5-1-2021

Mast Cell Infiltration in Liver of Cannabigerol Treated Methionine/ Choline Deficient Diet Induced Mice Non-alcoholic Steatohepatitis Model

Agathe Jacobsen
University of Northern Colorado

University of Northern Colorado
Greeley, Colorado

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A Thesis Submitted in partial fulfillment for Graduation with Honors Distinction and the Degree of Bachelor of Science

Agathe Jacobsen

School of Biological Sciences

College of Natural and Health Sciences

May 2021

PREPARED BY: _____
Agathe Jacobsen

APPROVED BY
THESIS ADVISOR: _____
Yuyan Han, Ph.D.

HONORS
DEPARTMENT LIAISON: _____
Stephen P. Mackessy, Ph.D.

HONORS DIRECTOR: _____
Loree Crow, M.A.

RECEIVED BY THE UNIVERSITY TEHSIS/CAPSTONE
PROJECT COMMITTEE ON:

May 1, 2021

Abstract

Non-alcoholic fatty liver disease (NAFLD) is the most common chronic liver disease across the world. Once it progresses to non-alcoholic steatohepatitis (NASH), little can be done to reverse the damage. A potential treatment for NASH is cannabigerol (CBG), as it has shown anti-inflammatory effects in other models, although, little is known about its effects on NASH. Mast cells (MCs) play a role in mediating the progression of NASH. Their concentration in the liver directly correlates to levels of fibrosis. Therefore, we aim to evaluate levels of MC infiltration under CBG treatment in a NASH-induced mouse model. C57BL/6 mice were fed with a methionine/choline-deficient (MCD) or control (CTR) diet for 3 weeks, then divided into 3 sub-groups. The mice were injected with vehicle, low CBG [2.46 mg/kg/day], or high CBG [24.6 mg/kg/day] for another two weeks. The livers were harvested and frozen to be sliced for placement on slides. Slides were stained for presence of MCs using Toluidine-blue (T-blue) stain and Naphthol A-SD chloroacetate-esterase (CAE) stain. The samples were observed under a microscope and evaluated for mast cell infiltration. Co-staining of FcεR1 (a MC biomarker) and TGFβ1 (a pro-inflammatory cytokine) was used to stain the co-localization of MCs and their inflammatory effect using immunofluorescence. We have found that treatment with low CBG decreased the numbers of MCs in MCD mice while high CBG treatment did not. There was no positive staining of MCs in the control groups. These results are consistent with the FcεR1/TGFβ1 immunofluorescence staining. There was no positive staining for mast cells in the control groups and treatment with low CBG decreased the presence of mast cells and TGFβ1 expression. In conclusion, low CBG treatment reduced MC infiltration caused by the MCD diet in the mice. High CBG treatment did not reduce MC infiltration in the mice.

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Introduction

As the prevalence of obesity and type 2 diabetes increases in Western culture, so does the risk of developing non-alcoholic fatty liver disease (NAFLD), the most common chronic liver disease worldwide (Yu, 2018). Non-alcoholic steatohepatitis (NASH) is the advanced stage of NAFLD, followed by potential fibrosis and cirrhosis or cryptogenic cirrhosis, which can develop into hepatocellular carcinoma (HCC), at which point a liver transplant is required (Yu, 2018). Following the development of NASH, chances of cardiovascular mortality increase, leaving the medical community searching for treatment methods (Sanyal, 2011). While beginning stages of NAFLD can be counteracted with diet and exercise, NASH is less reversible, and researching potential treatments with rising incidence levels is important (Oseini and Sanyal, 2017). One of the potential treatment options being researched for NASH is cannabinoids, as there are endogenous cannabinoid receptors in the liver that are expressed by immune cells that can be targeted to slow hepatic fibrosis (Caraceni et al., 2014). Mast cells are an important part of the body's immune response to damage, and their numbers in liver hepatocytes directly correlate to fibrosis levels in NASH (Lombardo et al., 2018). My research aims to evaluate the infiltration of mast cells in Methionine/Choline deficient (MCD) diet induced mouse MASH models and observe any changes that may result from treatment with high and low levels of cannabigerol (CBG).

