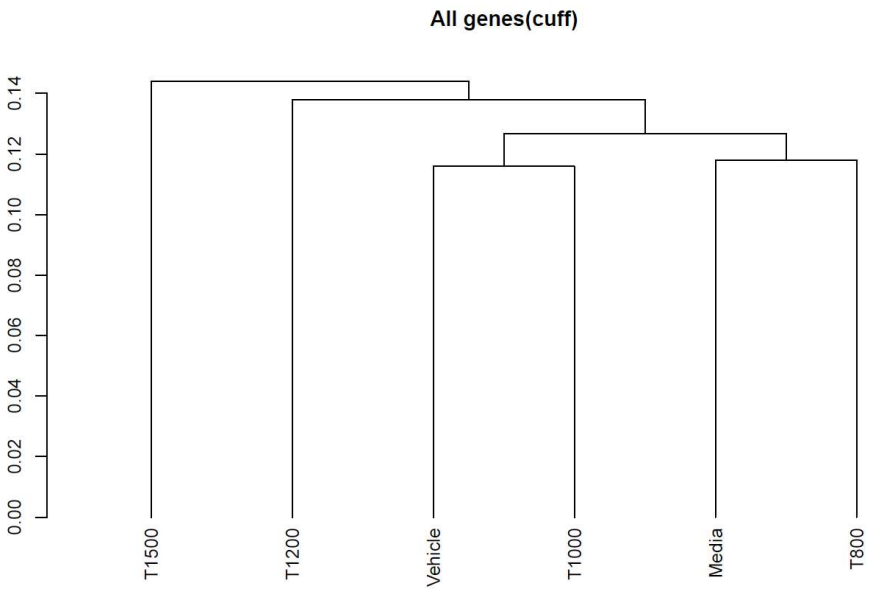


Table 3. Top 10 Dysregulated mRNAs in Δ-9-THC 24hr Exposure

Downregulated				
Concentration		Gene symbol	Log2 Fold change	Q Value
800	1	MCRIP1	-4.58684	0.001878
	2	SSBP1	-4.53692	0.001878
	3	GFUS	-3.81228	0.001878
	4	MED18	-3.44421	0.030653
	5	GSA	-3.39334	0.001878
	6	TADA2A	-3.37367	0.004818
	7	TAF9	-3.36146	0.017186
	8	SLC16A1	-3.06372	0.001878
	9	MRPS12	-3.06178	0.001878
	10	SGPP1	-3.02411	0.008371
1000	1	SLC39A4	-4.7043	0.006095
	2	GBA	-4.31396	0.001878
	3	MCRIPL	-4.02778	0.001878
	4	RPS25	-3.86789	0.001878
	5	FAN1	-3.76331	0.017186
	6	PCK2	-3.67426	0.003423
	7	GFUS	-3.54866	0.001878
	8	SLC25A24	-3.39671	0.006095
	9	EIF3K	-3.00309	0.001878
	10	DDT	-2.98129	0.001878
1200	1	SLC39A4	-5.29575	0.012503
	2	ATOH8	-4.461	0.001878
	3	NDUFS3	-3.81763	0.001878
	4	TNNI2	-3.42941	0.009452
	5	ECH1	-3.37068	0.001878
	6	DHRS4	-3.36445	0.004818
	7	PEG10	-3.11481	0.001878
	8	PCDH18	-3.04253	0.001878
	9	TAF9	-3.01899	0.022339

	10	RP L7A	-3.00786	0.001878
1500	1	SLC39A4	-4.36553	0.001878
	2	GFUS	-4.0102	0.001878
	3	MTCO2P12	-3.50199	0.02154
	4	SYT8	-3.24789	0.001878
	5	SURF2	-3.19974	0.009452
	6	KIFC1	-3.17516	0.031878
	7	LYSE	-2.93844	0.001878
	8	ANKRD2	-2.81355	0.011499
	9	SLC25A24	-2.74909	0.031878
	10	TNNI2	-2.68063	0.001878

Upregulated

Concentration		Gene symbol	Log2 Fold change	Q Value
800	1	ABR	4.92421	0.004818
	2	FAIN/120C	4.37887	0.001878
	3	SLCC5AL	4.13916	0.001878
	4	MMP1	3.81462	0.001878
	5	ATFSB	3.5628	0.001878
	6	SLC25A6	3.50842	0.001878
	7	NEDD8	3.39449	0.001878
	8	IL1RL1	3.18243	0.001878
	9	C1RL	3.07876	0.014481
	10	BRD2	3.0684	0.001878
1000	1	MT1G	inf	0.001878
	2	MMP1	6.28529	0.001878
	3	IL1RL1	6.12073	0.001878
	4	KLHDC7B	4.59062	0.008371
	5	LCN2	4.29486	0.001878
	6	RAD17	4.2562	0.011499
	7	HSPA6	3.95743	0.001878
	8	NEDD8	3.85935	0.001878
	9	HMOX1	3.54616	0.001878

	10	ZNF469	3.49049	0.001878
1200	1	CDC42EP5	inf	0.001878
	2	MT1G	inf	0.001878
	3	RN7SKP11	inf	0.001878
	4	MMP1	7.01104	0.001878
	5	IL1RL1	6.57479	0.001878
	6	LCN2	4.88431	0.001878
	7	HSPA6	4.42459	0.001878
	8	KLHDC7B	4.29577	0.012503
	9	NCF2	4.12327	0.004818
	10	HMOX1	4.07414	0.001878
1500	1	LOC646938	inf	0.026199
	2	TNKS2-AS1	inf	0.018979
	3	RPS27P9	inf	0.017186
	4	MT1G	inf	0.001878
	5	SPRR1B	inf	0.001878
	6	RPS18	8.50609	0.022339
	7	HSPA6	7.77894	0.001878
	8	MMP1	5.43214	0.001878
	9	HSPA1B	5.16909	0.001878
	10	HMOX1	4.82331	0.001878

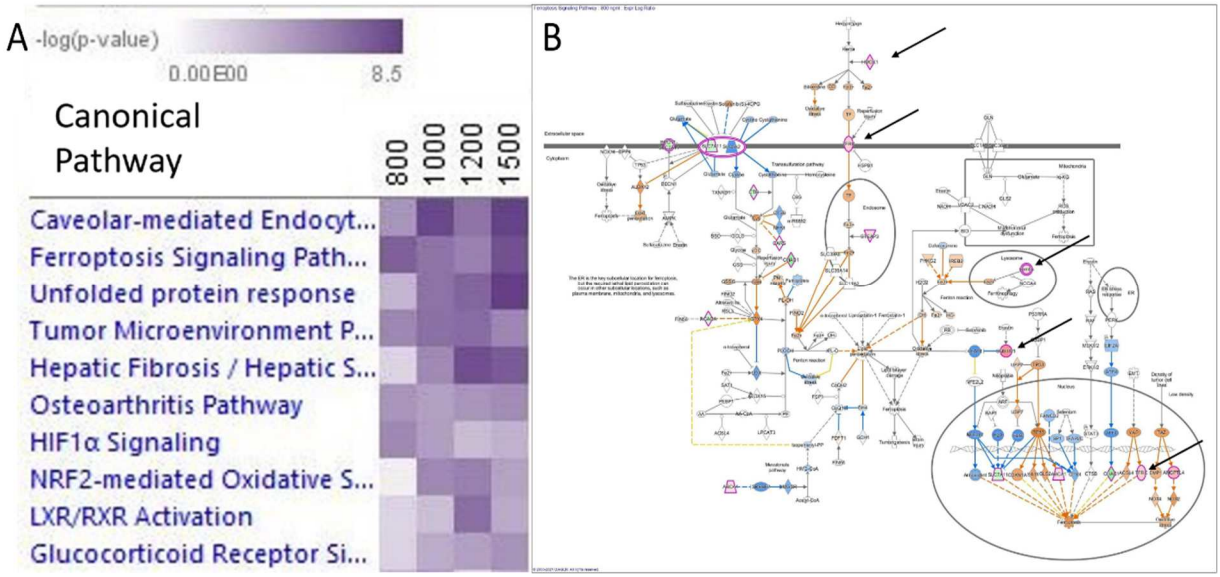
Molecular Pathway and Functional Network Analysis

