5-1-2021

Cannabigerol attenuate methionine/choline deficient diet- induced non-alcoholic steatohepatitis symptoms in C57BL/6 male mice via modulating the expression of cannabinoid receptors

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CANNABIGEROL ATTENUATE METHIONINE/CHOLINE DEFICIENT DIET- INDUCED NON-ALCOHOLIC STEATOHEPATITIS SYMPTOMS IN C57BL/6 MALE MICE VIA MODULATING THE EXPRESSION OF CANNABINOID RECEPTORS

A Thesis Submitted in Partial Fulfillment
of the Requirements for the Degree of
Master of Science

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College of Health and Natural Science
School of Biological Sciences

May 2021

This Thesis by: Nouf Abdulrahman Aljobaily

Entitled: *Cannabigerol attenuate methionine/choline deficient diet- induced non-alcoholic steatohepatitis symptoms in C57BL/6 male mice via modulating the expression of cannabinoid receptors*

has been approved as meeting the requirement for the Degree of Master of Science in the College of Natural and Health Sciences in the School of Biological Science

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ABSTRACT

Aljobaily, Nouf. *Cannabigerol attenuate methionine/choline deficient diet- induced nonalcoholic steatohepatitis symptoms in C57BL/6 male mice via modulating the expression of cannabinoid receptors.* Unpublished Master of Science thesis, University of Northern Colorado, 2021.

Non-Alcoholic Steatohepatitis (NASH) is the advanced and more aggressive form of Non-Alcoholic Fatty Liver Disease (NAFLD). NASH is associated with severe hepatic fibrosis and inflammation. Methionine/choline deficient (MCD) high fat diet is known to induce NASH in a short period of time without showing signs of metabolic syndrome. Cannabigerol (CBG) is a plant-derived, non-psychotropic cannabinoid that has a potential anti-inflammatory effect. Other hemp extracts reduce the progression of NAFLD to NASH, whereas the impact of CBG on NASH is still unknown. Therefore, these studies aim to 1. Evaluate the therapeutic potential of CBG on reducing hepatic steatosis and fibrosis. 2. Evaluate the anti-inflammatory effect of CBG in MCD-induced NASH C57BL/6 male mice. 3. Evaluate how CBG interacts with CB1 and CB2 receptors. Liver tissues were harvested from C57BL/6 mice (n = 36) fed with MCD or high fat control (CTR) diets for three weeks then the mice were divided into three sub-groups and injected with a vehicle solution, low or high dose of CBG for two additional weeks. Body weight, liver-to-body weight ratio, serum chemistry and H&E staining were also measured to evaluate the overall health of mice, liver function and morphology. Moreover, various histological tests were performed to evaluate collagen deposition, inflammation, and fat deposition. In addition, the expression of cannabinoid receptors was evaluated using immunofluorescence staining.

It is concluded that MCD diet caused a significant body weight loss in mice (55), while CBG administration showed a trend towards recovery of their body weight, liver-to-body weight ratio, as well as ALT levels in MCD diet group. Further, inflammation decreased with low CBG treatment but increased when treated with a high dose of CBG in both the CTR and MCD groups. Similarly, the expression of cannabinoid (CB) receptors 1 and 2 showed increased expression with high dose CBG, but alleviated expression with low CBG dose intervention in MCD group. Collectively, low dose of CBG can reduce fibrosis and inflammation in MCD-induced NASH. CBG is gaining traction as a commercially available supplement with a variety of health-related claims. These results will provide rigorously controlled pre-clinical data to guide future intervention studies in humans addressing the potential uses of CBG for inflammatory liver pathologies.

ACKNOWLEDGEMENTS

Throughout my year at UNC, I have received tremendous amount of educational and emotional support from many people.

First, I would like to thank my advisor, Dr. Yuyan Han, for giving me the opportunity to lead different projects and for the continuous feedback, valuable advices, and insights on how to improve my work. Thank you for being patient and understanding throughout my three years I have worked in your laboratory. I, also, would like to thank all members of the Han lab for their continuous help and for moving this project as well as other project forward.

I would like to express my deepest appreciation to my committee members, Dr. James Haughian and Dr. Nicholas Pullen, for all the skills and knowledge that I learned from them. I also cannot express how grateful and thankful I am to have both of you in my committee, as you have been a great listeners and for being there to help me with anything that I struggled with. I truly cannot thank you enough.

I also would like to thank my family who were the most supportive people through my graduate years, and without them I would have not been here today. Thank you for all the days and nights that you patiently listened to my problems. No matter when I called my parents who live in Saudi Arabia, they always were there to listen and encourage me to continue moving forward. I also would like to thank my siblings, Sara, Hend, Bader, and Yousef, for everything that you have done for me and for all the times that you got me tea and coffee when I was feeling down. I cannot express how much I love all of you and how much I am thankful for having you in my life.

I cannot express how grateful and thankful I am to my sister, Dr. Hend Aljobaily, who is the reason I am graduating and pursuing a PhD degree. Hend, I truly cannot express how much I love you and how much you mean to me. You have been the most supportive person I have ever known. Thank you for never going to bed before making sure that I have finished all of my assignment, and for all the times I called you crying telling you that I want to quit and you pushed to stand strong no matter what was going around, and for pushing me to fight for what I truly want. I also would like to thank you for staying up late to review my thesis and giving me insightful advises on how to improve my writing skills. You are truly the most amazing sister, I will always be thankful and grateful to have you in my life.

I also would like to thank our future brother in law and my best friend, Michael MacVean for being a great listener and for editing my thesis although you had other things you were working on. You are one of the kindest people I know, and I am thankful to have such a great friend.

Finally, I would like to thank the Graduate Student Association (GSA) at UNC, for funding all of my projects. All funding that I have received from GSA have improved my project and gave me the chance to investigate new questions.

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CHAPTER 1

INTRODUCTION

Overview of Non-Alcoholic Fatty Liver Disease Epidemiology, Diagnosis and Treatments

A person could develop a fatty liver by either excessive alcoholic consumption or excessive calorie consumption as part of an unhealthy diet (1). Non-Alcoholic Fatty Liver Disease (NAFLD) is one of the most common forms of chronic liver diseases (2), characterized by accumulation of more than 5% of fat in the liver (2) independent of alcoholic consumption (1). NAFLD is associated with obesity, type-2 diabetes, and metabolic syndrome (3). Globally, 25% of the world population and 24.13% of the United States population is estimated to have NAFLD (4). Further, it is projected that 100 million individuals in the United Stated will develop NAFLD by 2030 (4), as it is expected to increase by 33.5% in the next ten years (2). It is also projected that NAFLD incidence will increase in North America as obesity continues to rise (5).

Currently, there are limited ways to diagnose NAFLD, which include serum chemistry analysis and/or liver biopsy. Serum chemistry analysis includes measuring alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels (6). While both ALT and AST enzymes are present in various organs, they are primary found in the liver and the kidney and are responsible for the transfer amino acids. Change in serum ALT and/or AST levels could be used as an indicator for the presence of liver fibrosis. Hepatic steatosis is defined as the accumulation of fat in the liver cells (hepatocytes). NAFLD starts as simple steatosis but can progress to more severe stages (7). The second stage of NAFLD, called Non-Alcoholic Steatohepatitis (NASH), is characterized with inflammation and continuous liver scarring

(fibrosis) (8). NASH could further progress to liver cirrhosis and/or hepatocellular carcinoma. At that stage, the liver starts to fail, and patients require a liver transplant (9). Hence, a change in lifestyle, including continuous exercise and eating a healthy diet, is the only current way to reverse NAFLD, enabling a return to a healthy liver with no or less than 5% fat accumulation, which prevent the development of NASH. More specifically, it has been suggested that losing around 3% of total body weight might enhance the reduction of hepatic steatosis (8).

Non-Alcoholic Steatohepatitis Epidemiology, Diagnosis and Proposed Treatments

NASH is the more aggressive stage of NAFLD associated with hepatic inflammation and hepatic fibrosis (8). Fibrosis is defined as thickening of the connective tissues as a result of continues scarring. In NASH, the scaring is developed as a result to the accumulation of fat in the liver. Thus, it is estimated that 20-25% of NAFLD cases in the United States are at the NASH stage, of which 3-4% of those cases are estimated to have cirrhosis (10). Further, between 2007 and 2016, 2% of the US deaths were NAFLD/NASH-related (48). Like NAFLD, NASH is clinically diagnosed via either liver biopsy and/or serum chemistry analyses. However, unlike NAFLD, NASH is not known to be reservable (11). Yet, it is suggested that a 10% reduction in total body weight could help in reducing inflammation associated with NASH (8, 12). However, the change in lifestyle was found to be hard to maintain in NASH patients. Currently, there are no FDA-approved treatments for NASH, which highlights the importance of research on seeking effective treatments for NASH. Some NASH patients have required a liver transplant due to the development of liver cirrhosis and its related complications (9, 48, 49). However, this is not an adequate way to treat the disease as not all NASH patients have immediate access to a transplant liver. In addition, there are serious complications associated with the liver transplant such as bile leaking, infections, and sometimes it causes the mortality of the recipient patient (50). Further,

Liver transplant rejection is another major complication, which is a response of the immune system. Although there are lower transplant rejection incidences due to the improve in immune suppressive therapies, there are still a significant clinical liver transplant rejection rate (54). Various treatments have been suggested or are currently in clinical trials to treat or slow down the progression of the disease. Peroxisome proliferator-activator receptors (PPARs) have been investigated as a target for NASH. While PPAR-α agonist did not show an affect in reducing NASH, PPAR-γ agonist showed an alleviation in NASH through histological data but not in alanine transaminase (ALT) levels or body weights. However, PPAR-α/γ combination showed alleviation in serum ALT levels, insulin resistance and body weight (8, 13, 14). Further, Tumor Necrosis Factor alpha (TNF-α) is a NASH biomarker associated with hepatic inflammation. It is known that endoplasmic reticulum (ER) stress mediates TNF-α activation in response to NASH as well as hepatocyte injury and cellular apoptosis; thus, it has been proposed that a blockade of TNF-α using emricasan, a drug designed to inhibit caspase activity, reduces the morphological effect in NASH patients. This study ended in phase IIb clinical trials and emricasan did not improve NASH histology or liver fibrosis and liver ballooning (8, 15-17). Further, supplements such as omega-3 (51) and vitamin E have been used in NASH patients due to its antioxidant characteristic. It has been shown to decrease ER stress as well as inflammation (18), which is thought to be the start point to initiate hepatic fibrosis and inflammation.

