EXPLORING THE POTENTIAL THERAPEUTIC EFFECTS OF THE MEDITERRANEAN DIET ON THE LIVER-BRAIN AXIS IN C57BL/6J MICE

By

Miranda E. Jelinek

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Project Approved:

Supervising Professor: Michael Chumley, PhD

Department of Biology

Gary Bohem, PhD

Department of Psychology

Molli Crenshaw, MS

Department of Biology

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ABSTRACT

Alzheimer's Disease (AD) affects approximately 6.5 million Americans, and currently has no cure. Prior research has shown that a key pathology of AD is amyloid beta, a protein that aggregates and forms plaques in the brain, under pathological conditions. If amyloid beta cannot by the body, resultant plaques may disrupt proper cognitive and neuronal function. As the liver plays a crucial role clearing amyloid beta, liver damage may jeopardize the efficacy of the liver to clear amyloid beta in the periphery of the body, thereby enabling it to reach the brain. One way liver function can be disrupted is through poor diet, specifically the Western diet (WD), that has been shown to induce non-alcoholic fatty liver disease (NAFLD) and inflammation, both of which are associated with AD. A WD is classified as one that contains high amounts of refined sugars and saturated fats derived from animals. Conversely, the Mediterranean Diet (MD), a largely plant-based diet, containing few refined sugars, and high amounts of antioxidants, along with monounsaturated and polyunsaturated fatty acids. These dietary factors have been shown to decrease inflammation and increase antioxidant effects, further protecting the brain from AD pathology. Therefore, we hypothesize that the MD could protect the liver and be used as a potential prevention strategy for NAFLD and AD. The current study examined the effects of WD or MD on the relationship between the liver and the brain in wild-type mice. During tissue collection, livers were taken and histologically analyzed. The livers from each experimental group were processed, stained, and evaluated for their overall composition.

INTRODUCTION

Alzheimer's Disease (AD) is a fatal neurodegenerative disease that is the most common form of dementia. Although this disease currently affects over 6.5 million Americans, there is still no cure for AD. AD is characterized by several clinical symptoms, including cognitive dysfunction and memory loss (Breijyeh & Karaman, 2020).

Two hallmark pathologies of AD include hyperphosphorylated neurofibrillary intracellular tau tangles and extracellular amyloid-beta (Ab) plaques that accumulate in the brain and provoke oxidative stress and inflammation (Więckowska-Gacek, 2021). This accumulation of Ab plaques in the brain is caused by the impaired degradation and clearing of these compounds, as well as increased cleavage of the membrane protein, amyloid precursor protein (APP) (LaFerla & Oddo, 2005).

Part of AD pathology is the dysfunction seen at the blood-brain barrier (BBB). In a healthy brain, the BBB is maintained via tight junctions that closely regulate the transport of molecules into and out of the brain. If the BBB is disrupted, peripheral inflammatory triggers, such as pro-inflammatory cytokines and Ab, can potentially cross the BBB and enter the brain. Pro-inflammatory cytokines are proteins produced by immune cells in response to environmental risk factors that may provoke inflammation, such as pollution, sedentary lifestyle, and poor diet. Pro-inflammatory cytokines are released in response to Ab, and thus, an increase in this AD pathology creates a cyclic process of chronic inflammation that stimulates the body's immune system. Chronic stimulation of the immune response increases the production of these pathogenic proteins in the periphery of the body, and they can be transported into the brain. For example, Ab production in the periphery of the body is particularly harmful, as it can potentially enter the brain if it is not cleared by peripheral organs, or the BBB is impaired. This impaired transport at the BBB can lead to neuroinflammation and cognitive dysfunction seen in

AD patients (Xie, 2020). Many environmental risk factors, such as pollution, lack of exercise, chronic stress, chronic sleep loss, and poor diet, have been shown to affect the onset and progression of AD in prior research (Silva, 2019). Notably, diet has been shown to considerably affect cognitive function, including learning and memory, over time. Prior research has clearly demonstrated that the Western diet (WD), also known as the typical American diet, increases AD risk (Więckowska-Gacek, 2021), while the Mediterranean diet (MD) may potentially lower AD risk (McGrattan, 2019). Therefore, the current study aimed to examine the potential, protective effects of the MD in comparison to the WD.