Review of Relevant Literature

Mast Cells

Mast cells were originally viewed as allergy effectors, playing an important role in allergic reactions, and when being studied in 1878, could only be identified by their

morphological features (da Silva et al., 2014). As technology improved, mast cells were found to release histamine during anaphylactic shock, triggered by the presence of IgE antibodies that bind to receptors on the mast cell surface (da Silva, 2014). Derived from hematopoietic stem cells, mast cells are distributed to tissues of high vascularization and surfaces exposed to external environments (Galli, Nakae, and Tsai, 2005). Therefore, they are the first immune cells to interact with any released antigens, allergens, or pathogens, leading to the release of bioactivators which can mediate the effects of inflammation, anti-inflammation, or immunoregulation and more (Galli or Tsai, 2005). These mediators are stored in the granules of mast cells and can be released upon fusion to plasma membranes and extrusion of the granules into the tissue (da Silva et al., 2014).

In response to allergens, mast cells activate FcεRI which leads to protein phosphorylation, intracellular calcium mobilization, lipid metabolism and phosphorylation, and the activation of transcription factors (TF). Activating TF's can stimulate cytokine and chemokine production to influence inflammation, increase vascular permeability, initiate smooth muscle contraction, mucus secretion, and/or edema (da Silva et al., 2014). Pathogens can bind directly to the mast cells, initiating granule release, contributing to innate immunity, and supporting adaptive immunity (da Silva et al., 2014; Galli, Nakae, and Tsai, 2005). Mast cells enhance the recruitment of T-cells and initiate lymph node enlargement to avoid harm caused by the invaders (Galli and Nakae, 2003). By enlarging the lymph nodes, lymphocytes, T-cells, and antigen-presenting cells are collected and drained, aiding in adaptive immune response (McLachlan et al., 2003).

Mast cells also have important function in the maintenance of homeostasis and tissue repair. Certain mediators released by mast cells are growth factors which can initiate

proliferation of epithelial cells, endothelial cells, and fibroblasts, potentially remodeling tissue (da Silva et al., 2014; Galli and Nakae, 2003). The most common example of mast cells remodeling tissue is airway remodeling due to asthma, where changes are made to bronchial microvasculature, altering basement membrane thickness, hypersecretion of mucus and regulation of profibrotic growth factors (Hong, Kim, and Ro, 2014).

Tissue remodeling and other pathways regulated by mast cells can have a pathologic effect, resulting in harmful consequences to an organism, such as chronic inflammation in Crohn's disease, innate immune response towards own cells in autoimmune diseases, mastocytosis caused by the mutation of mast cells and released mediators, and even cancer as a result of tumor formation via tissue remodeling (da Silva et al., 2014; Williams and Galli, 2000; Maltby, Khazaie, and McNagy, 2009).

NAFLD and NASH

NAFLD has been on the rise in many westerns and Asian countries due to changes in lifestyle and diet, increasing the potential development of hepatocellular carcinoma (Hashimoto et al., 2015; Yu, 2018). The disease is characterized by rapid weight gain, the accumulation of fat in pathological amounts, and varying stages of fibrosis (Sanyal, 2011). Its classification relies on the alcohol consumption of the individual, classifying as non-alcoholic if less than 20-30g of alcohol is consumed per day (Hashimoto et al., 2015; Sanyal, 2011). Risk factors associated with NAFLD include obesity, diabetes, insulin resistance, hypertension, low levels of high density lipoprotein (HDL) cholesterol, or dyslipidemia (Yu, 2018). The risk is higher when these occur simultaneously in an individual and can be influenced by diet. Polyunsaturated fatty acids (PUFAs) have pro- and anti-inflammatory effects. Altered ratios of these PUFAs are associated with the development of NAFLD due to

excess fatty acids, and their manipulation by diet is used for treatment (Wree et al., 2013; Oseini and Sanyal, 2016). However, continued diet with altered PUFA ratios can advance NAFLD to NASH (Wree et al., 2013). Excess consumption of fructose also influences NAFLD development, as it is lipogenic and increases triglyceride formation in the liver (Wree et al., 2013; Oseini and Sanyal, 2016). Age, genetics, gender, and ethnicity are also risk factors in NAFLD development, Hispanic's having the highest prevalence and African Americans having the lowest (Yu, 2018).