Cannabidiol in Liver Diseases

Cannabinoids are commercially available and are being used by the general population in various forms for medical purposes due to their widely recognized anti-inflammatory effects. However, there are many unknowns about the side effects of cannabis, and it is still controversial whether *cannabis* could be safely used for a disease therapy. A preclinical study suggested that

CBD could be used to attenuate diseases such as anxiety, bowel disease (28) and NAFLD (19). When C57BL/6 mice were administered either 5 or 10 mg/kg/day CBD treatment, they showed a reduction in liver steatosis as well as liver inflammation. This treatment did not appear to have any impact on the brain (19). Even after these findings, the impact of CBD on the liver is still debatable due to the risk of drug-to drug interaction, as well as the associated hepatocellular injuries and hepatic abnormalities (52). Another complication that was observed in human liver was elevated serum chemistry function including, but not limited to, AST, ALT, and γ-glutamyl transferase (GGT) levels (52, 53). Thus, further study of the potential effect of CBD on the liver as well as the potential application of other cannabinoids as anti-inflammatory alternatives to CBD in liver treatment is needed. Despite the popularity of various forms of cannabis, there is still little known about their molecular mechanisms and effect on the liver. In addition, cannabidiol (CBD) is a form of *cannabis* derivative with anti-inflammatory characteristics that have been found to reduce liver steatosis, inflammation, and oxidative stress (19). Nevertheless, the therapeutic effect of *cannabis* is still not fully understood. A recent study by Abulseoud et. al. suggested that the therapeutic effect of *cannabis* is dose-dependent, suggesting that low dosages could be helpful while high dosages could have a harmful impact (20).

Cannabinoid Receptors and their Role in Liver Diseases

Endocannabinoid (EC) signaling has been widely investigated in the central nervous system (CNS) as it has been shown to regulate the appetite. Additionally, cannabinoid (CB) receptors are G-protein coupled receptors that have been found to be abundant in various mammalian tissues including the liver (21, 22). Currently, there are two known CB receptors in the liver CB1 and CB2. CB1 has been shown to be involved in hepatic steatosis, fibrosis and NASH; while CB2 is involved in NAFLD and Kupffer cells embryogenesis (22, 23). Lately, CB

receptors have been used as a therapeutic target in various liver metabolic related diseases such as NAFLD, NASH and hepatocellular carcinoma (24-26). In fact, the CB1 receptor is the receptor predominantly expressed in the CNS to regulate appetite, mood and pain, whereas CB2 is found to play a role in the immune system response and in the gastrointestinal tract. Further, a study suggested that CB receptors play a role in the development and the metabolism of the liver (27).

Recent Research Using Cannabigerol

CBD is derived from the precursor Cannabigerol (CBG), $C_{21}H_{32}O_2$, which is a form of cannabinoid found in the plant *cannabis* sativa that does not have the psychotropic effect that tetrahydrocannabinol (THC) has (29). Due to the novelty of CBG, few studies investigated the therapeutic mechanism of CBG (28, 30). It was shown that CBG has an anti-inflammatory, anti-proliferative and anti-bacterial effect on a variety of diseases and disorders (28) such as CNS diseases (29), breast cancer (31), antibiotic resistance (32, 33) and anti-glaucoma (28). Nevertheless, the impact of CBG on the liver has not yet been investigated. Therefore, this study aims to evaluate the role of CBG in improving liver function in diet-induced NASH using a mouse model.

Cannabigerol as Potential Treatment for Non-Alcoholic Steatohepatitis

This study will advance the medical and pharmaceutical field in various ways. CBG could target CB1 receptor which is expressed in the liver and could be a potential target when considering drug synthesis (29). Further, NASH reversibility is controversial; some studies suggest that NASH cannot be reversed back to NAFLD whereas other studies suggested that it can. Therefore, this study aims to investigate whether CBG could reverse NASH back to NAFLD by decreasing fibrosis, inflammation (34) and oxidative stress.

Methionine/Choline-Deficient Diet to Induce Non-Alcoholic Steatohepatitis in Rodents

Methionine/Choline-deficient (MCD) diet is a widely used high fat diet (Table 1) containing 455.294 g/kg of sucrose, 200 g/kg of corn starch, 30 g/kg of cellulose, and 100 g/kg of corn oil. It is known to induce NASH by inhibiting the synthesis of very-low-density lipoprotein (VLDL) as well as stearoyl-CoA desaturase-1 (SCD1), leading to failure in exporting lipid from the liver (35). The excessiveness of lipid accumulation in the liver decreases the regeneration of hepatocytes which leads to hepatic fibrosis (36), potentially triggered by oxidative stress. Although MCD diet does not fully replicate the human NASH symptoms because it fails to show metabolic syndrome, insulin resistance and body weight gain; it replicates other NASH symptoms such as hepatic steatosis, inflammation, ER stress, cellular apoptosis and fibrosis over a short period of time (37). It is important to keep this in mind while interpreting the data. In this study we are specifically interested in the main pathologic features of NASH which are hepatic steatosis, fibrosis, and inflammation.

Goal and Aims

The current understanding of NASH suggests that it is not reversible while NAFLD is known to be a reversible condition (11). A schematic representation is shown in Figure 1 summarizing the overall goal of this study. It was hypothesized that CBG would be used as a therapeutic agent to alleviate NASH symptoms as it has been suggested to have an anti-inflammatory effect (28) (Figure 1) and thus might help reducing the liver damage. Initially, body weight, liver to body weight ratio and ALT levels were obtained to assess the general health of the mice. Therefore, this study is intended to evaluate the efficacy of CBG in alleviating NASH major symptom. To accomplish this goal, we proposed the following three aims.

A1 Evaluate the effect of different dose of CBG on reducing hepatic steatosis and hepatic fibrosis in MCD-induced NASH C57BL/6 male mice.

Our working hypothesis was that CBG will not impact steatosis as other cannabinoids have shown to increase appetite. However, we expected that CBG would decrease hepatic fibrosis. In order to accomplish this aim, steatosis is measured using histological and immunohistochemical assays, and qRT-PCR; while fibrosis is measured using Pico-Sirius Red staining, immunofluorescence staining and qRT-PCR.

A 2 Evaluate the effect of different dose of CBG on alleviating inflammation in C57BL/6 mice that were induced with NASH by a MCD diet.

Our working hypothesis was that low CBG concentration would reduce inflammation while high CBG concentration would cause enhanced inflammation. Immunofluorescence staining was utilized to assess inflammation using NASH-inflammation related biomarkers. While gene expression at the mRNA level was evaluated using qRT-PCR.

A 3 Investigate the change in gene expression of the two known forms of CB receptors in liver tissues.

The working hypothesis was that the gene expression of the CB1 receptor would increase in groups receiving CBG. CB1 is related to controlling appetite, while CBG might be a precursor to trigger the expression of the CB1 receptor. In the liver, CB2 receptor was predicted to show similar trends to the inflammatory response where the expression decreased with the increase of CBG dose because it is known to regulate immune response. Immunofluorescence staining

against CB1 and CB2 receptors was used to evaluate the expression of the receptors with intervention of difference CBG doses.

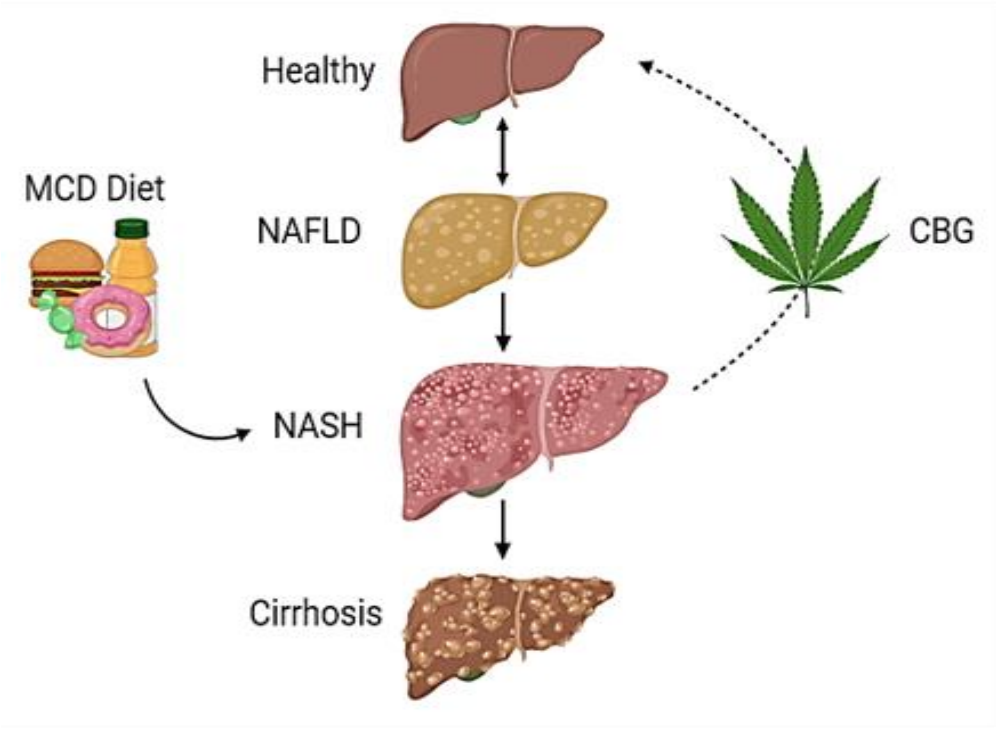


Figure 1. A representative illustration presenting the hypothesis of this study.

CHAPTER 2

METHODOLOGY AND MATERIALS

Diet, Reagents, and Primers

Control diet (CTR) and Methionine/Choline-Deficient diet (MCD) (table 1) were purchased from Envigo (Denver, CO, USA), while all other reagents and primers were purchased from Thermo Fisher Scientific (Denver, CO, USA), VWR (Radnor, PA, USA), Biolegend (San Diego, CA, USA), and Invertogen (Fredrick, MD, USA) unless otherwise indicated. CBG treatment was generously provided by Mile High Labs. (Broomfield, CO, USA).