Diet and Alzheimer's Disease

Prior research has demonstrated that several dietary factors in the WD, including refined carbohydrates and sugars, as well as animal-based proteins and saturated fatty acids (SFA), provoke inflammation and AD pathogenesis in rodents (Sullivan, 2020). Conversely, plant-based diets, such as the MD, may play a potential neuroprotective role. This diet is mainly plant-based, including many fruits and vegetables, moderate amounts of fish, olive oil, and some alcohol, such as red wine. Hallmarks of the MD are monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs), as opposed to SFA that dominate in the typical Western diet. MUFAs and PUFAs have been shown to increase antioxidant effects and aid in reducing oxidative stress and inflammation in mice (Castellanos-Tapia, 2020). More specifically, there have been multiple studies that indicate omega-3-polyunsaturated fatty acids (w3-PUFAs) are able to offer some protection against AD by reducing inflammation, oxidative stress, and cell death in the brain. Ab degradation and transport out of the brain has been shown to be enhanced by w3-PUFAs in mice (Xie, 2020). Furthermore, the Mediterranean Diet (MD) has been shown to protect individuals from cognitive illness and neural dysfunction, and thus could be utilized as

a potential preventative strategy for AD. Prior studies have found that individuals who adhere to a strict MD have lower incidence of Alzheimer's Disease (van den Brink, 2019).

Due to the large portion of vegetables that are staples in the MD, there are high amounts of phytochemicals, including carotenoids, phytosterols, polyphenols, and sulfur-rich compounds. Polyphenols, abundant in extra virgin olive oil and wine, have been well studied, and their ability to mitigate and counteract cognitive impairment is well documented (Lauretti et al., 2017; Hornedo-Ortega, 2018). Polyphenols have been shown to decrease microglial cell activation and inflammation. These polyphenolic compounds protect against and reduce amyloid beta plaques, decrease reactive oxygen species, and enhance chemical signaling pathways (Lauretti et al., 2017; Hornedo-Ortega, 2018). By decreasing the number of amyloid beta plaques that form in the brain, fewer microglia in the brain are activated and, therefore, less inflammation occurs (Hornedo-Ortega, 2018).

A typical western style diet is one that includes large amounts of simple carbohydrates, free fatty acids (FFA) that are saturated, and cholesterol. The WD increases risk of several illnesses related to metabolism, such as diabetes mellitus type II, cardiovascular disease, obesity, metabolic syndrome, and non-alcoholic fatty liver disease (NAFLD) (Castellanos-Tapia, 2020). Notably, many of these deadly diseases are typically comorbid with AD, such as cardiovascular disease, obesity, and diabetes mellitus type II (Beam, 2021; Hills, 2019).

A key risk factor for AD is obesity, which affects many bodily functions and has been known to diminish the efficiency of the immune system. Over-activation of the immune system is common in obese individuals and proinflammatory cytokines are produced at higher-than-average levels (Christ, 2019). These proinflammatory cytokines, such as interleukin-1 beta (IL-1b) and tumor necrosis factor-alpha (TNFa) not only circulate within the body's periphery, but also within the brain. Overactivation of the immune system itself has been

shown to induce cognitive impairment, specifically learning and memory deficits that are common in AD pathology (Baumgarner, 2014). As a result of consistent ingestion of WD, these metabolic pathologies begin to develop alongside chronic systemic inflammation. In a study in which mice were fed a WD, systemic inflammation was present through the increased levels of cells in the innate immune system, namely granulocytes and monocytes. Additionally, the WD contains a large amount of cholesterol which has been shown to provoke inflammation. Hypercholesterolemia can ensue and increase the production of pro-inflammatory cytokines and Ab, and thus exacerbate AD pathology (Więckowska-Gacek, 2021).

Furthermore, prior research has demonstrated that chronic consumption of WD induces a chronic inflammatory state (Christ, 2019). Inflammation has also been shown to be present in the microglia – immune cells of the brain responsible for Ab clearance – of mice who were on a WD. The phagocytic function of microglia was also shown to be diminished in mice that were fed the WD (Więckowska-Gacek, 2021).