To further compare the molecular effects associated with Δ-9-THC exposure in bronchial epithelial cells, the canonical pathways and networks associated with Δ-9-THC exposure were examined. The differentially expressed genes were analyzed for canonical pathway and network associations. The most affected pathways in Δ-9-THC 800 ng/mL treated cells were the Ferroptosis Pathway (P- 9.6E-08), Tumor Microenvironment Pathway (P-5.3E-06), HIF1A Signaling Pathway (2.13E-05), Osteoarthritis Pathway (9.6E-08) and the Caveolar-mediated Endocytosis Pathway (P-2.27E-05) (Figure 11A). The pathways that were most affected in 1000ng/ml treated cells were the Tumor Microenvironmental Pathway (P- 4.66E-07). Caveolar-

mediated Endocytosis Pathway (P-8.52E-09), Tumor Microenvironment Pathway (P- 4.66E-07), Unfolded Protein Response (P- 6.30E-07), Ferroptosis Pathway (P- 7.26E-07), and the NRF2-mediated Oxidative Stress Response Pathway (P- 5.48E-06). (See Figure 11A). The pathways that were most impacted in the Δ-9-THC 1200 ng/mL exposed cells were the Hepatic Fibrosis/ Hepatic Stellate Cell Activation Pathway (P- 1.60E-08), Unfolded Protein Response (P- 1.611E-07), Tumor Microenvironment Pathway (P-3.10E-07), LXR/RXR Activation Pathway (P- 4.02E-07), and the Caveolar-mediated Endocytosis Pathway (P- 6.72E-07). The pathways most impacted in the 1500 ng/mL exposed cells were the Unfolded Protein Response Pathway (3.52E-09), Caveolar-mediated Endocytosis Pathway (P- 4.49E09), Ferroptosis Pathway (P- 2.02-08), Hepatic Fibrosis/ Hepatic Stellate Cell Activation Pathway (P- 5.53E-08) and the Super pathway of Cholesterol Biosynthesis (P- 9.39E-07) (See Figure 11A).

The Molecule Activity Predictor (MAP) tool was used to predict how the up-regulated and down-regulated genes in the database could impact the activity of the other molecules within specific pathways (See Figure 11B). This analysis suggests that exposure to Δ-9-THC leads to activation of Ferroptosis with many genes being upregulated as a result of exposure.

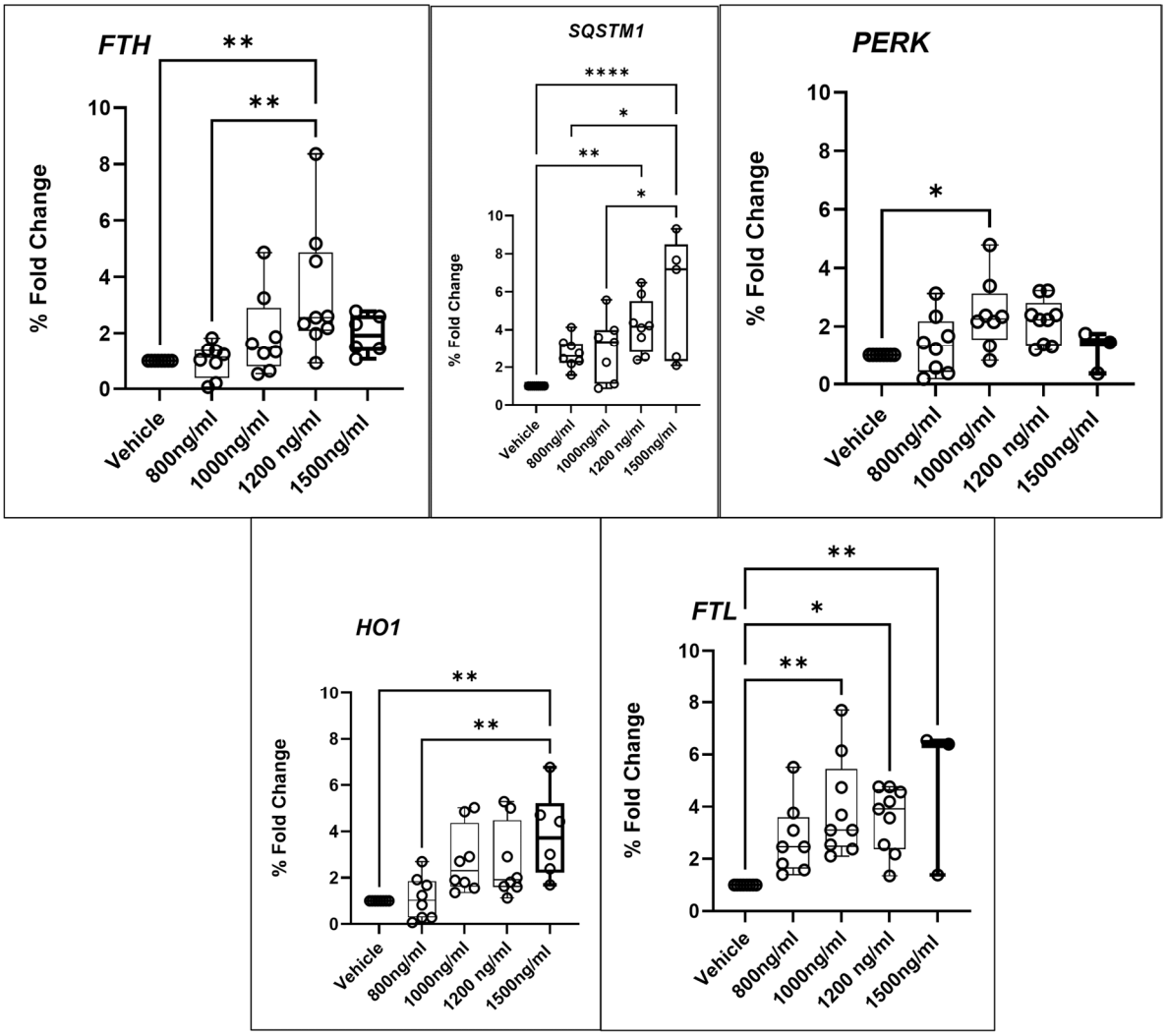
Figure 11. Common Canonical Pathways Disrupted in Bronchial Cells Exposed to Different Concentrations of Δ-9-THC



Validation of Gene Expression Data by Real-Time Reverse Transcription Quantitative PCR (RT-qPCR)

To validate the gene expression data, several up-regulated targets identified by RNA seq data were selected and their transcription levels by RT-qPCR were examined. An up-regulation of essential ferroptosis enzyme, *HO1* and iron storage proteins ferritin heavy chain (*FTH1)*, ferritin light chain *FTL1* and *SQSTM1* associated with autophagy were all detected, in parallel with the RNA-sequencing results.

Figure 12. RT-qPCR Analysis Confirming the Validity of the RNA-seq Results



Discussion

Because marijuana use for medicinal and recreational purposes is increasing rapidly, understanding the alterations the constituents of marijuana make in the lung epithelium is important for dealing with potential lung issues. The present study investigated the impact of 24-hour Δ-9-THC exposure on transcriptional profiles of bronchial epithelial cells by RNA sequencing. Cell viability data shows that exposure to Δ-9-THC induces cytotoxicity and decreases cell viability.

The cell viability was confirmed by MTT assay. Several top canonical pathways were altered by Δ-9-THC, among which ferroptosis, "tumor microenvironmental" and the "unfolded protein response" pathways were the major pathways. A gene ontology enrichment analysis showed that the genes that were significantly altered as a result of exposure were, *H01, CDK7, HLA-C,* and *SLC39A4*. Finally, to validate the gene expression data, RT-qPCR was used to examine upregulated genes, *H01, FTL1, FTH1,* and *PERK*, which are associated with ferroptosis and unfolded protein response pathway.

Reasearch has shown that Δ-9-THC exposure can lead to cell death in various cell types, including airway cells. Specifically, exposure causes the initiation of apoptosis in oral cancer cells and cortical neurons, as well as in immune cells (Campbell, 2001; Goncharov et al., 2005; Sarafian et al., 2001; Semlali et al., 2021). In the present study, bronchial airway cells that were exposed to Δ-9-THC concentrations lower than the human relative concentration suffered cell death. Although a potential link between Δ-9-THC and cell death has been shown in airway cells (Sarafinian et al., 2001), no molecular mechanism has been clearly identified thus far. The present study reveals for the first time a link between Δ-9-THC and the ferroptosis pathway that was consistently represented in all treatment groups, although in a non-monotonic manner. A low concentration and high concentration of Δ-9 THC caused similar changes in the transcriptome profiles.