Animal, Diets and Cannabigerol Treatments

All proposed procedures have been approved by the Institutional Animal Care and Use Committee at the University of Northern Colorado (protocol no. 1910CE-YH-M-22). Male C57BL/6 mice have been used in this study due to the fact that they show NASH symptoms faster than female C57BL/6 female mice (56). Male C57BL/6 mice were obtained from the University of Northern Colorado Animal Facility (n=36) and were randomly assigned into either the control group (TD.94149, n=18) or the MCD diet group (TD.90262, n= 18) and were fed the assigned diet for 3 weeks as the MCD diet has the ability of inducing NASH by the end of the third week. Then, they were randomized in three-subgroups and intraperitoneally (I.P.) injected with a vehicle, a low dose (2.46 mg/kg/day) (L. CBG) or a high dose (24.6 mg/kg/day) (H. CBG) of CBG for three times a week for two additional weeks during the course of the diet. All animals were housed in the animal facility at the University of Northern Colorado for 5 weeks and euthanized on the last day of the study using the recommended dose of EUTHASOL® and heparin, which was I.P. injected. Animals were monitored on a daily basis in accordance with the

Institutional Animal Care and Use Committee and the Collaborative Institutional Training Initiative guidelines.

To prepare high dose CBG treatment, 6.642 mg of anhydrous CBG was dissolved in 10µl of tween-80 and then added in 37.5 µl of Dimethyl sulfoxide (DMSO). Upon injection, Phosphate-buffered saline (PBS) was freshly mixed with the reagents described above to avoid cross-contamination. The low dose of CBG was 10x less concentrated than the high dose CBG, diluted in PBS (1:10 dilution). The vehicle solution was the same solution for dissolve the high dose CBG but without the CBG.

Liver Harvest and Blood Collection

Upon sacrifice, roughly 500µL of peripheral blood was collected and centrifuged at a speed of 123 xg for 5 minutes to isolate the serum. Isolated serum was snap frozen in liquid nitrogen and stored in -80^{o} C for further analyses. In order to assess liver health, serum samples were sent to the Veterinary Teaching Hospital Diagnostic Laboratories at Colorado State University to analyze the levels of ALT. Post sacrifice, liver tissues were harvested and weighed. Then, they were either embedded in an Optimal Cutting Temperature (OCT) medium and snap frozen in liquid nitrogen or directly snap frozen in liquid nitrogen. All samples were stored in -80^{o} C until used except of tissues used for RNA-related analyses which were stored in 4^{o} C.

Histology

Hematoxylin and Eosin Staining

To evaluate overall liver health, Hematoxylin and Eosin staining was performed. 8µm frozen liver sections were fixed in 10% Neutral buffered formalin (NBF) for 10 minutes followed by a wash in 95% ethanol and 2-3 changes of tap water rinse. Tissues were then stained with Hematoxylin for 40 seconds and washed in ammonia water. They were then mordant in

95% ethanol followed by eosin stain for 10 seconds. After that, they were dehydrated in 2 changes of 95% ethanol followed by 3 changes of 100% ethanol. Finally, tissues were washed in 2 changes of xylene and mounted with a mounting medium. All images were taken at 20x magnification.

Oil-Red-O Staining

Lipid accumulation was evaluated using Oil-Red-O staining. 8µm frozen liver sections were fixed in 10% Neutral buffered formalin (NBF) for 10 minutes then immediately washed in 3 changes of distilled water. Tissues were then placed in 100% propylene glycol for 5 minutes followed by pre-heated Oil-Red-O staining for 8 minutes. After that, they were placed in 85% propylene glycol for 5 minutes and rinsed in 2 changes of distilled water. Further, tissues were counterstained with Hematoxylin for 40 seconds and washed afterward in running tap water for 3 minutes. Last, tissues were mounted in aqueous mounting media. All images were taken at 20x magnification.

Pico-Sirius Red Staining

Liver fibrosis was assessed using Pico-Sirius Red staining. 8µm frozen liver sections were fixed in 10% Neutral buffered formalin (NBF) for 10 minutes followed by xylene for ten minutes. Then they were rehydrated in 100%, 90% then 70% ethanol. Then, the nucleus was stained with hematoxylin for 40 seconds followed by a rinse in tap water for 10 minutes. Then, tissues were stained for collagen deposition using Pico-Sirius Red stain for 1 hour followed by 2 washes in 0.5% acidified water and dehydrated in 70%, 90% and 100% ethanol. Finally, tissues were placed in xylene and mounted in mounting media.

Gene Expression

Total RNA was extracted using Pure Link™ RNA Mini Kit obtained from Invitrogen and converted into cDNA using High-Capacity cDNA Reverse Transcription Kit obtained from Applied Biosystems™, according to manufactures' recommendations. qRT-PCR was performed to analyze the mRNA expression of ATGL (triglyceride), CD36 (fatty acid transferase, steatosis), α-SMA (alpha smooth muscle actin, fibrosis), IL-6 (interleukin 6, inflammation), TGF-β1 (transforming growth factor, inflammation), CB1 (cannabinoid receptor 1) and CB2 (cannabinoid receptor 2) and normalized to GAPDH (Table 2- primer sequences). Six biological replicates were used to measure relative gene expression at the transcriptional level unless otherwise indicated. All genes expressions were normalized to GAPDH.

Immunofluorescence Staining

8µm frozen liver sections were fixed in 10% NBF for 10 minutes then washed with 1X PBS. Sections were then blocked for 20 minutes with 10% normal goat serum diluted in 1X PBS, followed by a rinse in 1X PBS. Thereafter, sections were incubated in primary antibody overnight in a 4^{o} C incubator except for the Bodipy dye which was incubated for 30 minutes. The next day, specimens were washed with 1X PBS and stained with the secondary antibody for 45 minutes in the dark at room temperature. Finally, tissues were mounted with DAPI mounting medium and stored in -20^{o} C until analyzed with a Zeiss 700 confocal microscope. All images were taken at 20x magnification and quantified using Fiji ImageJ software (Table 3).

Statistical Procedures

All data were analyzed using GraphPad Prism 9 software and reported as mean ± SEM (Standard Error Mean). The Shapiro-Wilk test was performed to test the normality assumption and indicated that all data were normal, thus parametric statistical tests can be used. Following

that, a one-way analysis of variance (ANOVA) was performed to test significant difference between group means for each experiment in this study. After performing ANOVA tests, Tukey's post hoc tests were performed comparing every pair of groups to indicate which group is causing the significance in the ANOVA tests, when significance is detected. Finally, Pearson's correlation was performed to test if a significant relationship exists between the liver weight and body weight of mice. For all tests used, a p-value ≤ 0.05 was considered significant.

Table 1. MCD diet and MCD control diet components.

Formula	CTR	MCD
L-Amino acids (g/kg)	156.4	156.4
L-Methionine (g/kg)	8.2	0.0
Choline chloride (g/kg)	350 g/kg*	0.0
Sucrose (g/kg)	443.597	455.294
Corn starch (g/kg)	198.783	200.0
Cellulose (g/kg)	30.0	30.0
Corn oil (g/kg)	100.0	100.0
Salt mix (g/kg)	35.5	35.0
Vitamin mix (g/kg)	10.0	5.0

***** information was taken from PMID: 26267291.

Table 2. Primer sequences for qRT PCR.

Gene	Forward	Reverse
AGTL	CAACGCCACTCACATCTACGG	GGACACCTCAATAATGTTGGCA
CD36	AATTAGTAGAACCGGGCCAC	CCAACTCCCAGGTACAATCA
α-SMA	ACTGGGACGACATGGAAAAG	AGAGGCATAGAGGGACAGCA
IL-6	CATCTGTGAGTGGCGTCCGA	AACGCTTCGTTGTGGCTGGA
TGF-β1	GAGCCCGAAGCGGACTACTA	CACTGCTTCCCGAATGTCTGA
F4/80	TGACAACCAGACGGCTTGTG	GCAGGCGAGGAAAAGATAGTGT
CB1	CCAAGAAAAGATGACGGCAG	AGGATGACACATAGCACCAG
CB2	TCGCTTACATCCTTCAGACAG	TCTTCCCTCCCAACTCCTTC
GAPDH	TGCACCACCAACTGCTTAGC	GGCATGGACTGTGGTCATGAG

Table 3. Antibodies and fluorescent dyes dilution and clones

Antibody/Fluorescent dye	COMPANY	Dilution	Clone
DNMT1	Invitrogen	1:100	PA3-16556
a-SMA	Invitrogen	1:100	1A4
CNR1 (CB1)	Invitrogen	1:1000	PA1-743
CNR2 (CB2)	Invitrogen	1:200	PA5-18428
F4/80	Biolegend	1:100	BM8
Alexa Fluor 647	Biolegend	1:100	MAR-1
Alexa Fluor 594	Biolegend	1:100	Poly4053
FcεRI	Biolegend	1:200	MAR-1
Alexa fluor 488	Biolegend	1:100	Poly4054
CD45	Biolegend	1:100	OX-1
a-SMA	Biolegend	1:100	1A4
Bodipy	ThermoFisher	1:1000	N/A
Alexa Fluor® 647	ABCAM	1:200	N/A
Alexa Fluor® 555	ABCAM	1:200	N/A
Alexa Fluor® 488	ABCAM	1:200	N/A

CHAPTER 3

RESULTS

Methionine/Choline-Deficient Diet Changed the Liver Morphologies While Low Dose of Cannabigerol Seem to Restore the Damaged Liver