Additionally, the WD can also alter the microbiome within the gut. For example, high sugar and SFA content in the WD significantly changes the gut microbiome and microbial diversity (Hills, 2019). These negative changes in the gut microbiome have been linked to cognitive decline and deterioration (Bruce-Keller, 2014). Further, consumption of a typical WD is associated with an increased risk for inflammatory bowel disease (IBD). The mucus lining of the gut and lungs are adversely altered by the western style diet. The WD and inflammation inherently negatively impact the composition of microorganisms in the gut and can lead to a disruption in beneficial commensal bacterial species that are able to fight off pathogens. A disruption in the microbiome composition can lead to systemic inflammation and can leave an individual susceptible to disease (Statovci, 2017).

In addition to these consequences, the WD has also been shown to harm the liver, an organ that is crucial for the clearance of peripheral Ab (Lian, 2020). The composition of fatty acids in liver tissue is directly related to diet (Castellanos-Tapia 2020). In individuals with NAFLD, oxidative stress is the consequence of mitochondrial damage resulting from a diet with a high cholesterol level (Castellanos-Tapia, 2020). Liver pathology that results from WD diminishes the healthy function of the liver which includes degrading and clearing Ab from the body. This decreased clearance resulting from a damaged liver is associated with increased levels of Ab in the brain, which ultimately leads to higher levels of oxidative stress and inflammation within the brain. This is a recurring cycle that occurs between the liver-brain axis, and thus, protecting the liver through dietary strategies could potentially be utilized to protect the brain from inflammation and Ab accumulation (Więckowska-Gacek, 2021).

In previous studies using the Western style diet, these diets usually have extremely high levels of fat and sugar. This particular study utilizes the Typical American Diet (TAD) which is lower in fat content compared to Western diet studies, but the TAD is more representative of what an average American consumes. The major difference between the TAD and MD diet used in this study is the types and sources of macronutrients while the percentages of macronutrients are the same.

Hypothesis

Given previous findings showing that consumption of the MD causes an increase production in antioxidants and decrease in inflammation, we hypothesize that diet could be a potential target therapeutic measure for protecting against AD. With the liver's crucial role in the liver-brain axis and facilitation of proinflammatory cytokine and Ab circulation in the brain, we chose to look at how the MD and the TAD affected liver composition and markers of AD pathogenesis. Consumption of the TAD can cause inflammation, increased Ab, and liver

pathologies related to increased fat deposition. Further, we hypothesized that consumption of the MD would have a therapeutic effect on the liver through decreased fat deposition in the liver. We expect increased amounts of fat deposition in mice administered the TAD.

MATERIALS AND METHODS

Animals and Diet

This study utilized 7-month-old C57BL/6 mice housed in the Texas Christian University vivarium in accordance with approved protocols of the Institutional Animal Care and Use Committee (IACUC). Mice are placed onto their assigned diets, Typical American diet or Mediterranean diet, at weaning (postnatal day 21) and are on the diet for 6 months with access to water *ad libitum*. Mice had a schedule of 12 hours of light and dark each day and were housed in polycarbonate cages in groups of two to four animals. At 7 months of age, liver tissue was collected from the mice.

Our laboratory collaborated with Dr. Jada Willis (TCU, Department of Nutritional Sciences) to design two experimental rodent diets, including a Typical American rodent diet (TAD) and Mediterranean rodent diet (MD). The custom rodent diets were created in pellet form, and purchased from Research Diets, Incorporated (New Brunswick, New Jersey).