Ferroptosis is a cell death process that is iron dependent and characterized by lipid peroxidation. This process is mediated by phospholipid peroxidation in association with free iron-mediated fenton reactions (Yang & Stockwell, 2016). A correlation between Δ-9-THC exposure and cell death has been demonstrated. However, iron-dependent cell death has not yet been demonstrated. In a study with the same type of cells as those used in the present study,

ferroptosis was found to be one of the major pathways affected by exposure to cigarette smoke condensate (Yosida, et al., 2019). Another study that exposed airway cells to the cigarette smoke showed an up-regulation in *HO1* as seen in the present studies (Park et al., 2019). HO1 is a widely used biomarker for oxidative stress and plays a critical role in ferroptosis, as it processes heme and release Fe^{2+}. This is highlighted in a study that used EF24 to induce apoptosis in osteosarcoma cells and determined ferroptosis was involved in cell death that was mediated by HO1 (Lin et al., 2021). When *HO1* was upregulated, it resulted in ferroptosis in the osteosarcoma cells and had the exact opposite effect when *HO1* was knocked down, thus showing its involvement in ferroptosis. This confirms the earlier study in this dissertation showing the up-regulation of HO1 due to Δ-9-THC exposure and the associated cell death.

Another key component of ferroptosis is ferritin, Ferritin is a major intracellular iron storage protein that is involved in intracellular iron homeostasis. Ferritin is a heteropolymer that includes FTL1 (Ferritin light polypeptide 1) and FTH1 (Ferritin heavy polypeptide 1) and protects cells from iron-mediated damage (Miotto et al., 2020). In the present study there was an increase in both *FTH1* and *FTL1* expression in Δ-9-THC exposure cells. However, studies from Hou *et al.* showed a decrease in FTH1 levels in HA-exposed leukemia cells as a result of degradation of ferritin by autophagy, leading to an increase and accumulation of free iron (Acharya et al., 2020). In contrast, and similar to the present studies, FTL1 expression was increased in RAW264.7 cells that were exposed to LPS (Murphy et al., 2018). In addition to being an iron storage protein, FTL1 is also known to be an antioxidant. The significant increase in *FTL1* gene expression observed in the present study may be the result of the oxidative stress. Specifically, lipid peroxidation may be occurring because of Δ-9-THC exposure. It is reasonable to assume that if the level of lipid peroxidation increases, the FTL1 and FTH1 proteins may

increase in a protective manner as shown in this study. Similarly, HeLa cells that were exposed to Doxorubicin (DOX) had an increase in ferritin mitochondrial expression to reduce the cytotoxicity that occurred as a result of DOX exposure (Watson et al., 2015). Deregulation of ferroptosis resulting from airway cell exposure to cigarette smoke extract has been linked to Chronic Obstructive Pulmonary Disease (COPD) (Haugenbuchner et al., 2012). As cigarette smoke and marijuana utilize the same mechanism to penetrate through the airway cells into the body, this can be a possible problem. Taken together, it appears that Δ-9-THC exposure may lead to ferroptosis, which may play a role in COPD pathogenesis.

The current studies showed an opposing relationship in the nuclear factor E2-related factor (NRF2) and osteoarthritis. Osteoarthritis is characterized by the death of chondrocytes and the increase of oxidative stress and inflammation (Martinasek et al., 2016; Tan et al., 2019; Tashkin, 2013). The present results are consistent with a study in which IL-1β-stimulated human chondrocytes overexpressed NRF2, resulting in a decrease in oxidative stress and apoptosis markers (Tetrault et al., 2007). Similarly, Volkow et al. (2014) showed IL-1β-stimulated human chondrocytes that were treated with eriodyctiol (that has antioxidant and anti-inflammatory properties) inhibited the anti-inflammatory and activated the NRF2 signaling pathway. This illustrates the connection between the upregulation of the NRF2 pathway attenuating the inflammation, and oxidative stress driven by upregulation of the osteoarthritis pathway. It appears that Δ-9-THC is working in a similar manner by upregulating NRF2 expression and leading to a decrease in inflammatory expression in bronchial cells.

NRF2 pathway is an inducer of cytoprotective enzymes such as *HO1, GSH, FTH1* and FTL1: all major components in the ferroptosis pathway and are highlighted in our study. NRF2 is activated by oxidative stress resulting in an increase of antioxidants to detoxify and eliminate the

reactive oxidants (Howden &Naughton, 2011; Ghasemiesfe et al., 2018). Δ-9-THC has been shown to induce oxidative stress in different cell types such as human placental, neuron and glioma cells (Golwala, 2012; Megarbane & Chevillard, 2013; Moore et al., 2005). NRF2 plays a significant role in the mitigation of ferroptosis and lipid peroxidation (Johnson et al., 2000). Specifically, NRF2 activates the antioxidant responsive element-dependent genes that counteract and balance oxidative mediators to restore and maintain cellular redox homeostasis (Rieder et al., 2010). NRF2 also mediates the Hepatic Fibrosis/ Hepatic Stellate Cell Activation Pathway, that is characterized by a net accumulation of extracellular matrix and scar tissue as a result of chronic liver injury (Sarafian et al, 2003). The process is mediated by several novel pathways such as oxidative stress, autophagy and endoplasmic reticulum stress (Zhu et al., 1998). NRF2 can also induce the unfolded protein response pathway that was upregulated in the current studies via activation of *PERK* (Campbell, 2001; Fiandalo et al., 2012).

The results from this study suggest that Δ-9-THC induces the NRF2 pathway when bronchial epithelial cells are exposed to various concentrations of Δ-9-THC. NRF2, that is typically upregulated as a response to stress, may be playing an essential role in this study. It has also been demonstrated from previous studies that NRF2 regulates multiple pathways. Thus, it appears that Δ-9-THC is dysregulating many pathways as demonstrated by the results of this study, and in response, NRF2 becomes activated and functions accordingly.

Unfolded protein response (UPR) is another pathway that was up-regulated in Δ-9-THC exposed cells. UPR is a cellular stress pathway that is associated with endoplasmic reticulum (ER) stress. When a cell undergoes UPR an increase in expression of *PERK* can be expected as it is one of the primary signal transducers of the UPR pathway. This effect was found in our studies, as they showed an increase in *PERK* expression as a result of bronchial epithelial cells

exposed to Δ-9-THC. The ferroptosis and unfolded protein response pathways are connected as half of the lipids in the cells are in the endoplasmic reticulum (Cianchi et al., 2008) and therefore the ER may play a major role in the initiation of ferroptosis. *PERK* is a known mediator of ferroptosis and has been shown to have a direct impact on NRF2. It causes phosphorylation and increasing levels of HO1, thus activating ferroptosis (Greenhough et al., 2007). The UPR is also known to be activated and upregulated in tumor microenvironments (Ramirez et al., 2020). The tumor microenvironment consists of the tumor mass with surrounding blood vessels, fibroblast, immune cells, signaling molecules, lymphatic cells and the extracellular matrix (Balkwill et al., 2012; Whiteside, 2008). This environment has been shown to be modulated in response to cigarette smoke exposure, not only resulting in phenotypic changes and inducing autophagy, but also contributing to tumor cell aggressiveness *in vitro* as well as *in vivo* (Domingo-Vidal et al., 2019; Hao et al., 2018). Ferroptosis has also been shown to contribute to the tumor microenvironment by driving the polarization of macrophages within the tumor microenvironment (Dai et al., 2020).

The caveolar-mediated endocytosis pathway is responsible for internalizing, uptake, and regulation of signaling organelles and bulk membrane (Kiss & Botos, 2009). As a result of cigarette smoke exposure, airway cells have been shown to induce the caveolar-mediated endocytosis pathway, with the increase of this pathway being associated with lung infections in smokers (Duffney et al., 2020). Similarly, overexpression of HIF1-alpha has been associated with lung cancer, and is correlated with cigarette smoking (Sies, 1991). Specifically, genes linked to the HIf1- alpha signaling pathway are shown to have a significant impact on lung carcinogenesis and genomic instability (Liu et al., 2014). Moreover, activation and overexpression of the HIf1-alpha signaling pathway has also been linked to COPD and decreased

lung function (Fu & Zang, 2018). In addition, the LXR/RXR activation pathway also has a negative impact on lung function. The LXR/RXR activation pathway was slightly increased at most Δ-9-THC concentrations used in the current studies. This pathway regulates cholesterol homeostasis, and lipid and carbohydrate metabolism (Cha & Repa, 2007; Larrede et al., 2009; Ulven et al., 2005). When cells are dying there is typically an increase in lipid production in which the LXR/RXR pathway breaks down and removes the surplus of lipids (Ulven et al., 2005). LXR/RXR activation pathway also can induce airway inflammation and airway hyperresponsiveness. Researchers used an agonist against the LXR/RXR activation pathway to attenuate airway inflammation, asthma, and pulmonary emphysema in murine and rat models (Fujii et al., 2017; Morichika et al., 2019).