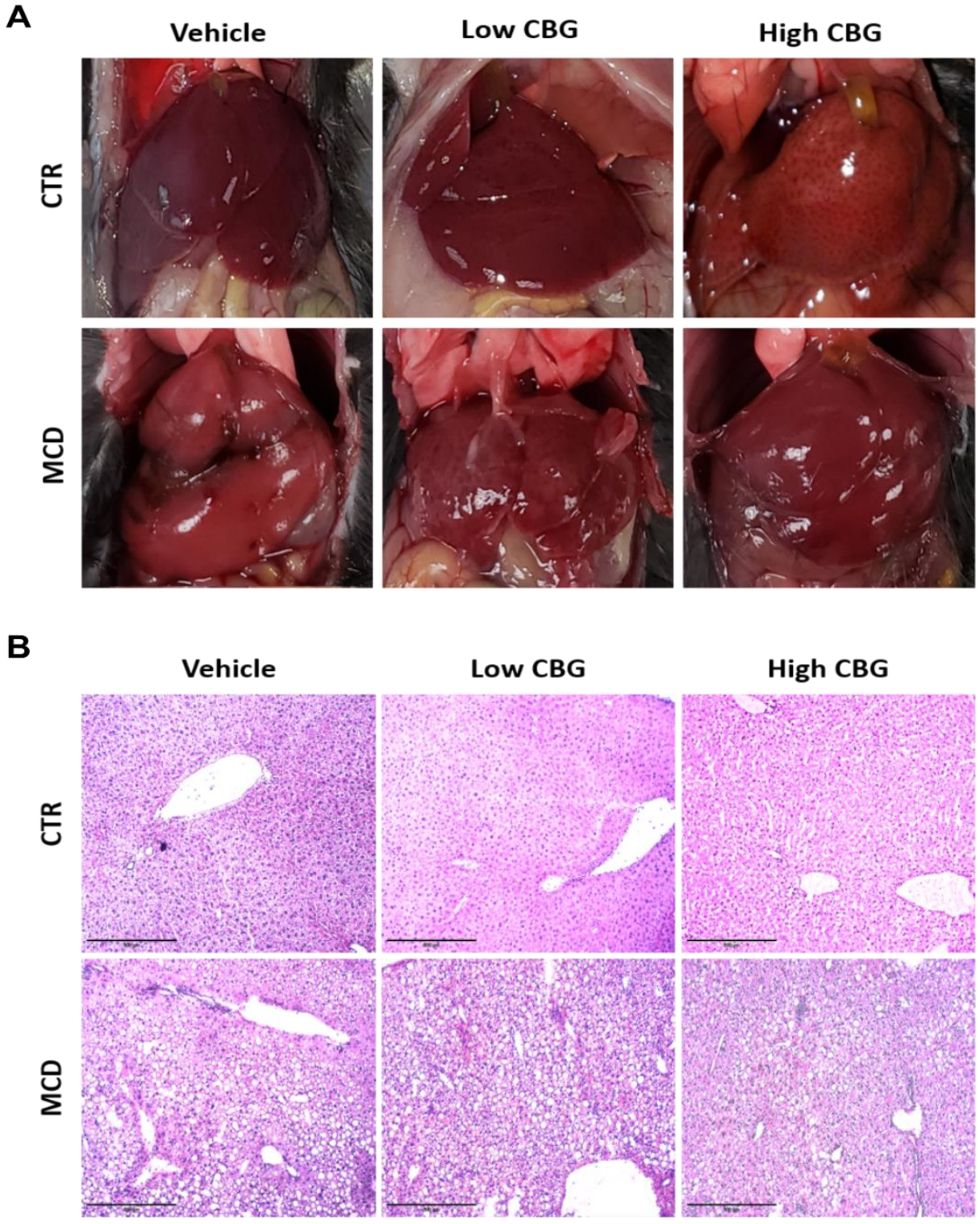
Gross morphologies of the subjects' livers were obtained to further assess the general health of the liver as well as whether NASH was induced in the mouse models described above. The liver pictures along with the corresponding H&E staining (Figure 2A and 2B) indicate there was not a morphological difference between the CTR and CTR L. CBG. However, there was an indication of white blood cells infiltration shown as small blue clusters of cells, surrounding the blood vesicles in groups fed with MCD diet. Also, an accumulation of lipids shown as white circles were observed in CTR H. CBG, MCD, MCD L. CBG and MCD H. CBG (Figure 2B, top right and bottom row). There was a significant change among groups in both the liver weight ($p< 0.001$, Figure 2C) and the body weight ($p< 0.0001$, Figure 2D) but not in the liver to body weight ratio ($p= 0.4535$, Figure 2E). This trend led to the investigation of whether there is a correlation between the liver weight and the body weight. Interestingly, there was a positive correlation between the liver and body weight in the CTR ($r= 0.6134$), CTR L. CBG ($r= 0.8053$) and MCD L. CBG group ($r= 0.4383$), meaning, as the liver weight increases, the body weight increases as well. On the other hand, the CTR H. CBG ($r= -0.1365$), MCD ($r= -0.5237$) and MCD H. CBG ($r= -0.1059$) showed a negative correlation between the liver weight and the body weight, indicating that as the liver weight increases, the body weight decreases in those groups. Last, serum samples were obtained to analyze the ALT levels which is commonly used as a

biochemical marker for NASH (9). Although there was not a statistical difference detected among groups, MCD-treated groups had higher ALT levels (Figure 2G). In general, there was a damage associated with the MCD diet and the high dose of CBG in both the CTR and the MCD group, but not in the MCD L. CBG. This could be a result of either the high dose of CBG or the tween-80 used to dissolve the anhydrous CBG, as it is an oil-based solution which could contribute to the lipid accumulation.

Methionine/Choline-Deficient Diet Increased Steatosis Accumulation in Hepatic Tissues as Fatty Acid Import Increased

To further assure that steatosis was induced in mice and to assess whether CBG reduced the steatosis associated with NASH, Oil-Red-O staining was performed. The data showed that there was not an indication of lipid accumulation in all the control groups, whereas there was a clear lipid accumulation in all the groups that were fed the MCD diet (lipid is shown in red). Within the MCD groups, there was a variation in the size of the lipid droplets. The MCD group showed larger lipid droplets when compared to the MCD L. CBG and MCD H. CBG group (Figure 3A). Similarly, immunofluorescence staining of neutral lipids (bodipy dye) showed no or minimal positivity of lipids in the control groups, while the MCD-fed groups showed a significant increase in lipids positivity ($p< 0.0001$, Figure 3B and 3C). At the transcriptional level, CD36 (NASH lipid translocase biomarker) (63, 64) relative gene expression significantly changed among groups ($p< 0.0001$). More specifically, there was a significant increase in CD36 expression in the MCD group when compared to the CTR group ($p< 0.01$) indicating that more fatty acids were imported into the liver tissue with the MCD diet (Figure 3D). However, the CBG treatment did not change the profile of the fatty acid import into the liver tissues. Furthermore, there was not a statistical difference noted in the gene expression of triglyceride-

related genes, yet ATGL mRNA levels were noted to be the highest in the MCD group (Figure 3E). Overall, the MCD diet induced NASH by increasing the lipid accumulation observed in the liver sections and CD36 gene expression, while CBG treatment did not decrease the lipid accumulation and the gene expression of CD36 in the liver tissues.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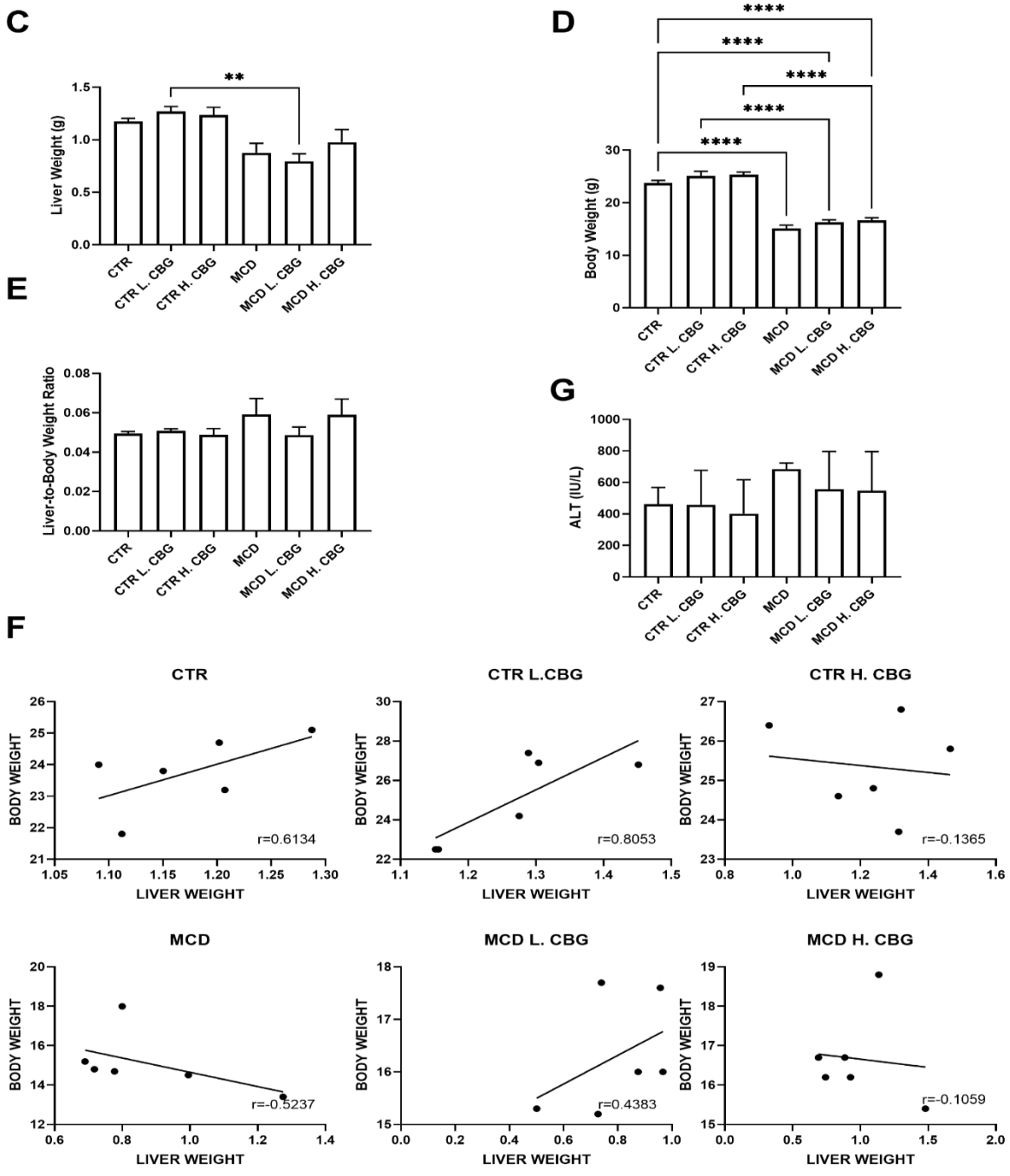

Figure 2. Evaluation of the overall liver health with the induction of NASH using MCD diet and role of CBG in reducing the damage. Macroscopic (A) and Microscopic (B, 20X) representation of the liver and after being stained with H&E, showing lipid accumulation and white blood cells infiltration with the MCD diet (bottom row). Average liver weight (n=6, C) and average body weight (n=6, D), and average liver-to-body weight ratio (E) were measured as an initial step in evaluating the overall health of the liver. Correlation between the liver and body weight (F) was measured to further investigate trends in liver and body weights.