Table 1: Experimental Diet Macronutrients

Macronutrients	**Mediterranean Diet**	**Traditional American Diet**
Macronutrients (grams)		
Protein (gms)	147.2	147.3
Carbohydrate (gms)	495.3	495.5

Fat (gms)	153.2	153.1
Fiber (gms)	153.7	50.0
Macronutrients g%		
Protein	14.3	16.0
Carbohydrate	48.0	53.7
Fat	14.8	16.6
Fiber	14.9	5.4
Macronutrients kcal		
Protein	*589*	*589*
Carbohydrate	*1981*	*1982*
Fat	*1379*	*1378*
Total kcal	***3949***	***3949***
Macronutrients kcal%		
Protein	*15*	*15*
Carbohydrate	*50*	*50*
Fat	*35*	*35*

Fats (grams)		
Saturated Fatty Acids	29.5	74.4
Monounsaturated Fatty Acids	86.2	54.0
Polyunsaturated Fatty Acids	24.4	14.1
n-6	15.9	13.4
n-3	8.0	0.9
n-6/n-3 Ratio	2.0	15.6
Fats kcal%		
Saturated Fatty Acids	6.7	17.0
Monounsaturated Fatty Acids	19.6	12.3
Polyunsaturated Fatty Acids	5.6	3.2

Tissue Processing

At 7 months of age, livers were isolated from mice in each of the following experimental groups: males fed Typical American Diet diet, males fed Mediterranean diet, females fed Typical American diet, females fed Mediterranean diet. Livers were kept refrigerated in vials of 10% formalin and retrieved as needed for processing and sectioning.

Livers were obtained when the mice had been on their assigned diets for 6 months and were 7 months old. Whole livers were fixed in 10% formalin to prevent tissue degeneration and

preserve cellular structures and liver sections were retrieved as needed for sectioning. Livers were cut into approximately 1cm cubes and placed in plastic cassettes labeled with the animal ID for processing. Tissue specimens were processed using a Leica TP1020 automated tissue processor (Leica Microsystems, Bannockburn, IL) in which the tissue encased in a plastic cassette underwent dehydration, clearing, and wax infiltration by passing through separate wells containing different reagents for pre-programmed periods of time. Since paraffin is immiscible with water, the tissue was first dehydrated in an ascending series of ethanol concentrations (American MasterTech Scientific, Lodi, CA) to allow for complete infiltration of the paraffin wax. The ethanol must also be removed since it is largely immiscible with paraffin. In this study, an ethanol clearing agent known as Sub-X (Leica Microsystems), a less toxic substitute for Xylene, was used. The tissue was placed in the two subsequent Sub-X wells for one hour. After dehydration and clearing, histological paraffin can infiltrate the tissue. The specimen was placed in three paraffin stations at a temperature of 60° C. The order and duration for each processing step are outlined in the procedure below:

Table 2: Paraffin Processing Procedure

Reagent	**Time (minutes)**
Water	15
70% Alcohol	15
80% Alcohol	15
95% Alcohol	15
100% Alcohol	20
100% Alcohol	20

100% Alcohol	25
Clearing Reagent (Xylene or substitute)	25
Clearing Reagent (Xylene or substitute)	25
Paraffin 1	25
Paraffin 2	25
Paraffin 3	25

Tissue Embedding

After thorough paraffin infiltration, the tissue was embedded in a paraffin wax block using a Leica HistoCore Arcadia C paraffin embedding device (Leica Microsystems). The specimen was carefully oriented in a mold to determine the plane of section and topped with a plastic cassette. The mold was then filled with molten paraffin and allowed to cool into a solid block.

Tissue Sectioning

First a warm water bath (40°C) was prepared by filling the Leica HI1210 with distilled water and is allowed to warm up while preparing the microtome for cutting. The warm water bath was necessary to relax the sliced paraffin wax and tissue sections before mounting on a slide.

After the tissue has been allowed to cool into a solid block of paraffin wax in a cassette, excess wax surrounding the tissue was cut away using a razor blade to allow smaller sections to be cut and more sections to fit onto each slide. The cassette was transferred to the Leica RM2235 Manual Rotary Microtome. Then a blade was inserted into the microtome so that thin sections of the paraffin tissue block can be cut. Once the blade is inserted, the angle of the blade is positioned to effectively cut the tissue block. Using the coarse speed wheel on the left side of the

machine, the tissue block was moved forward until it is nearly touching the blade. At this point, the thickness setting was adjusted to a range of 8-15 microns and the hand wheel on the right side of the machine was used to incrementally move the paraffin block toward the blade. Once the microtome begins cutting the paraffin block, the initial wax layer was removed until the liver tissue is exposed. When needed, the tissue block was removed from the microtome and placed into a beaker of cold ice water in order to rehydrate the tissue and prevent the tissue from crumbling when cut. After approximately 1–2 minutes, the tissue block was repositioned on the microtome. Additional rehydration in cold water was administered as needed throughout the sectioning process. Using forceps or gloved hands, sections were placed into a warm water bath.