The glucocorticoid signaling pathway was also deregulated in bronchial epithelial cells exposed to Δ-9-THC. While the pathway does not directly relate to Δ-9-THC exposure, Δ-9-THC has been shown to induce a variety of respiratory symptoms. Glucocorticoids - a class of corticosteroids - are typically prescribed to treat chronic inflammation and asthma in the respiratory tract (Adcock & Mumby, 2016; Kadmiel & Cidlowski, 2013). However, research has shown that an increasing number of people do not respond to the use of glucocorticoids to treat inflammation associated with asthma. In contrast, glucocorticoid inhibitors may decrease asthma associated inflammation, although studies are still ongoing (Enweasor et al., 2021; Palumbo et al., 2020). Studies are now trying to find new therapeutic strategies to treat different types of inflammation. A recent study combined glucocorticoid with ferroptosis inducing agents in order to find a novel therapy for eosinophilic airway inflammation and found that the induction of ferroptosis-like cell death relieved the allergic airway inflammation in mice (Wu et al., 2020). As

a result of that study, subsequent studies are looking into this pathway to cell death as a novel pathway in drug abuse.

In summary, our RNAseq transcriptome analysis of bronchial epithelial cells exposed to Δ-9-THC revealed genes with distinctly altered patterns of expression, particularly those specific to ferroptosis, unfolded protein response, and tumor microenvironment pathways. The extent of the gene deregulation was dependent on the concentration of Δ-9-THC. To our knowledge, and based on literature, the ferroptosis pathway is a novel pathway associated with Δ-9-THC - induced cytotoxicity in bronchial epithelial cells. Many pathways were highlighted in this study as a result of Δ-9-THC exposure, and many were seen across multiple concentrations. Because of the cell death phenotype in the Δ-9-THC exposed airway cells, the follow-up study will focus on the ferroptosis pathway and the mechanism behind Δ-9-THC-induced cell death.

CHAPTER IV: AIM 3: MECHANISM BEHIND Δ-9-THC-INDUCED CYTOTOXICITY IN BRONCHIAL EPITHELIAL CELLS

Introduction

Cannabis sativa, commonly known as Marijuana, is a widely used recreational drug in the United States (Bonini et al., 2018; Welty et al., 2019). Many states have legalized marijuana for recreational or medical use, and during the COVID-19 pandemic usage significantly increased (Bartel et al., 2020; Graupensperger et al., 2021). As more people are potentially impacted, questions about its effects on human health become more relevant. Most people who use marijuana do so by smoking it. The details of how marijuana constituents interact with the epithelial lining of users' lungs, and what happens as a result of exposure, are not fully understood.

Regulated cell death is an essential process and plays an important role in a variety of physiological processes. Conventionally, cell death has been divided into two process, apoptosis, and necrosis. However, a novel type of cell death called "ferroptosis" has recently been discovered. Ferroptosis is an iron-dependent and reactive oxygen species (ROS)-reliant form of cell death characterized by an increase of intracellular iron that results in lipid peroxidation (Cao & Dixon, 2016; Chen et al., 2020; Tao et al., 2020).

Iron is a required and necessary trace element in the human body because it contributes to oxygen transport and many other mechanisms. Iron typically circulates in the body as ferric iron and is transferred into cells by the transferrin receptor 1 (TFR1). If there is an excess of iron in a cell, iron can be sequestered by the iron storage protein ferritin. Ferritin proteins increase as a

due to an increase in the concentration of iron (Anderson & Frazer, 2017; Arosio et al., 2017). However, an excess amount of iron is known to be detrimental to cells. When in surplus, the ferric ions (Fe+3) can bind to superoxide (O2-) ions produced as a result of energy production in the mitochondria. This will result in ferrus ion (Fe2+), that can then couple with hydrogen peroxide to form superoxide ion radical $(O2 \bullet -)$ and hydroxyl radical l (OH•) (Awadallah, 2013; Fibach & Rachmilewitz, 2010). The increase in reactive oxygen species results in the activation of glutathione-dependent antioxidant mechanism, and consequently overwhelms glutathione (GSH). GSH is an integral part of the glutathione system and an antioxidant defense mechanism produced by most mammalian cells. The depletion of GSH levels leads to inhibition of glutathione peroxidase 4 (GPX_4). GPX_4 converts lipid peroxide to lipid alcohol. When GSH is depleted, conversion cannot take place, resulting in lipid peroxidation (Catala, 2009; Yin & Xu, 2011). In turn, these events result in degradation of lipids in membranes, loss of permeability, and ultimately, cell death.

Nuclear factor erythroid 2-related factor 2 (NRF2) is a key regulator of antioxidant response element-dependent genes. When there is an imbalance of oxidants/radicals and antioxidants, resulting in oxidative stress, the NRF2 pathway will become activated (Ma, 2013; Nguyen et al., 2009). Research has also shown that SQSTM1 protein modulates NRF2 expression and can contribute to NRF2 expression levels in ferroptosis (Sun et al., 2016). In ferroptosis, NRF2 has been found to play a key role in the mitigation of lipid peroxidation by inducing iron metabolism genes such as ferritin (FTH1) and heme-oxygenase 1 (HO1)[136, 137]. NRF2 also initiates the expression of GSH to help maintain and diminish oxidative stress [138].

Literature suggest that airway cells exposed to Δ-9-THC can suffer cell death, although the mechanism behind cell death is not clear. Many pathways are altered as a result of delta-9

THC exposure, as seen in Chapter 2. Ferroptosis was one of the pathways that was significantly altered and directly correlated with cell death. RNAseq analysis also suggested several marker genes of the ferroptosis pathway that can be used as endpoints in the subsequent studies concerning Δ-9-THC exposure. HO1, FTH1, GSH and NRF2 are candidate marker genes. Utilizing these marker genes as endpoints, the present study examined the involvement of ferroptosis pathway in Δ-9-THC-induced cell death in bronchial BEAS-2B cells using an antagonist and agonists of ferroptosis pathway in the 1200 ng/mL concentration.

Materials and Methods

Cell Culture, Exposure and Sample Collection and Reagents

Cell culture was conducted using immortalized human bronchial epithelial cells (BEAS-2B), which were obtained from ATCC in Manassas, Virginia. Cells were grown in LHC-9 (1x) media and maintained at 37° C in a humidified incubator with 5% CO_2 and 95% air in vented T-75 flasks (Corning). Delta-9 THC was purchased from Sigma Aldrich. When cells were 95-100% confluent they were seeded in 24 well plates and 100 mm dishes and exposed to 1200 ng/ml Δ-9-THC, vehicle (0.24% DMSO), ferrostatin-1 (Fer-1 5μM), Erastin (ERA 0.1 μM) and ERA + Fer-1, for 24 HR days. The cells were harvested after a 24 HR exposure. Delta-9 THC (T4764), Δ-9-THC (SML0583) and Erastin (E7781-1mg) were purchased from Sigma Aldrich (St. Louis, MO). Fer-1 and ERA were both dissolved in dimethyl sulfoxide (DMSO) Fer- 1 treatment 1 HR before exposure. All other treatments were simultaneous with Δ-9-THC.

Cell Viability

Cell viability was assessed using a 3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide (MTT) cell proliferation assay (Thermo Fisher) according to Volkow et al. (2014). Briefly, the MTT assay measures the conversion of MTT to an insoluble formazan, which

produces a color. The MTT stock solution (2 mg/ml in 1 x PBS) was diluted 1:9 with media and added to washed cells in the culture plate. Incubation was for two hours. After incubation, the MTT solution was decanted, and cells were rinsed with 1X PBS before DMSO was added. The plates were scanned in a Biotek microplate reader at an absorbance of 540 nm. The absorbance values were calculated at percent control, graphed using graphpad prism, and analyzed using One-way ANOVA followed by Dunnett's post-hoc test for multiple comparisons.

RNA/DNA Extraction and cDNA Synthesis

Total RNA and DNA were extracted from the cells using the ZR Duet kit (D7003, Zymo) according to the user's guide. Following extraction, cDNA was synthesized from 2 µg of total RNA per sample using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, 43668814). Oligo(dT) and random primer mix were used following the guidelines provided by the user's guide.