NASH is the advanced stage of NAFLD, characterized by cell injury and fibrosis, occurring in 30-80% of NAFLD patients (Yu, 2018; Sanyal, 2011). It is expected to become the leading cause for liver transplantation in the US, passing hepatitis C virus infection (Oseini and Sanyal, 2016). High levels of scarring from NASH can cause cirrhosis, increasing chances of HCC development (Eguchi et al., 2021). With such high NAFLD and NASH incidence rates, HCC is now the fifth most common cancer among males globally with 0.5-2.3% of NALFD patients developing the cancer (Eguchi et al., 2021; Yu 2018). NASH also independently increases risk of cardiovascular mortality, particularly in adults with type 2 diabetes, due to the effect of the excess adipose on glucose and lipid metabolism (Targher et al., 2007; Sanyal, 2011; Lim and Meigs, 2014).

Treatment for NAFLD and NASH begins with changes to diet and lifestyle, such as losing weight, exercising regularly, and restricting calorie intake (Oseini and Sanyal, 2016; Yu, 2018). Pharmaceutical options include peroxisome proliferator-activator receptor (PPAR) agonists, synthetic bile farnesoid X receptor (FXP) agonists, lipid altering agents, incretin-based therapy, antifibrotic therapy, microbiome therapy, and agents that target inflammation, cell injury, apoptosis, and oxidative stress, such as Vitamin E (Oseini and

Sanyal, 2016). Vitamin E is used first when diet and exercise yield little results, reducing steatosis, inflammation, and alanine aminotransferase levels (Oseini and Sanyal, 2016; Sanyal et al., 2010). PPAR agonists target PPAR receptors which regulate metabolic processes such as β-oxidation, gluconeogenesis, and lipid transport. A PPARα/δ agonist has shown promise in NASH treatment, improving hepatic insulin sensitivity, and in some cases, resolving NASH cases with no cirrhosis (Oseini and Sanyal, 2016). One study found that a PPARγ/α agonist was effective in NASH treatment of fructose induced rats, inducing effects on gene expression responsible for insulin resistance, fibrosis, and the synthesis of fatty acids (Abd El-Haleim et al., 2016).

Role of Mast Cells in NAFLD and NASH

NALFD an NASH induce hepatic injuries, damaging hepatocytes and recruiting mast cells as part of the immune response (Jarido et al., 2017). Mast cells were initially not thought to play a role in liver fibrosis (Sugihara et al., 1999). Multiple studies have since determined that mast cell numbers correlate directly to levels of fibrosis in NASH as they are mediators of inflammation (Lombardo et al., 2018; Lewandowska et al., 2020). In human and rodent livers, mast cells are mainly associated with connective tissue that is found by arteries, veins, and bile ducts, particularly those that have been injured (Jarido et al., 2017; Kennedy et al., 2021). Infiltration by mast cells is preceded by proliferation of cholangiocytes, which are cells that line biliary tracts (Kennedy et al., 2021). Mast cells and cholangiocytes are thought to regulate one another, and chronic inflammation of cholangiocytes can lead to cholangiocarcinoma, the second most prevalent liver cancer following HCC (Jarido et al., 2017).

Mast cells release mediators into the liver in response to proinflammatory cytokines, adipokines, oxidative stress, endoplasmic reticulum stress, mitochondrial dysfunction, and other damage factors (Jarido et al., 2017; Lewandowska et al., 2020). Mediators released include histamine, tryptase, heparin, cytokines, TGF-β1, vascular endothelial growth factor, and many fibroblast growth factors (bFGF and FGF), which contribute to fibrosis (Jarido et al., 2017; Lewandowska et al., 2020). As NAFLD progresses to NASH, higher levels of mast cells were recorded, directly affecting levels of fibrosis in patients (Lewandowska et al., 2020). A study that observed mast cell deficient mice reintroduced to mast cells displayed higher levels of fibrosis, and that inhibiting mast cell mediators can reduce levels of fibrosis (Kyritsi et al., 2020).