Hepatic Fibrosis Decreased with Administration of Low Cannabigerol Dose but not with High Dose of Cannabigerol

Along with steatosis, NASH is generally known to be associated with liver fibrosis. In the present study, fibrosis was first evaluated by staining for collagen deposition in the liver using Sirius Red staining. The data showed that collagen deposition was the highest in the CTR H. CBG ($p<0.01$), MCD ($p< 0.01$) and MCD H. CBG ($p< 0.0001$) when compared to the CTR group. Further, in was observed that the fibrosis significantly decreased in MCD L. CBG when compared to the MCD group ($p< 0.05$) and MCD H. CBG groups ($p< 0.0001$) (Figure 4A and 4B). This observation was consistent with α-SMA immunofluorescence findings, as α-SMA expression increased in the MCD group when compared to the CTR group ($p<0.0001$), while the α-SMA expression decreased in both the MCD L. CBG ($p< 0.0001$) and MCD H. CBG ($p< 0.05$) when compared to the MCD group. Unlike the Sirius Red staining, there was no change between the CTR group and CTR H. CBG ($p= 0.7879$, Figure 4C and 4D). Although no statistical significance was detected ($p= 0.2347$), mRNA expression of α-SMA showed similar patterns to the α-SMA immunofluorescence expression. There was an increasing trend in the gene expression in both MCD and MCD H. CBG, whereas the gene expression was reduced in the MCD L. CBG group (Figure 4E). Taken together, liver fibrosis decreases with a low dose of CBG and increases with a high CBG dose in the MCD groups but not in the control groups.

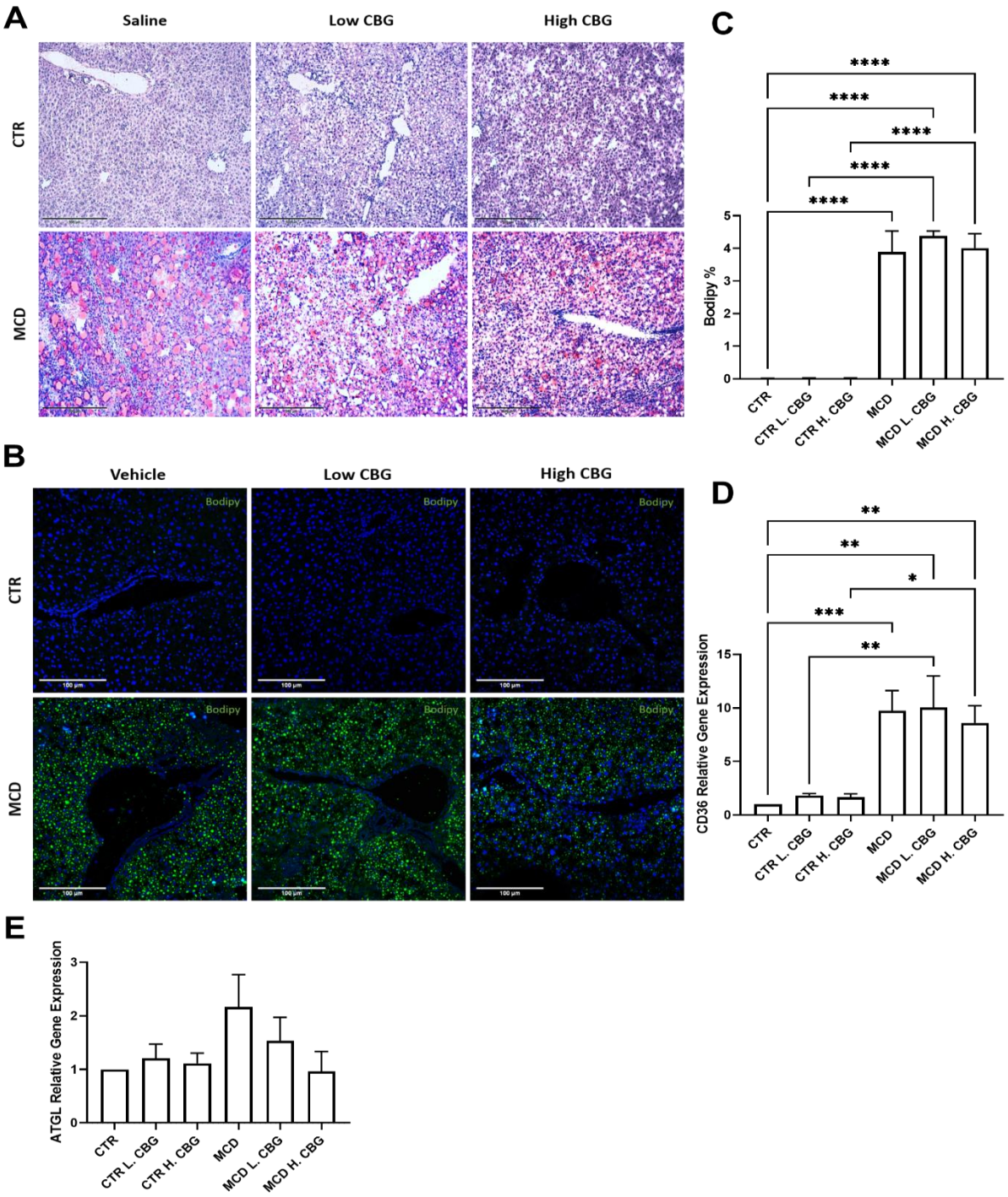

Figure 3. Steatosis was evaluated using histology and qRT-PCR. Microscopic representation showing Oil-Red-O staining (A, 20X) and immunofluorescence staining of bodipy (green) and DAPI (blue) antibodies (B, 20X). Immunofluorescence staining was quantified using ImageJ (C). mRNA expression of CD36 (D) and ATGL (E) were evaluated to measure lipid import and triglycerides.

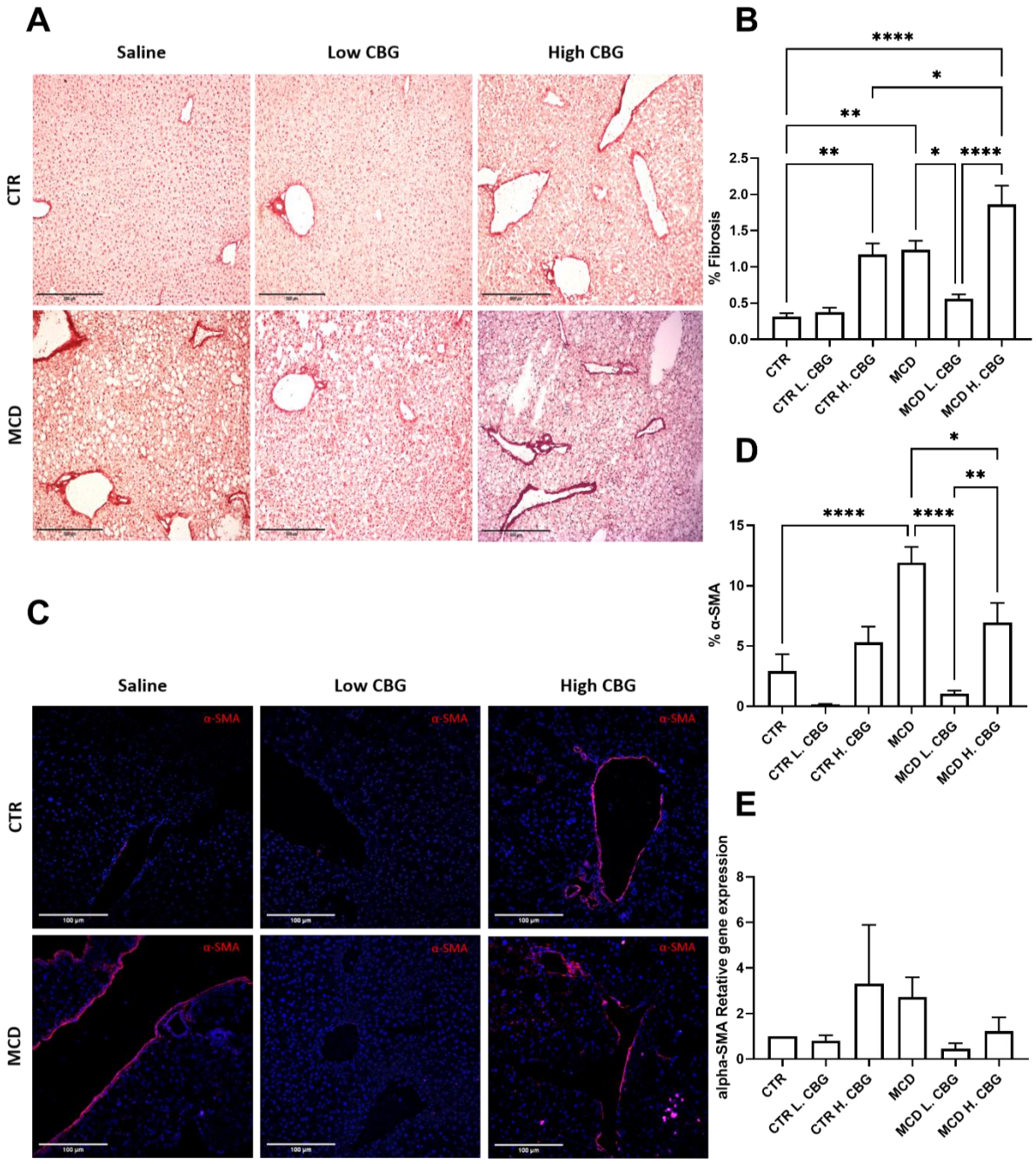

Figure 4. Liver fibrosis was measured by Picro-Sirius Red staining, α-SMA staining and mRNA gene expression. Representative pictures and quantification of collagen deposition in liver frozen section (A and B, 10X). α-SMA immunofluorescence staining and quantification (C and D, 20X) showing α-SMA in red and DAPI (nucleus) in blue (D). Relative gene expression of α-SMA was evaluated (E).