Real-Time Quantitative PCR (qPCR)

Realtime qPCR was conducted using PowerUp SYBR ™ Green PCR Master Mix (Applied Biosystems) with a QuantStudio 3 (Applied Biosystems). Primers specific to Ferroptosis (*HO1, FTH1, TRFC, SQSTM1)* and ROS scavenging proteins *(NRF2, GCLC*) were examined. (See Table 4).

Table 4. Ferroptosis Primer Sequences

FTH1	5' GCA CGA GCA AGT CAA GAC CAT 3'	5' CTT GTC GAA CAG GTA CTC AGC 3'
SQSTM1	5' GCC ATA CCC TCT TCG ACT ACG 3'	5' GAT TCT GGC ATC TGT AGG 3'
PERK	5' CTT ATG CCA GAC ACA CAG AA 3'	5' TCC ATC GTG CTG AAT GGA ATA C 3'
HO1	5' CTC TGA AGT TTA GGC CAT TG 3'	5' AGT TGC TGT AGG GCT TTA TG 3'
FTL	5' CAG CCT GGT CAA TTT GTA CCT 3'	5' GCC AAT TCG CGG AAG AAG TG 3'

Thiobarbituric Acid Reactive Substances Assay (TBARS)

Lipid peroxides were detected as malondialdehyde (MDA) reacting with thiobarbituric acid (TBA) to form a 1:2 adduct (color complex, TBARS) measurable by spectrofluorometric analysis. Following exposure, cells were collected by centrifugation at 1000 RPM for 7 minutes and resuspended in 200⌈l of tissue buffer. The cell suspension was sonicated on ice for three 5-sec intervals at setting 7.0 (Fisher scientific sonic dismembrator F550, USA). Whole homogenates were used to quantify lipid peroxides using the TBARS assay. Cell homogenates or standard (MDA) were mixed with 100 ⌈l of sodium dodecyl sulfate in test tubes and swirled to mix. Then, 2.5 ml of a TBA/Buffer reagent mix was added by pouring down the side of each tube. The mixture was incubated in a boiling water bath at 95°C. After cooling the tubes in a water bath, the reaction mixture was centrifuged at 3000 RPM for 10 minutes. The fluorescence in the supernatants was read by a Biotek microplate reader at an absorbance of 530 nm. The concentrations of TBARS were calculated using MDA as a reference standard. The quantities of TBARS were expressed in terms of amount (pmol) per milligram of sample protein.

Protein Isolation and Protein Quantification

After the 9-day exposure, cells were collected and re-suspended in ice-cold 50 mM potassium phosphate buffer. The cell suspension was sonicated on setting 2 in the sonicator on 30 second interval for 4 pulses, followed by centrifugation at 13,000 x g for 10 minutes at 4°C to remove cell debris. The supernatants were collected, and the protein concentration quantified using the Bio-Rad protein assay (23236, Bradford, Hercules, CA) using bovine serum albumin (BSA) as the standard.

Glutathione Assay

The protein lysate from treated cells were used for the measurement of total cellular glutathione (GSH) according to the published procedures (Jia et al., 2008). Fluorescence intensity was measured at an excitation wavelength of 350 nm and an emission wavelength of 420 nm. The sample GSH content was calculated using a GSH (in-house protocol) standard curve and expressed as nanomoles of GSH per milligram of sample protein.

Cellular Reactive Oxygen Species Detection Assay (Orange Fluorescence)

After exposure was completed using a CellROX Orange kit (Thermo fisher, C10443) the presence of intracellular reactive oxygen species was determined. Cells were stained with the ROS orange working solution and incubated for 60 minutes at 37°C. After incubation, fluorescence was measured in a Biotek microplate reader at an absorbance of 540/570nm. The measured values were expressed as a percent of control, graphed using graphpad prism, and analyzed using One-way ANOVA, followed by Dunnett's post-hoc test for multiple comparisons.

Results

Δ-9-THC Induced Cell Death in BEAS-2B Cells Is Linked to the Ferroptosis Pathway

To characterize the role of ferroptosis in Δ-9-THC treated cells, the effects of inhibiting or enhancing ferroptosis in BEAS-2B cells exposed to Δ-9-THC were examined (Figure 13). Cells were treated with 1200 ng/mL, with and without the ferroptosis inhibitor Fer-1 or agonist ERA for 24 hr. As expected, Δ-9-THC, alone, decreased cell viability at 1200 ng/mL. Treatment with Fer-1 at 5 μM increased cell viability in the Δ-9-THC exposed cells but not to control (without Δ-9-THC) levels. In contrast, the ferroptosis agonist ERA caused a further reduction in cell viability in Δ-9-THC exposed cells but only at 0.1 μM. At the higher concentrations (0.5 and 1 μM) there was no significant effect compared to the Δ-9-THC only control (Figure 14).

Overall, these results suggest that the ferroptosis pathway is at least partially involved in the response of the BEAS-2B cells to Δ-9-THC exposure.

Figure 13. Alterations in Cell Viability Caused by Agonist and Antagonist of Ferroptosis

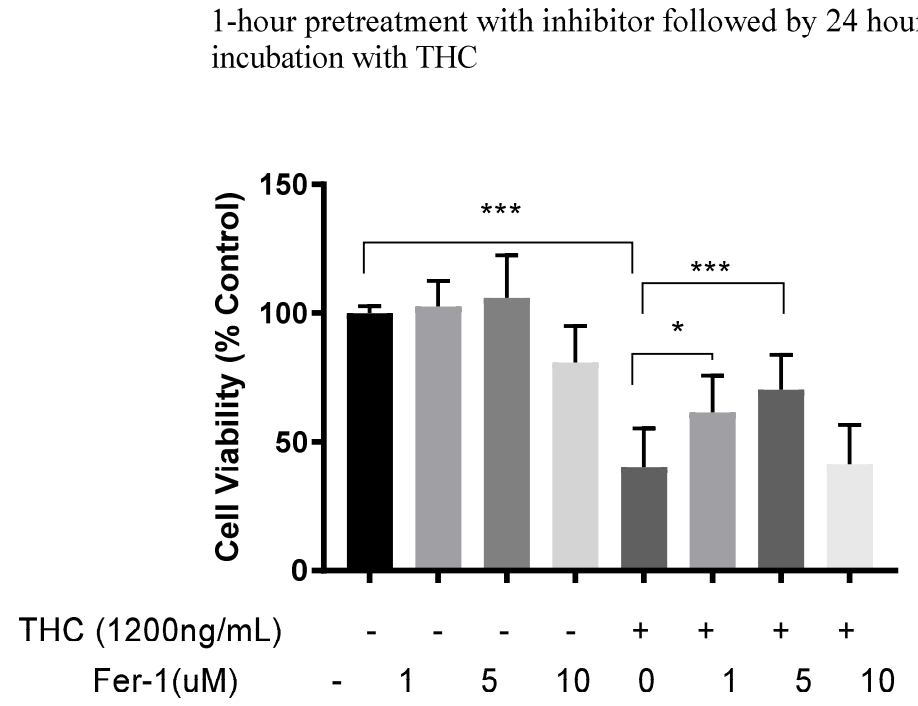
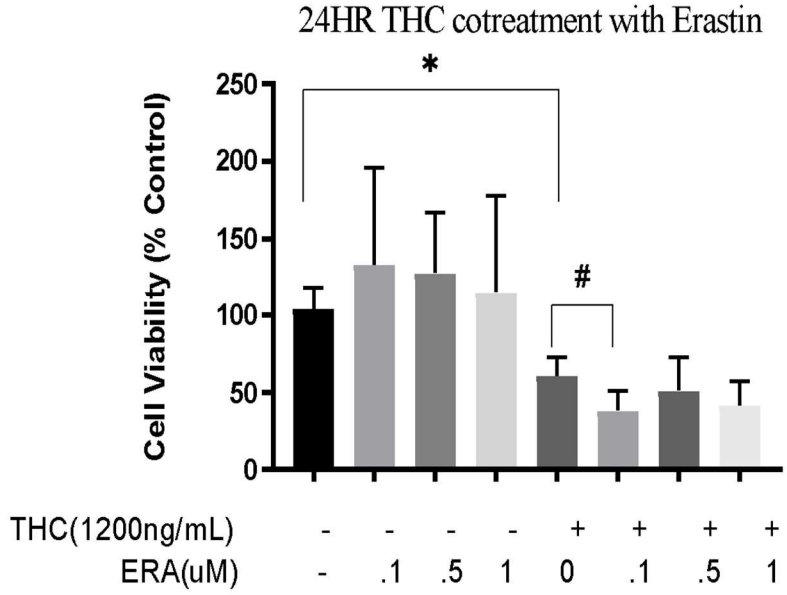


Figure 14. Expression of Genes Downstream in the Ferroptosis Pathways Following Delta-9-THC



The expression of several genes downstream in the ferroptosis was examined to determine whether the ferroptosis pathway is associated with the Δ-9-THC exposure-induced cell death. BEAS-2B cells were treated with Δ-9-THC at 1200 ng/mL for 24 HR and RNA was isolated for analysis of gene expression by RT-qPCR. There was not a significant increase in any of the ferroptosis related genes in 1200 ng/mL (Figure 15) or 1500 ng/mL (Figure 16). Due to a lack of the standard cell culture medium as a consequence of the Covid-19 pandemic, the experiments were altered. An alternative medium (BEGM) was obtained from UNC Chapel Hill. Cell viability assay after Δ-9-THC exposure were conducted with this new medium and results proved different from previous assays. The measured % cell death at 1200 ng/mL Δ-9-THC in the original medium was now found at 1500 ng/mL in the new medium. This complicated the experimental design as the next steps required that the cells be exposed to both 1200 ng/mL and 1500 ng/ml Δ-9-THC.