The growth factor TGF-β1 is an anti-inflammatory cytokine that is secreted from mast cells upon activation of the FcεRI receptors (Lyons et al., 2018). It initiates damage pathways that lead to hepatic fibrosis, biliary senescence, and biliary angiogenesis (Kyritsi et al., 2020). Through the activation of hepatic stellate cells (HSC), TGF-β1 imitates HSC proliferation and the production of collagen and other extracellular matrix proteins (Kiss et al., 2018).

Therapeutic Role of CBG

Cannabigerol (CBG) is a *Cannabis Sativa* terpenophenol, utilizing a similar mechanism of action to cannabidiol (CBD), that is being explored as a medical treatment for many different diseases (Giacomo et al., 2020). CBG acts as a competitive partial agonist to CB2 receptors and binds to the orthosteric center of CB1 receptors, effectively acting as a regulator for endocannabinoid receptors (Navarro et al., 2018). These receptors are located throughout the body at different concentrations, and CB2 receptors are expressed on immune

cells (Caraceni et al., 2014). CBG has been found to use antiproliferative, antibacterial, and anti-glaucoma actions, displaying potential therapeutic effects on neurodegenerative diseases, similar to CBD which has been widely studied (Borrelli et al., 2013; Giacomo et al., 2020).

A CBG derivative, VCE-003.2, was observed to improve antioxidant brain responses and inhibit proinflammatory marker upregulation in Huntington's disease, alleviating symptoms and preventing neuronal loss (Diaz-Alonso et al, 2016). Mice models in a study using VCE-003.2 had improved motor performance, less neuron damage from Huntington disease induced mutations, and reduced inflammation under the CBG treatment (Aguareles et al., 2019). When exploring neuroprotective and antioxidant effects of cannabinoids on astrocytes, CBG was found to decrease apoptosis occurrence due to oxidative stress and prevent serotonin depletion (Giacomo et al., 2020). CBG has shown to enact neuroprotective effects and decrease levels of neuroinflammation and oxidative stress, making CBG a promising drug for other diseases in the body (Gugliandolo et al., 2018).

Diseases in the gastrointestinal tract and liver have been targeted using cannabinoids as the endocannabinoid system has been detected in both humans and mice (Caraceni et al., 2014). CBD and THC were found to have many beneficial effects on diseases such as irritable bowel syndrome (IBS) and GERD (Caraceni et al., 2014). When CBG was used for IBS treatment, nitric oxide production from macrophages and reactive oxygen species formation in intestinal epithelial cells was reduced, potentially relieving IBS symptoms and prompting more research on this treatment (Borrelli et al., 2013).

Methods

This study was conducted at the University of Northern Colorado in the Department of Biology and the School of Biological Sciences. 8 week old C57BL/6 mice were fed a methionine/choline deficient (MCD) diet to induce NASH or a control (CTR) diet 3 times per week for 3 weeks. Then the mice were divided into 3 experimental subgroups for each diet. Mice were then injected with vehicle, high CBG [24.6 mg/kg/day] or low CBG [2.46 mg/kg/day] for 2 weeks while being fed the same diets. The mice were sacrificed, and their livers were harvested and frozen for further experimentation. This was conducted with 6 male mice and 4 female mice for each treatment group.

Slide Preparation

For preparation of the frozen slides, the mouse livers were stored at -80°C embedded in tissue freezing media. Slices were sectioned at 8μm using a cryostat microtome at -20°C. The slides were stored at the same temperatures as the livers.

For preparation of paraffin slides, the mouse livers were fixed in 10% formalin for 24 hours, then sent to a commercially available company for tissue processing, wax embedding, cutting and slide placement (iHisto Inc., CA). The slides were then sent back for staining use.

Toluidine Blue Staining

Slides with sections of frozen liver were stained with T-blue to attempt visualization of mast cells. The toluidine blue stain was used and prepared according to the provided protocol. Slides were fixed in neutral buffered formalin (NBF), dipped in HCl for 10 seconds, stained for 30 seconds, dipped in increasing isopropanol concentrations, then dipped

in Xylene as the clearing agent. The slides were mounted with a non-aqueous mounting media and observed under the microscope.