Low Dose of Cannabigerol Decreased Inflammation while High Dose of Cannabigerol Promoted Inflammation

NASH is thought to be initiated by the oxidative stress in the liver, which promotes the activation of proinflammatory cytokines. Thus, it was vital to investigate the potential anti-inflammatory effect of CBG in the MCD-induced NASH mouse model used in this study. The presence of white blood cells (leukocytes) biomarker CD45 was evaluated as an initial step to check the inflammation profile in the liver tissues, and whether CBG aid in reducing the inflammation. Figure 5A and 5B showed an increase in the white blood cells biomarker, CD45, expression in the CTR H. CBG and MCD groups when compared to the CTR group ($p<0.05$). While in the MCD L. CBG group, the gene expression of CD45 significantly decreased when compared to the MCD group ($p<0.01$), while increasing in the MCD H. CBG group when compared to the MCD group ($p<0.01$) and the MCD L. CBG ($p<0.0001$). Likewise, the expression of the liver macrophages, F4/80, showed similar patterns in immunofluorescence staining where there was an increase in expression in the CTR H. CBG group when compared to both the CTR and CTR L. CBG groups ($p<0.05$). Also, there was a decrease in the mRNA and protein expression of F4/80 in the MCD L. CBG when compared to the MCD H. CBG group ($p<0.05$); but not when compared to the MCD group (Figure 5C and 5D). Interestingly, there was not a change in the gene expression of F4/80 at the transcriptional level between the control groups; however, the expression of F4/80 increased in the MCD H. CBG group when compared to the MCD ($p<0.05$) and the MCD L. CBG group ($p<0.05$, Figure 5E). In addition, there was not a change detected among the above-described groups at the transcriptional level of TGFβ1 and IL-6 (Figure 5F and 5G). Overall, white blood cells infiltration was highest with the intervention of high dose of CBG as well as the MCD. While group low dose of CBG decreased

the white blood cells infiltration in the liver tissues, as well as it reduced the expression of pro-inflammatory cytokines in both the CD45 and F4/80 immunofluorescence staining.

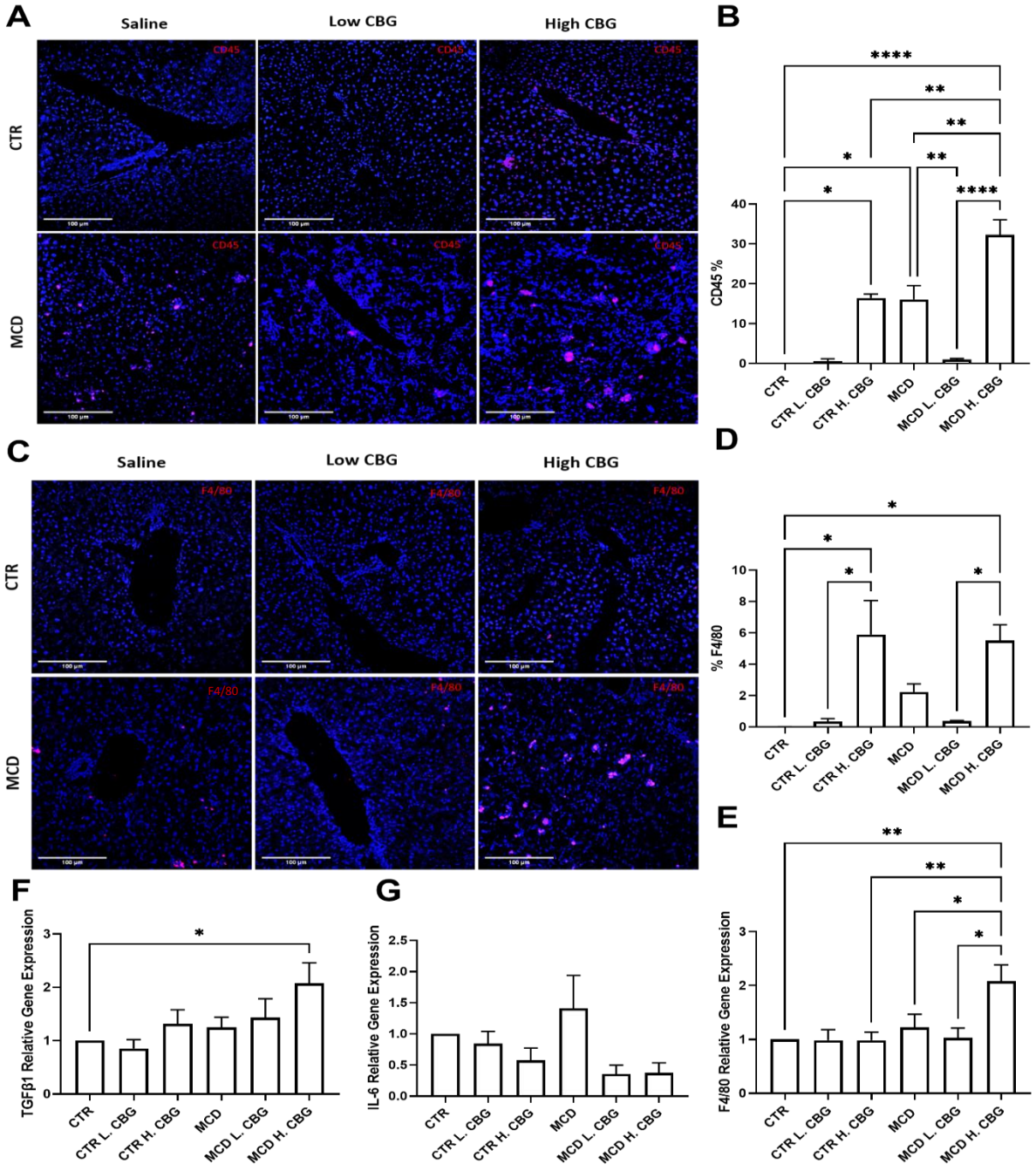

Figure 5. Immunofluorescence staining and qRT-PCR were utilized to measure inflammation in liver tissues. A representative image of liver frozen sections stained for white blood cells showing CD45 in red and DAPI in blue (A, 20X), which was quantified using ImageJ (B). After, the expression of liver localized macrophages (F4/80) in Frozen liver section was evaluated (C) shown in red F4/80, while nucleus is shown in blue. Images of F4/80 was also quantified using ImageJ (D). mRNA expression of F4/80 (E) and TGFβ1 (F), and IL-6 (G) were evaluated using qRT-PCR.

Cannabinoid Receptors' Expression is Promoted due to Methionine/Choline-Deficient Diet but not with the Cannabigerol Administration

Cannabinoid (CB) receptors 1 and 2 have been identified to play a role in the liver metabolism and immune system defense, respectively. More specifically, they play a role in various liver diseases such as hepatocellular carcinoma, NAFLD, and NASH (22, 23). Here, the expressions of both CB receptors were evaluated using immunofluorescence (Figure 6A), which have revealed no or minimal expression of both receptors in the control groups. On the contrary, the MCD diet-fed groups showed an increase in the gene expression of both CB1 and CB2 (Figure 6B and 6C). Although the CB2 receptor had higher expression, interestingly, when subjects were treated with the low dose of CBG, there was a significant decrease in the gene expression of both the CB1 ($p<0.0001$) and CB2 receptors ($p<0.01$) when compared to the MCD group.. Further, there was a general trend of co-localization of CB1 and CB2 receptors (shown as white dots). Overall low dose CBG treatment seems reduced the expression of both CB1 and CB2 while high dosage of CBG did not change CB1 and CB2 expression induced by MCD; Meanwhile, the CBG treatment only is not enough to induce expression of CB1 or CB2. Yet, more investigations are still needed to obtain a better understanding of their roles in the liver, and more specifically, in the NASH progression.

These observations lead us to investigate whether the CB receptors expression was enriched in immune cells that induced by the MCD diet. Thus, a co-staining against CB1, CB2, FcεRI was employed. FcεRI is a cell-surface receptor for the immunoglobulin IgE and is known to be the start point of a cascade of responses (57) such as the regulation and activation of mast cells (58). Recently, it was noticed that mast cells are found to be highly expressed during liver injury thus mediating fibrosis (59). Figure 7 is demonstrating the relation between CB1, CB2,

and FcεRI by the increase of gene expression in the MCD-fed groups. Interestingly, we noticed that both CB1 and CB2 are expressed in Mast cells (FcεRI+). No other cell types showed either CB1 or CB2 positive staining. This indicating the Mast cells might be activated by MCD-diet in mice liver via activation of both CB1 and CB2 receptors; while low CBG treatment inhibit the infiltration or activation of Mast cells in MCD group via decreased expression of CB1 and CB2. It is worth to notice that the expression of CB1 and CB2 expression decreased in MCD H. CBG group as well, while the Mast cell number seems not decreased, which might indicate the high CBG treatment might cause the Mast cell activation via a CB1 and CB2 independent pathway. However, more experiments are needed to confirm the findings.