Figure 15. Expression of Ferroptosis Related Genes in Δ-9-THC Exposed BEAS-2B Cells

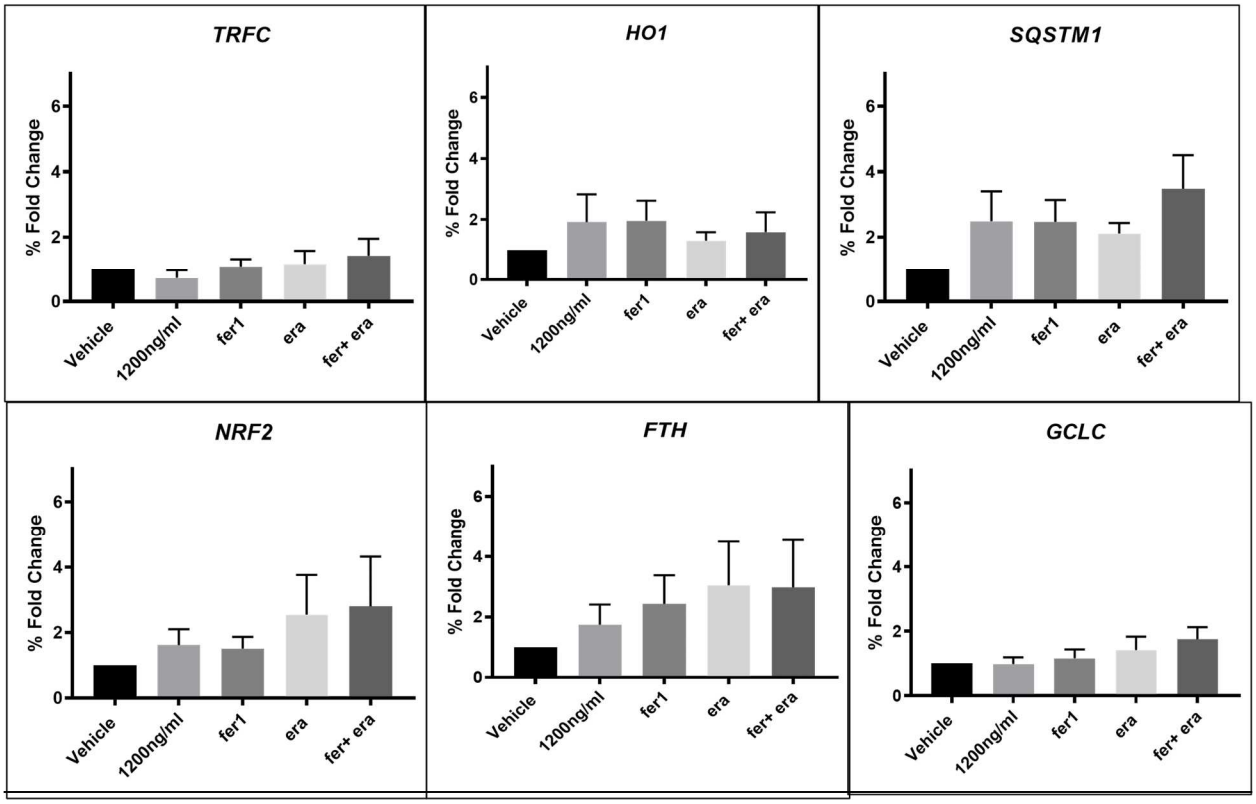
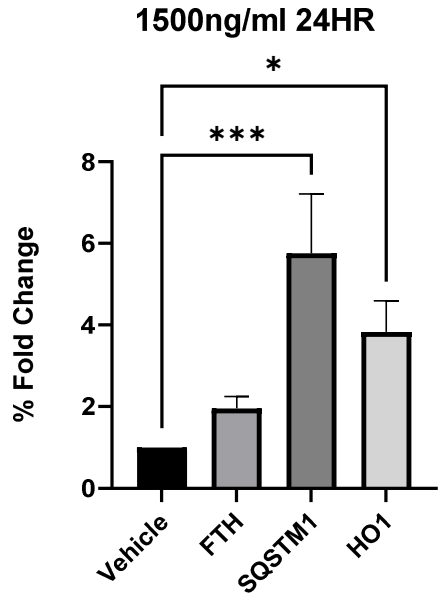


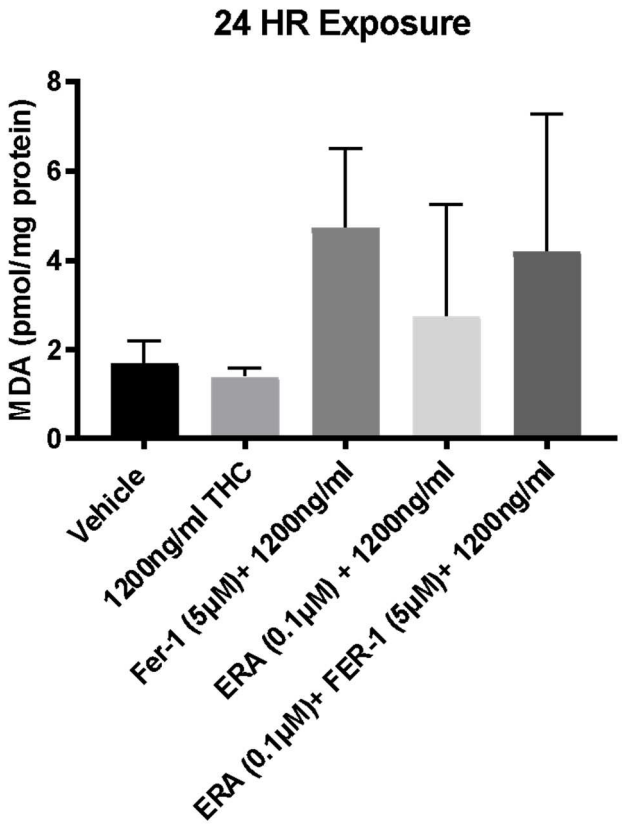
Figure 16. Expression of Ferroptosis Related Genes in BEAS-2B Cells Exposed to 1500 ng/mL Δ-9-THC



Lipid Peroxide Levels in BEAS-2B Cells Exposed to Δ-9-THC and Ferroptosis Agonist and Antagonist

To confirm the involvement of lipid peroxidation in Δ-9-THC induced cellular death, the TBARS production rate was determined in BEAS-2B cells after Δ-9-THC exposure for 24 hours and following a 24hr cotreatment of Δ-9-THC + ERA, and an 1hr pretreatment of Fer-1 followed by a 24hr exposure to Δ-9-THC, in bronchial epithelial cells. The levels of MDA, an indicator of lipid peroxidation, were measured. Δ-9-THC exposure did not significantly increase MDA levels compared to the control (Figure 17). Furthermore, there was not a significant difference in MDA levels in cells exposed to Δ-9-THC together with Fer-1 inhibitor and the ERA agonist in comparison to the control (Figure 17).