Chloroacetate Esterase Staining

Frozen slides and paraffin slides were stained using CAE staining. The Sigma-Aldrich Naphthol AS-D Chloroacetate Esterase staining kit was used, and the stain was prepared according to provided protocol. Frozen slides were fixed according to protocol, then stained with the CAE solution for 15 minutes at 37°C incubation, and counterstained with hematoxylin for 30 seconds. Paraffin slides had to be dewaxed prior to staining. No fixation was necessary. Slides were heated to 55°C for 5 minutes, dipped in 2 separate fresh xylene solutions, dipped in isopropanol of descending concentrations, and rinsed with distilled water. Staining protocol was followed with alteration to the staining times. The slides were incubated with the CAE solution for 30 minutes, then counterstained for 1 minute. Both frozen and paraffin slides were mounted with aqueous mounting media and observed under the microscope. For best results, slides should never dry during entire staining process until mounted.

FcεR1/TGFβ1 Staining

The FcεR1 antibody used was conjugated, already containing fluorescent dye. It was used with a dilution of 1:50. The TGFβ1 antibody was unconjugated, so a second staining step with fluorescent dye was performed. It was used at a dilution of 1:100. Frozen liver slides were fixed with 10% NBF for 10 minutes, then treated with 10% goat serum diluted in PBS to block the FC domain and incubated for 20 minutes at room temperature. Slides were washed with 1X PBS in between each step. The two antibodies were mixed together in 1%

goat serum and 100 µL of the mixture was applied to each slide followed by overnight incubation at 4°C. The following day, the secondary antibody was mixed in 1% goat serum and slides were stained with the mixture for 1 hour at room temperature in a dark environment. The slides were mounted with DAPI mounting media and observed using a confocal microscope.

Statistical Analysis

Mast cells were quantified by counting visualized mast cells from photos taken through the microscope using CAE slides. Mast cell counts were compared between MCD and CTR groups, and between low CBG and high CBG groups. A one-way ANOVA was used for comparing the experimental groups. Once the differences were determined, the appropriate post-hoc analysis was performed using two-tailed t-tests. Values where $p<0.05$ were treated as significant.

Results

Toluidine Blue Staining

The desired results of the T-blue staining were not achieved. There was poor visualization of any mast cells and differentiation from artifacts was difficult. This can be seen in Figure 1. Tissue morphology was poor and there were inconsistencies of stain darkness even with a controlled staining time.