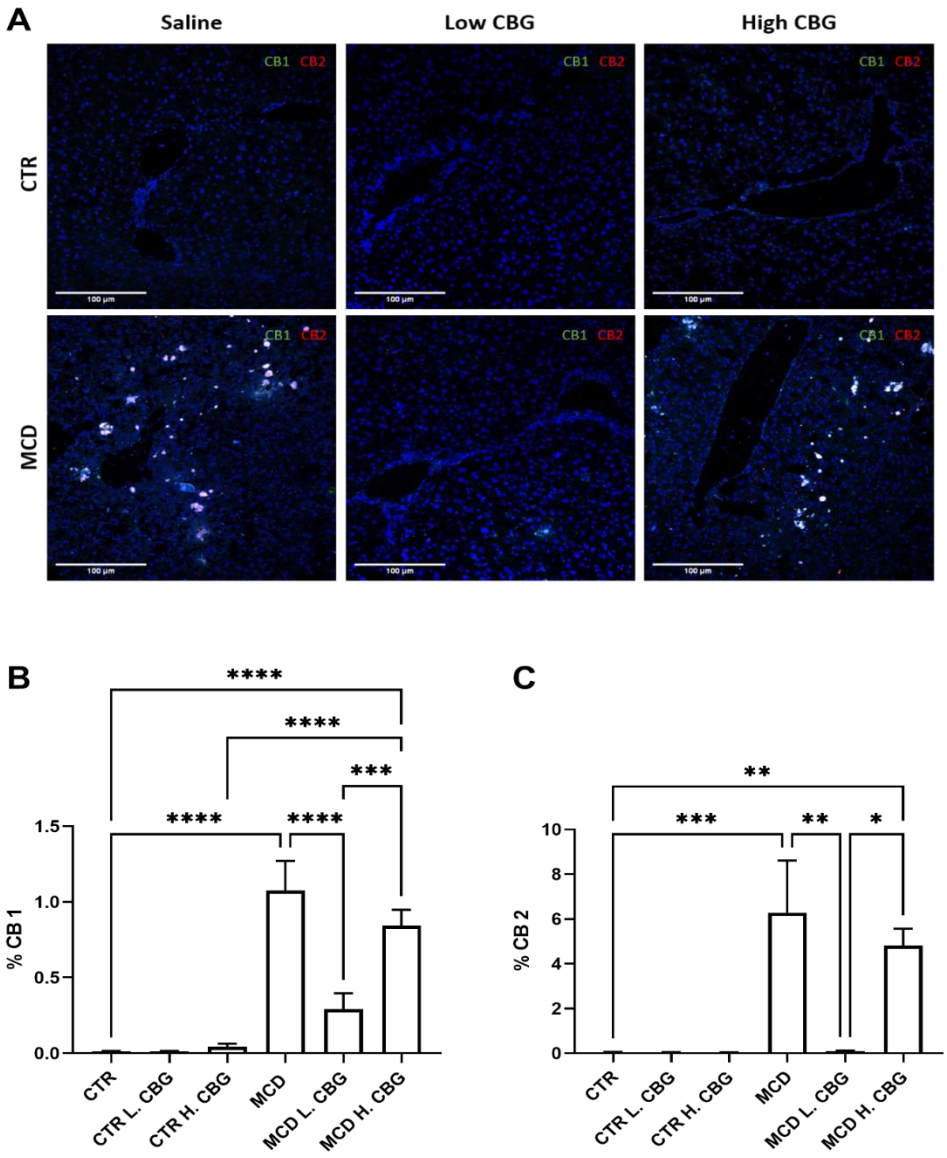

Figure 6. Evaluation of CB receptors in liver frozen sections.
Immunofluorescence staining of frozen liver sections for cannabinoid receptors 1 and 2 (A, 20X), illustrating CB1 in green and CB2 in red. All images were quantified using ImageJ (B and C).

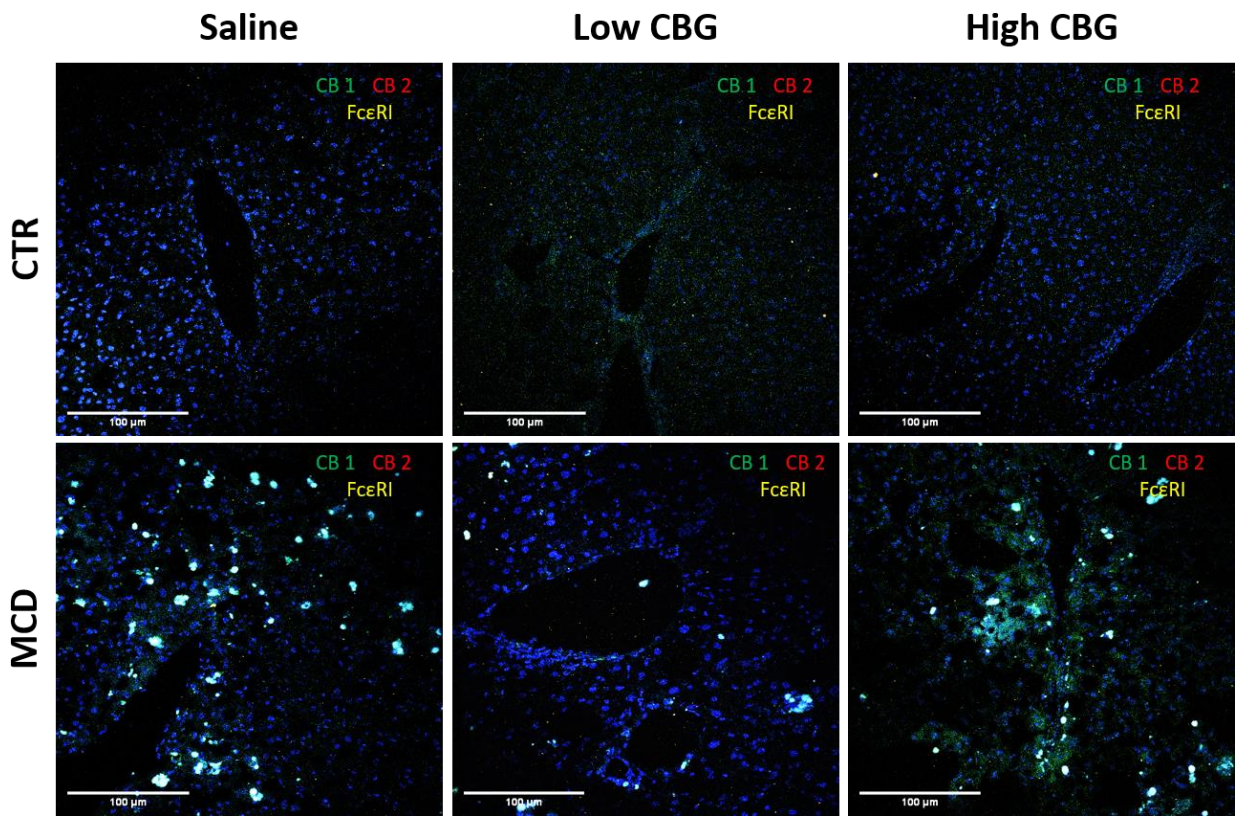

Figure 7. Evaluation of CB receptors with FcεRI co-localization in liver frozen sections. Immunofluorescence co-staining of frozen liver sections against CB1, CB2, and FcεRI (20X).

CHAPTER 4

SUMMARY AND CONCLUSIONS

NASH is one of the most common liver diseases globally and is becoming a worldwide concern as cases continues to rise (9). The fact that there are no FDA-approved treatments for NASH makes the rise in cases more concerning. NASH patients are left with no treatment options and require liver transplants in most cases (9) as the disease keeps progressing to liver cirrhosis and/ or hepatocellular carcinoma. Pathologic features of NASH such as hepatic steatosis, fibrosis and inflammation are interconnected. This means that these pathologic features influence each other via positive feedback (38, 39). The current study has used MCD diet to induce NASH in three weeks and has confirmed the development of NASH by the increase levels of steatosis levels, CD36 expression, fibrosis, and inflammation levels. Furthermore, this study has shown the therapeutic potential of CBG, as it reduced NASH-related pathologic features when administrated in low dose.

Various studies have used other forms of *cannabis* such as CBD as a treatment for NAFLD (19, 40), but the effect of CBG in alleviating NASH has not been previously investigated. Interestingly, there was a positive correlation between the liver weight and the body weight when MCD-fed subjects were treated with a low dose of CBG, suggesting a trend in liver weight recovery. This trend could be explained by the fact that there is less fibrotic tissues and pro-inflammatory cytokines in the liver. Thus, this was an initial indication of the potential positive therapeutic effect of CBG in the liver. Yet, more investigations are still needed to confirm the cause for the recovery trend in the liver weight.

However, we did not find significant differences in ALT levels (data not shown), which is consistent with other findings (8, 13, 14). Nevertheless, the ALT levels we measured might not be precise due to the increase levels hemolysis, which is a contributor to the levels of ALT in the mice serum. The increase levels of hemolysis were due to the improper blood collection, which lead to skewed ALT levels (60-62) Further measurements are still needed to confirm the effect of CBG on ALT levels. Likewise, we did not find significant changes on steatosis and involved biomarker CD36 gene expression in male mice. Taken together, we found CBG plays minimal role in altering the ALT levels as well as the steatosis in NASH ice model. The recovery trend and the positive correlation observed between the liver and body weight in the MCD L. CBG group could be due to another factor.

This study is consistent with other studies that have used CBD, where they did not observe a statistical difference in the ALT levels using low dose of CBD (40). Another study by Wang Y. et al., found that 5mg/kg of CBD reduced hepatic steatosis in alcoholic fatty liver disease (19). Whereas our study did not observe any difference in hepatic steatosis with either the low dose (2.46 mg/kg) nor the high dose (24.6 mg/kg) of CBG in male mice. These findings were consistent with another study that used 0.05 mg/kg of Abn-CBD (atypical form of CBD) to treat NAFLD (40). Further, there was not any change in the import of fatty acids to the liver in any of the MCD-fed groups; indicating that CBG did not reduce the steatosis in the liver. Hence, this suggests that the recovery trend and the positive correlation observed between the liver and body weight in the MCD L. CBG group could be due to another factor.

This study showed that liver fibrosis was induced as a result of the MCD diet and was alleviated with the administration of a low dose of CBG. However, the high dose of CBG also increased the levels of fibrosis in liver tissues. More specifically, both collagen deposition shown

by the Pico-Sirius Red staining and α-SMA protein and mRNA expression decreased with the use of low dose CBG, but did not with high dose of CBG, which is consistent with other studies that have investigated liver fibrosis using low dose (0.05 mg/kg) of atypical CBD (40). Although these studies did not use similar doses, they have shown the potential of *cannabis* in reducing liver fibrosis when used in low amounts such as the dose used in this study. Despite the fact that low dose of CBG reduced fibrosis in the liver tissues, it is still important to acknowledge the fact that high dose of CBG have opposite effect. This is critical to take into consideration when administering CBG in future studies.