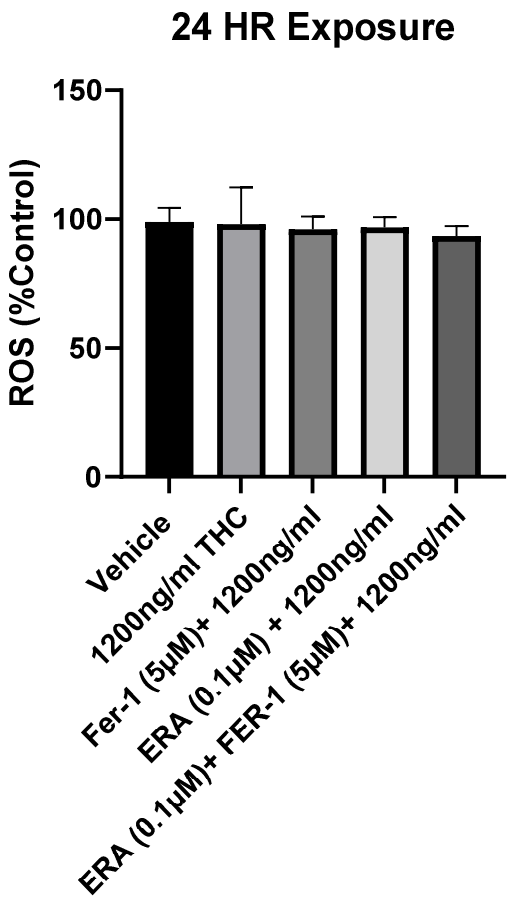
Figure 17. Lipid Peroxide Levels in BEAS-2B Cells Exposed to Δ-9-THC and Ferroptosis Agonist and Antagonist



ROS Levels in BEAS-2B Cells Exposed to Δ-9-THC and Ferroptosis Agonist and Antagonist

To determine if there was a significant increase in reactive oxygen species as a result of Δ-9-THC-induced cell death, cells were treated with Δ-9-THC or together with ferroptosis inhibitors and ferroptosis agonist. CellROX assay was used to determine ROS levels. As shown on Figure 18, ROS levels were not altered in any of the conditions compared to the vehicle control.

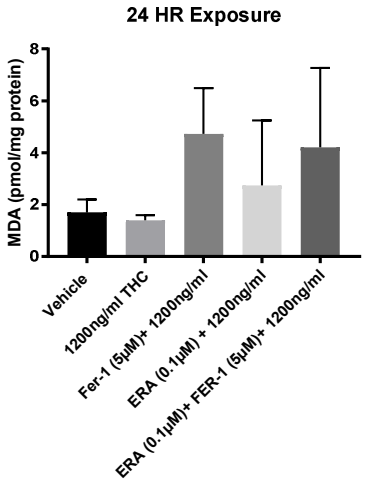
Figure 18. The Effect of Δ-9-THC and Ferroptosis Antagonist and Agonist in ROS Levels



GSH Levels in BEAS-2B Cells Exposed to Δ-9-THC and Ferroptosis Agonist and Antagonist

The next question was if cellular levels of GSH are affected in BEAS-2B cells treated with Δ-9-THC and in combination with Fer-1 and ERA. None of the treatments significantly altered GSH levels in comparison to the control (Figure 19). However, three of the treatment groups (Δ-9-THC only, Δ-9-THC + Fer-1, and Δ-9-THC + Fer-1+ERA) trended to increase relative to the control.

Figure 19. GSH Levels in BEAS-2B Cells Exposed to Δ-9-THC and Ferroptosis Agonist and Antagonist



Discussion

Inhalation is a major route of exposure for marijuana users allowing for direct contact with the epithelium cells of the lungs. Δ-9-THC is the major constituent of marijuana and is responsible for the euphoria or high associated with use. Literature suggests that Δ-9-THC exposure can result in cell death, although the mechanism behind it is poorly understood. The present study explored the possible involvement of ferroptosis in Δ-9-THC-induced cytotoxicity. When BEAS2B airway cells were exposed to Δ-9-THC it induced cytotoxicity, oxidative stress and resulted in cell death (Sarafinian, et al., 2003; Sarafinian, et al., 2005; Aguira, et al., 2019). To further evaluate the mechanism behind cell death, BEAS-2B cells were treated with FER-1 (5μM) to ameliorate Δ-9-THC induced effects. When airway cells were pretreated with Fer-1 for one hour and then immediately treated with Δ-9-THC for 24 hours, there was a significant increase in cell viability compared to treatment with Δ-9-THC alone (see Figure 13). These observations supported previous studies in that Fer-1 prevents oxidative lipid damage and ferroptosis in auditory hair, neuronal, and airway cells (Hu, et al., 2020; Li, et al., 2017: Liu, et

al., 2020). Ferroptosis is a form of non-apoptotic cell death primed by lipid peroxidation. Fer-1 is a potent ferroptosis inhibitor that prevents lipid peroxidation. Recent studies have shown that Fer-1 has ROS scavenging capabilities, particularly scavenging alkoxyl radicals (Miotto, et al., 2020). The current results suggest Δ-9-THC could induce ferroptosis in airway cells; and that Fer-1 - a potent inhibitor of ferroptosis - can inhibit cell death associated with Δ-9-THC. These findings provide insight into the cell death pathway associated with Δ-9-THC -induced cytotoxicity and suggests that ferroptosis is involved.

Erastin (ERA) is an inducer of ferroptosis as it inhibits cystine import thus depleting GSH, resulting in a decrease of *GPX4* expression. *GPX4* is a key regulator of ferroptosis as it mediates lipid peroxidation ((Seibt, et al., 2019; Ursini & Maiorino, 2020). In this study, when airway cells were cotreated with Δ-9-THC and ERA for 24 HRs, there was a noticeable but not statistically significant decrease in cell viability compared to exposure solely to Δ-9-THC. Similar to our results, human pancreatic, neuroblastoma, and airway cells when exposed to ERA, as the exposure led to an increase in cell death and ferroptosis (Pan et al., 2019; Shintoku et al., 2017; Zhao et al., 2020). The visible decrease in viability in BEAS2B cells cotreated with ERA (0.1μM) and Δ-9-THC resulted in a significant increase in viability, when pretreated with Fer-1, indicating that ferroptosis could be the mechanism underlying Δ-9-THC induced cytotoxicity.

The role of ferroptosis was futher studied by measuring the expression levels of the genes encoding corresponding proteins that both promote and inhibit ferroptosis. HO-1 regulates cellular iron and ROS levels during ferroptosis (Chiang et al., 2019; Suttner & Dennery, 1999). In this study, there was not a significant increase in *HO1* expression in BEAS-2B cells exposed to Δ-9-THC or cotreated with ERA; nor was there a decrease in *HO-1* expression in BEAS-2B cells pretreated with Fer-1. However, with the change in cell media from LHC-9 to BEGM, the

cell viability response got shifted in such a way that the responses seen in cells exposed to 1200 ng/mL Δ-9-THC were found in the cells exposed to 1500 ng/mL Δ-9-THC. Expression of *FTH1, SQSTM1*, and *HO1* significantly increased, confirming the shift. HO-1 expression is mediated by NRF2 which is shown to inhibit ferroptosis when upregulated (Dodson, et al., 2019; Shin, et al., 2018). NRF2 also modulates levels of GSH and FTH1 expression in cells that are experiencing oxidative stress and toxicity (Harvey et al., 2009; Li et al., 2021; Ma, 2013; Nguyen et al., 2009; Qaisiya et al., 2014; Sun et al., 2016). There must be an adequate amount of GSH present to prevent ferroptosis; otherwise, a depletion of GSH inhibits GPX4 production, resulting in ferroptosis (Ursini & Maiorino, 2020). FTH1 levels are upregulated when cells are undergoing ferroptosis as FTH1is a main iron storage site for cells, storing 70%-80% of imported iron (Xie et al., 2016). Studies showed that overexpression of FTH1 lead to a decrease in erastin-induced ferroptosis in neural cells (Yuan et al., 2016). Gene expression levels of NRF2, FTH1, and *HO-1* were measured to determine if NRF2 was upregulated, leading to upregulation of ferroptosis genes. NRF2 expression was visibly upregulated in the ERA (0.1 μM) + Δ-9-THC cotreatment compared to the Δ-9-THC and the Fer-1 exposure, however, results were not significant in any of the treatments. Similar patterns were observed in the GCLC (GSH) and *FTH1* expression. TFR1 is a receptor that allows for iron to be imported into the cell. When TFR1 is reduced, there is a decrease in siramesine and lapatinib-induced ferroptosis (Ma et al., 2016). Moreover, the loss of TFR1-suppressed cystine starvation and erastin-induced ferroptosis, illustrating that TFR1 plays an important role in the induction of ferroptosis (Gao et al., 2015; Yang & Stockwell,, 2008). In the present study, TFR1 was not significantly altered due to exposure. There was a visible increase in ERA (0.1μM) + Δ-9-THC cotreatment that was not significant. SQSTM1 is a cargo for autophagic degradation and is upregulated by various stressors activates NRF2. In this study

the increased expression of *SQSTM1* was not significant. These results suggest that NRF2 regulated genes are not being altered by Δ-9-THC induced cytotoxicity and NRF2 is not being activated by SQSTM1.