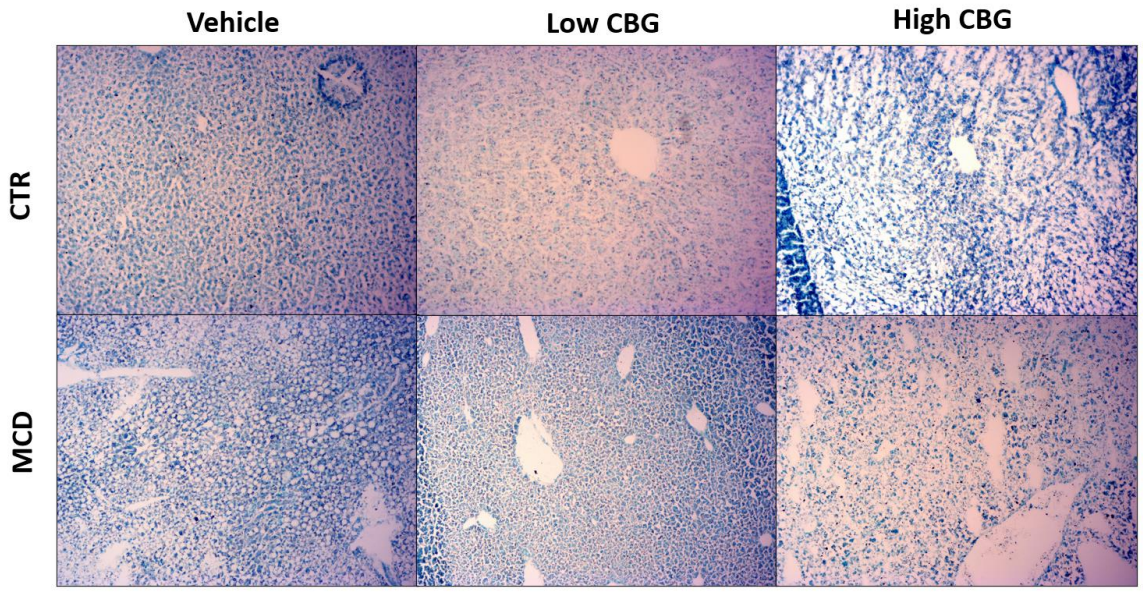

Figure 1 - Results from T-blue staining of frozen slides. Mast cells were not visualized with T-blue staining in mouse tissue.

Chloroacetate Esterase Staining

Frozen slides stained in CAE had poor tissue morphology, making mast cell visualization difficult. Using paraffin slides solved that issue and mast cells were easily visible. As shown in Figure 2, there were no mast cells observed in the CTR group, as there was no induced liver disease in the mice. There were higher levels of mast cells in the MCD-fed groups. The highest occurrence of mast cells was found in the control group treated with vehicle. There were also higher visual levels of fat deposition in the hepatocytes present in the tissue. Mast cells were observed in the CBG treatment groups but at lower amounts. There were less mast cells and visible fat deposition in the low CBG group compared to the high CBG group. There were higher visual levels of fat deposition in the high CBG group compared to the low CBG group. Figure 3 visualizes the average number of mast cell counts

found in the MCD livers. A trend of differences was found between vehicle and both CBG groups. There was no trend of differences between the low CBG and high CBG groups.

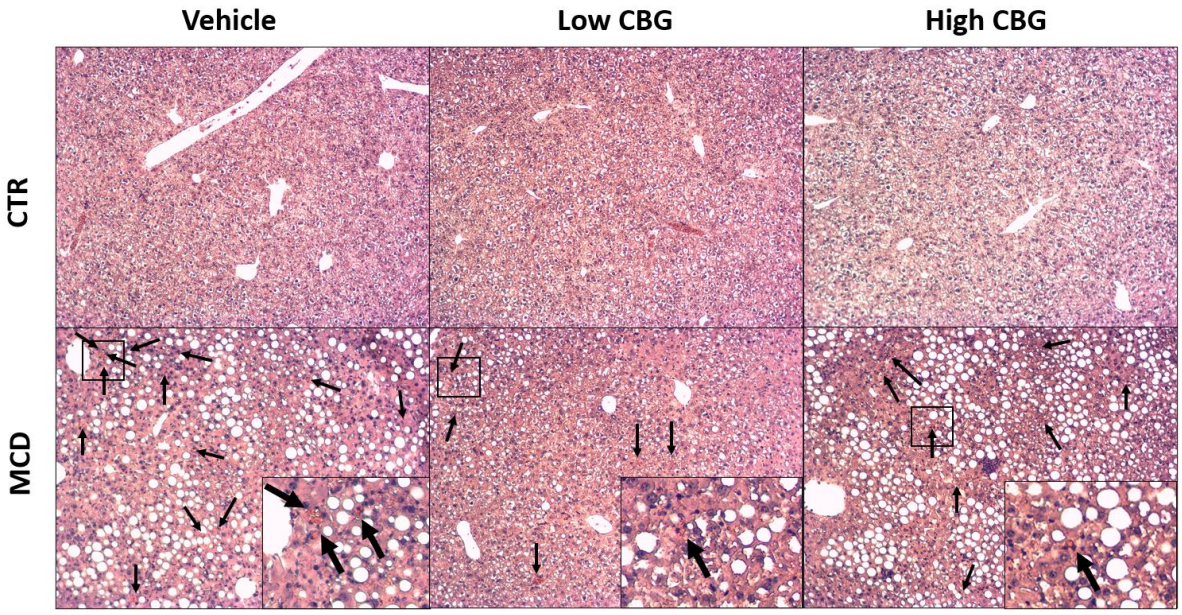

Figure 2 - Results from the CAE staining of paraffin slides. Liver samples were from female mice. Arrows point to mast cells found within the hepatic tissue. No mast cells were observed in the CTR group, and the highest mast cell counts were observed in the MCD-fed vehicle treatment group. Lowest mast cell numbers were observed in the MCD-fed low CBG treatment group.