Hepatic inflammation is one of the pathologic phenomena of NASH that could lead to liver dysfunction in NASH patients. A previous study has reported that hepatic inflammation plays an important role in the progression of hepatic steatosis and fibrosis (41). In this study, it was found that a low dose of CBG reduced the inflammation induced by the MCD diet, which was initially assessed using immunofluorescence staining for two of the white blood cells biomarkers, CD45 and F4/80, in frozen liver sections. The findings suggest that the low dose of CBG reduced infiltration of white blood cells in MCD diet-induced mice, while the high dose of CBG resulted in enhanced infiltration of white blood cells. Likewise, mRNA expression of NASH inflammation biomarkers, F4/80, was alleviated with intervention of low dose of CBG, but not TGFβ1 and IL-6. These findings are consistent with previous study showing an anti-inflammatory effect of CBG (33). Similarly, other reports that found that F4/80 expression was reduced with the intervention of atypical CBD, yet they found reduction in IL-6 mRNA expression which is not consistent with the findings in this study (40). Overall, the findings suggest that CBG has a potential in reducing inflammation associated with NASH.

The endocannabinoid systems involving CB 1 and CB 2, where CB1 receptor is primarily expressed in the brain and as well as other peripheral tissues (42). In the brain, CB1 receptor controls appetite (24), but its antagonist showed evidence suggesting the ability to reduce obesity due to the fact that it reduces the food intake in rodents (43). On the other hand, the CB2 receptor is primarily expressed in the immune system. This study has found that the expression of CB1 and CB2 receptors showed similar pattern to the inflammatory response in Figure 7, which lead us to investigate which cell is expressing these receptors. Our findings, indeed, confirmed that increased infiltration of Mast cells into the liver by MCD diet accompanied with enhanced CB receptors, while low dose CBG treatment reduced this trend. However, more mechanistic investigations are still needed to better understand the observed results.

In summary, this study observed the protective effect of CBG in MCD diet-induced NASH in mice. A low dose of CBG reduced hepatic fibrosis and hepatic inflammation but not hepatic steatosis while a high dose of CBG worsened the pathologic features of NASH. In conclusion, this study provides initial findings and a foundation for future studies on the efficacy of CBG on NASH.

Limitations

This thesis investigated the role of CBG in alleviating the pathologic features associated NASH such as steatosis, fibrosis and inflammation in C57BL/6 male mouse model, and there were several important limitations.

First, the MCD diet is known to induce NASH in a short period of time, yet it does not replicate metabolic syndrome seen in human NASH patients. In fact, the MCD diet shows reversed metabolic syndrome etiology in rodent models. However, since this study's main interest is focused on steatosis, fibrosis and inflammation, the MCD diet was found to be

appropriate. Additionally, this diet is considered the most accessible and affordable diet for the purpose of this study.

Further, the reversibility of the NASH with the administration of different doses of CBG was not answered in this study due to the lack of mice availability. This could be measured in the future by measuring the levels of steatosis, fibrosis, and inflammation in the liver prior to the administration of CBG. Once the course of CBG have finished, the reversibility could be assessed by comparing the liver morphologies before and after the intervention of CBG.

Although, biological sex of the mice was taken into consideration during the experimental design; only male C57BL/6 mice were used. Although the biological sex factor would be of interest, due to the time restriction as we only had 3 months to treat the mice and collect tissues, only C57BL/6 male mice were used for evaluating the role of CBG in alleviating the pathologic features associated with NASH, since male C57BL/6 mice show NASH symptoms more that the female ones (56).

Lastly, there were only 6 mice in each group in this experiment which is considered a relatively small sample size. The small sample size could explain the lack of significance detected between some groups which is due to the low power of detecting statistical difference as well as to the increase in the variation between group. In this thesis, small sample sizes (n=36) were used due to time and financial resources restraints.

Future Directions

While this study demonstrated the potential in using CBG as a therapeutic agent in MCD diet-induced NASH in C57BL/6 male mice, further rigorous study is needed to confirm the efficacy of CBG in NASH symptoms before conducting clinic trial.

In order to determine the potential use of CBG as a treatment for NASH, further understanding of oxidative stress and cellular senescence is needed. Oxidative stress is defined as the imbalance of free radicals and antioxidant particles in the body (44). It was suggested to likely be the start point of the damage in the liver caused by the high fat diet (45). Cellular senescence is defined as the cell cycle arrest that is combined with proinflammatory cytokine secretion. Further, hepatic cellular senescence was shown to be correlated with fat accumulation and the progress of NAFLD to NASH (46). Investigating oxidative stress and cellular senescence pathways could aid in understanding the progression of the disease in both human and non-human models.

Even though our study has showed promising change in expression of both the CB1 and CB2 receptors with the use of a low dose of CBG in MCD-fed mice, the endocannabinoid system is a possible area of investigation. As previously mentioned, the CB1 receptor is mainly expressed in the central nervous system while being expressed in lower levels in various peripheral tissues. A previous study showed that blocking the CB1 receptor reduced liver fibrosis in mouse models though the CB1 receptor/β-arrestin1/Akt pathway (47). This indicates that the CB1 receptor plays a role in the progression of NASH, meaning that it is important to understand the impact of CBG on CB1 receptor-involved pathways.

Further, the long-term impact of CBG on the liver and on the progression of NASH have not yet been investigated. Generally, the lack of long-term effect of any treatment leads to the treatment being considered controversial and uncertainty in the effectiveness of the proposed treatment is likely to arise. This study administrated CBG to NASH mouse models for only two weeks, but it has did not investigate the impact of using CBG for longer than two week, and whether fibrosis and inflammation would still be reduced, or whether opposite trend would be

seen with low dose of CBG. Therefore, it is vital to understand the long-term impact and safety of CBG on the liver and other organs such as the brain and heart.

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APPENDIX A

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE APPROVAL

UNIVERSITY OF
NORTHERN COLORADO

Institutional Animal Care and Use Committee

Date: *August 5, 2020*

Principal Investigator: *Yuyan Han*

Committee Action: **IACUC Protocol- Annual Continuation Approval #1- 2020**
Action Date: August 5, 2020

Protocol Number: 1910CE-YH-M-22
Protocol Title: *The Effect of Circadian Rhythm in High-Fat Diet Fed Mouse Model*

Expiration Date: August 28, 2022

The University of Northern Colorado Institutional Animal Care and Use Committee (IACUC) has completed an annual review and APPROVED the continuation of animal use protocol *The Effect of Circadian Rhythm in High-Fat Diet Fed Mouse Model*– #**1910CE-YH-M-22** on August 5, 2020 for another year. Since no changes were incorporated into this protocol at this time, animal use may continue for another year as previously approved.

The committee's review was based on the requirements of the Government Principles, Public Health Policy, USDA Animal Welfare Act and Regulations, the Guide for the Care and Use of Laboratory Animals, as well as university policies and procedures related to the care and use of animals at the UNC. Based on the review, the IACUC has determined that all review criteria have been adequately addressed. The PI is approved to perform the experiments or procedures as described in the protocol as approved by the committee for another year. It is the responsibility of the PI to be familiar with and comply with the protocol and all pertinent institutional, state, and federal rules and policies. Until this protocol expires, annual IACUC review of the protocol is required.

If you have any questions, please contact the UNC Animal Care and Use Program (ACUP) Director, Laura Martin, at 734-730-6631 or via e-mail at laura.martin@unco.edu. Additional information concerning the requirements for the welfare and use of animal subjects can be found at the websites for the University of Northern Colorado ACUP https://www.unco.edu/research/research-integrity-and-compliance/iacuc/, the NIH's Office of Laboratory Animal Welfare https://olaw.nih.gov/, and the USDA's Animal Plant and Health Inspection Services https://www.aphis.usda.gov/aphis/home/.

Sincerely,

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APPENDIX B

CREATINE ALLEVIATES DOXORUBICIN-INDUCED LIVER DAMAGE BY INHIBITING LIVER FIBROSIS, INFLAMMATION, OXIDATIVE STRESS, AND CELLULAR SENESCENCE

CREATINE ALLEVIATES DOXORUBICIN-INDUCED LIVER DAMAGE BY INHIBITING LIVER FIBROSIS, INFLAMMATION, OXIDATIVE STRESS, AND CELLULAR SENESCENCE

Published in *Nutrients*, DOI number: https://doi.org/10.3390/nu13010041

Treatment with the chemotherapy drug doxorubicin (DOX) may lead to toxicities that affect non-cancer cells including the liver. Supplementing the diet with creatine (Cr) has been suggested as a potential intervention to minimize DOX-induced side effects, but its effect in alleviating DOX-induced hepatoxicity is currently unknown. Therefore, we aimed to examine the effects of Cr supplementation on DOX-induced liver damage. Male Sprague-Dawley rats were fed a diet supplemented with 2% Cr for four weeks, 4% Cr for one week followed by 2% Cr for three more weeks, or control diet for four weeks. Animals then received either a bolus i.p. injection of DOX (15 mg/kg) or saline as a placebo. Animals were then sacrificed five days-post injection and markers of hepatoxicity were analyzed using the liver-to-body weight ratio, aspartate transaminase (AST)-to- alanine aminotransferase (ALT) ratio, alkaline phosphatase (ALP), lipemia, and T-Bilirubin. In addition, hematoxylin and eosin (H&E) staining, Picro-Sirius Red staining, and immunofluorescence staining for CD45, 8-OHdG, and β-galactosidase were performed to evaluate liver morphology, fibrosis, inflammation, oxidative stress, and cellular senescence, respectively. The mRNA levels for biomarkers of liver fibrosis, inflammation, oxidative stress, and senescence-related genes were measured in liver tissues. Chromosomal stability was evaluated using global DNA methylation ELISA. The ALT/AST ratio and liver to body weight ratio tended to increase in the DOX group, and Cr supplementation tended to

attenuate this increase. Furthermore, elevated levels of liver fibrosis, inflammation, oxidative stress, and senescence were observed with DOX treatment, and Cr supplementation prior to DOX treatment ameliorated this hepatoxicity. Moreover, DOX treatment resulted in chromosomal instability (i.e., altered DNA methylation profile), and Cr supplementation showed a tendency to restore chromosomal stability with DOX treatment. The data suggest that Cr protected against DOX-induced hepatotoxicity by attenuating fibrosis, inflammation, oxidative stress, and senescence.

Contribution of Authors and Co-Authors

Manuscript in Chapter V

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Contributions: data curation, formal analysis, investigation, methodology, visualization, writing—original draft, writing—review & editing

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