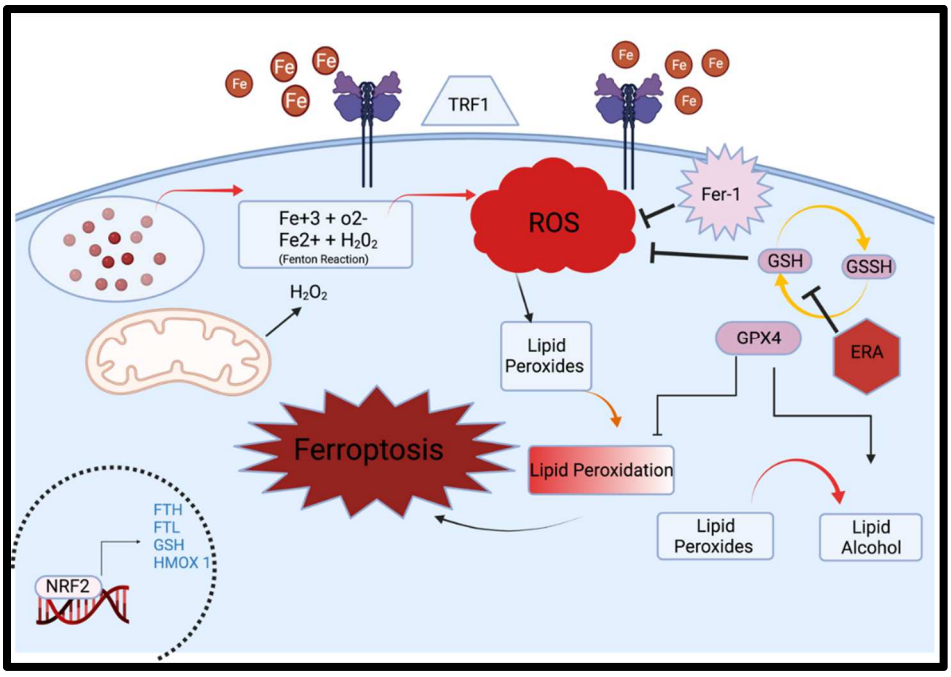
Lipid peroxidation is another key element that leads to the induction of ferroptosis. The results did not show a significant increase in lipid peroxidation in ERA (0.1μM) + Δ-9-THC, nor did it show a decrease in lipid peroxidation in with pretreatment of Fer-1 (5 μM), compared to Δ-9-THC alone in BEAS-2B cells. Lipid peroxidation is a known inducer of ferroptosis leading to iron-dependent death in both T cells and colon cancer cells (Matsushita et al., 2015; Ye et al., 2020). However, the results imply that no lipid peroxidation is took place in any of the treatments. Nor did it take place in other treatment groups exceeding 1200 ng/mL treatment groups that were not measured in the present study. This statement is supported by the observation that the expression of *FTH1, SQSTM1*, and *HO1* were significantly increased with 1500 ng/mL THC exposure. Further experiments were not possible during the Covid-19 pandemic as the cell culture media used for the studies in Aim 1 and 2 were not available. Ideally, all experiments should be repeated using the new BEGM media. Future studies will focus on the use of BEGM media, and the involvement of ferroptosis pathway will be confirmed.

ROS plays a role in ferroptosis, contributing to the depletion of GSH levels (Bouaboula et al., 1992; Lu & Mackie, 2016). GSH levels were measured in the current studies as an assessment of ferroptosis. None of the treatments lead to a significant increase or decrease in GSH levels as a result of exposure to Δ-9-THC, neither the inhibitor nor the agonist. A decrease in GSH levels in Δ-9-THC and cotreatment of Δ-9-THC and ERA (0.1μM), and an increase in GSH levels in cells pretreated with Δ-9-THC + Fer-1 (5μM) were expected. Follow-up measurements of ROS were made to determine if ROS were produced as a result of Δ-9-THC

exposure, and if the inhibitor and agonist could modulate these levels. There were no significant changes in any of the treatments.

In conclusion, the present findings show that Fer-1, inhibitor of ferroptosis can protect airway cells from Δ-9-THC-induced cell death. However, technical difficulties related to the Covid-19 pandemic blocked the exploration of the mechanism underlying the increase in cell viability. Figure 19 is a hypothesized schematic of the pathway that shows how Δ-9-THC can induce changes BEAS-2B cells when they are exposed to Δ-9-THC for 24 HRs.

Figure 20. Δ-9-THC-Induced Ferroptosis Pathway



Conclusion

Marijuana is a popular drug that is widely used due to the euphoric feeling that is associated with its use. Δ-9-THC is the major psychoactive ingredient and contributes to the plant's properties. Because smoking is the delivery method of choice for many users, the impact of smoke inhalation on airway cells is a critical health consideration. The objective of this study

was to determine if Δ-9-THC exposure leads to adverse outcomes in the respiratory epithelium, using normal human bronchial epithelial cell line (BEAS-2B). There were three major aims. The first aim was to determine if Δ-9-THC-induced cytotoxicity in human bronchial epithelial cells. This was accomplished by time- and dose-response studies coupled with the measurement of cellular and molecular endpoints. The second aim was to determine the molecular pathways associated with Δ-9-THC exposure in human bronchial epithelial cell lines. This was accomplished by global profiling of RNA transcripts by RNA sequencing, functional network analysis, and confirmation of observed changes by a second detection method (RT-qPCR). The third aim was to determine the role of the observed pathway found in Aim 2 as a driver of Δ-9-THC-induced decreased cell viability.

Regarding aim one, the results showed that Δ-9-THC significantly decreased cell viability in 24 HRs, 96 HRs, as well as in 9 days of culture. In the 9-day study, genes associated with oxidative stress were significantly decreased and global methylation markers were significantly increased as a result of exposure. Overall, there was a time and dose dependent decrease in the viability of BEAS-2B cells exposed to Δ-9-THC in a 24 HR study. Due to its success, the 24-hour study method was used thereafter. For aim two, transcriptome analysis of the Δ-9-THC-exposed BEAS-2B cells revealed genes with distinctly altered patterns of expression, particularly those specific to ferroptosis, unfolded protein response, and tumor microenvironment pathways. Despite several pathways were significantly altered, based on the results observed in cell viability, this aim led to the selection of the ferroptosis pathway as a pathway of interest to follow up. Lastly, for aim 3, it was demonstrated that Δ-9-THC-induced cell death can be ameliorated by the inhibition of the ferroptosis pathway, and that the agonist of ferroptosis exacerbates the process of cell death. However, the mechanism underlying these

observed phenomena could not be determined due to technical difficulties imposed by the Covid-19 pandemic. Specifically, the cell culture medium (LHC-9) that was being used was not available for more than a year and half. The use of a new cell medium (BEGM) resulted in a shift in Δ-9-THC exposure effect, requiring replications of all the prior experiments to insure reliability. Because time did not allow for replications, future studies can explore the molecular mechanisms underlying the role of ferroptosis in Δ-9-THC-induced cell death.

In summary, Δ-9-THC decreased the viability of BEAS-2B cells. While the transcriptome analysis suggests that ferroptosis may be the contributor to the increased cytotoxicity, this study is inconclusive. For future studies the use of BEGM cell media from UNC Chapel Hill, is recommended. This supplier can supply the media consistently without disruption and reference Δ-9-THC from NIDA. The Δ-9-THC from commercial vendor, Sigma, seemed to lose potency of the Δ-9-THC soon after its opening and there seemed to have batch effects, as well.

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BIOGRAPHICAL SKETCH

Megan Doldron is a native of the beautiful twin islands of Trinidad and Tobago. She earned her Bachelors degree at Hollins University in Roanoke Virginia with a double major in Biology and Dance and a minor in Psychology. She then earned a Masters in Biology Education at North Carolina A&T State University where she was awarded the North Carolina A&T State University Graduate Fellowship 2014-2015. Megan then taught high school Biology for 3 years at Reidsville High where she was awarded Teacher of the Year in her 2nd year. She then decided to return to school and pursued and earned her Master of Science in Biology at North Carolina A&T State University. Her research focused on natural alternatives such as antioxidant-rich plants and how they can reduce the signs of airway inflammation and oxidative stress caused by exposure to dust from a swine confinement facility.