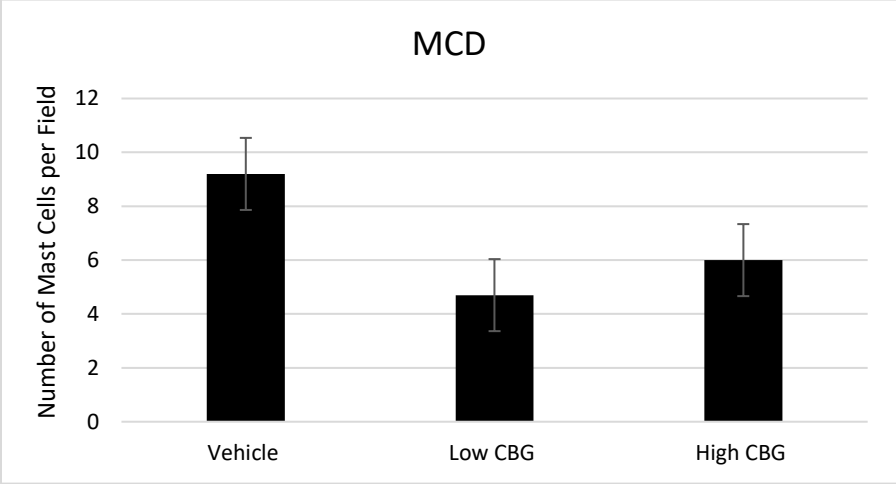

Figure 3 - Quantification of mast cells within the CAE stained MCD-fed treatment groups. Highest mast cell numbers were found in the vehicle control group, while lowest numbers were found in the low CBG group. Quantification was calculated based on 12 randomly selected fields per treatment group, n=1 for each treatment. Values are expressed as means ±SD.

FcεR1/TGFβ1 Staining

Immunofluorescence staining using FcεR1/TGFβ1 antibodies yeilded similar results to the CAE staining. There were no mast cells present in the CTR groups, with the exception of two areas of colocalization which were determined to be insignificant. Red immunofluoresence indicates the presence of mast cells. Figure 4 shows that mast cells were mostly present in the vehicle MCD group. There were almost no mast cells present in the low CBG group. There were higher levels of mast cells in the high CBG group compared to the low CBG. Green immunofluoresence indicates the presence of TGFβ1 and areas of colocalization, marked by white arrows, incicates mast cell release of TGFβ1. There was

high colocalization in the high CBG treatment group. Low levels of cololcalization were seen in the veichle MCD group, and even less were observed in the low CBG treatment group.

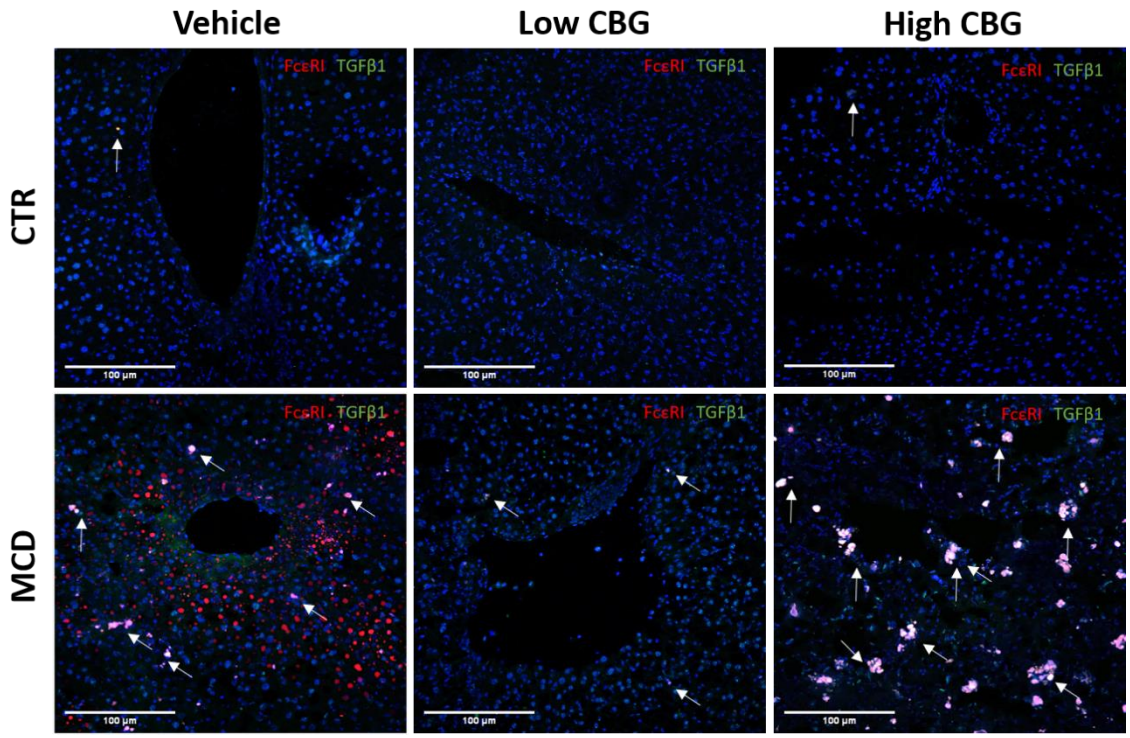

Figure 4 - Results from the FcεR1/TGFβ1 Immunofluorescence. Liver samples were from male mice. Red indicates FcεR1 positive staining (mast cell marker). Green indicates TGFβ1 positive staining. Arrows point to colocalization, indicating presence of mast cells expressing TGFβ1. Blue indicates hepatocyte nuclei, stained with DAPI.

Discussion

Mast cells were not observed under the CTR diet. They were observed under the MCD diet in high amounts with no treatment. The CAE staining showed that mast cell infiltration in the MCD groups did change under CBG treatment, decreasing the most under low CBG treatment. While mast cell infiltration also decreased under high CBG treatment, high levels of fat deposition in the hepatocytes was still observed inferring that low CBG had

the best effect on reducing mast cell numbers, aiding in the potential reversal of NASH. The results from the FcεR1/TGFβ1 immunofluorescence co-staining also showed that mast cell infiltration in the MCD groups decreased the most under low CBG. While there were lower levels of mast cells under high CBG compared to the vehicle group, the mast cells were expressing higher levels of TGFβ1, indicating that TGFβ1-dependent fibrosis may still be occuring under the high CBG treatment. The low levels of mast cell TGFβ1 expression under low CBG supports potential NASH reversal as seen with lower fat deposition presence under CAE staining.

The lower levels of fat deposition, decreased infiltration of mast cells, and decreased evidence of mast cell TGFβ1 expression under low CBG indicates that it is a viable treatment for NASH in MCD induced mice models. It has the potential to reverse the effects of NASH and aid in NAFLD treatment.

Of the methods used, CAE staining and immunofluorescence co-staining were the most functional methods, providing observable data. CAE staining worked best with paraffin slides, as they provided the clearest morphology under the microscope. It was crucial to keep the slides wet throughout the de-waxing and staining process for the best results. However, the mast cells were easier to visualize when stained with immunofluorescent protein. Co-staining allowed us to see where mast cells were activated, which CAE staining could not show us. Immunofluorescence provided the best information regarding the levels of mast cells in the tissue, while CAE staining provided the best information regarding changes in morphology, such as fat deposition in the tissue.

Future steps in this research include study replication with a larger sample size, further exploration of CBG's role in NASH reversal in multiple different models, and

exploration of off-target effects and other effects of long term CBG usage. CBG treatment has shown potential in neurodegenerative diseases, and now shows potential in hepatic diseases as well. With incidence rates of NAFLD and NASH rising, discovering treatment options is an important focus.

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