After sections were placed in a warm water bath to all them to relax they were placed on a slide. While the section were floating in the warm water bath, a slide was dipped under the surface of the water below the floating section and then positioned so that the section would stick onto the slide when it is pulled out of the water. The slides were then labeled with the animal ID that corresponds to the liver tissue embedded in the original paraffin block. The slides were then allowed to dry overnight in preparation for staining.

Hematoxylin and Eosin Tissue Staining

The liver sections were then stained using a Hematoxylin and Eosin (H&E) staining process so that the anatomical structure and composition of the liver tissues could be later viewed under a microscope. The slides are first placed in an upright position in a slide holder that will be dipped into the solutions during the staining process. Firstly, the paraffin wax that is surrounding the liver tissue was removed because wax is hydrophobic and will interfere with the aqueous stains. The paraffin was removed through dissolving it in a xylene substitute, Sub-X. The xylene was next removed from the slide with the use of alcohol. The alcohol was finally

washed off with water. At this point in the staining process, the tissue did not contain any hydrophobic wax, and was able to be stained by aqueous fluid. The slides were placed in Hematoxylin stain. The Hematoxylin stain is a darker violet/indigo color and highlights structures in the nucleus. The slides were rinsed with water to remove excess Hematoxylin stain. After the water wash, blueing solution was used to change the hematoxylin stain into an indigo color. A differentiation step was then used to "destain" some of the liver tissue to remove background stain that was not specifically staining nuclear material. The slides were then immersed in Eosin, which is considered a counterstain and used to highlight cytoplasmic structures in a pink color. After Eosin staining, the slides went through a series of alcohols to remove any hydrophilic substances. Finally, the slides went through a series of Sub-X immersions to remove all water. A generous amount of Stat Lab Submount Mounting Media was then placed on the slide and a glass coverslip placed on top. Once all slides were sealed with a coverslip, they were allowed to dry and harden overnight. The H&E staining protocol is outlined below.

Table 3: Hematoxylin and Eosin Staining Procedure

Reagent	**Time**
Sub-X	4 minutes
Sub-X	4 minutes
Sub-X	4 minutes
100% Ethanol	2 minutes
100% Ethanol	1 minute
95% Ethanol	1 minute
Water	1 minute

Hematoxylin	5 minutes
Water wash	3 minutes
Differentiate	30 seconds
Water wash	1 minute
Bluing	30 seconds
Water wash	1 minute
95% Ethanol	1 minute
Eosin	1 minute
Water	1 dip
95% Ethanol	2 minutes
100% Ethanol	1 minute
100% Ethanol	1 minute
100% Ethanol	1 minute
Sub-X	2 minutes
Sub-X	2 minutes
Sub-X	2 minutes

Oil Red O Tissue Staining

Using an Oil Red O Stain Kit from StatLab (Mckinney, TX), male and female liver tissue from the MD diet were stained for lipids. Initially, paraformaldehyde fixed liver tissue was sliced using a vibratome for rapid sectioning and analysis. The liver tissue was approximately 20–25 microns thick. Because the tissue was unable to remain affixed to the microscope slides due to

the thickness, the tissue was stained in 24-well dishes. The sections were placed on slides, a coverslip affixed, and the slides were imaged immediately.

Imaging

Imaging analysis for both H&E and ORO stained liver sections was done using bright field imaging on a Nikon Eclipse 90i microscope using NIS Elements Software (Nikon Instruments Inc., Melville, NY).

RESULTS

After the livers were removed from the mice, they were photographed, weighed, and measured. The photos below depict photos of a liver from each experimental group: Male MD, Female MD, Male TAD, and Female TAD. Visual comparisons can be made between animals in the MD experiential groups and the TAD experimental groups. Livers obtained from both male and female animals on the TAD were lighter and lighter in color compared to the MD animals.

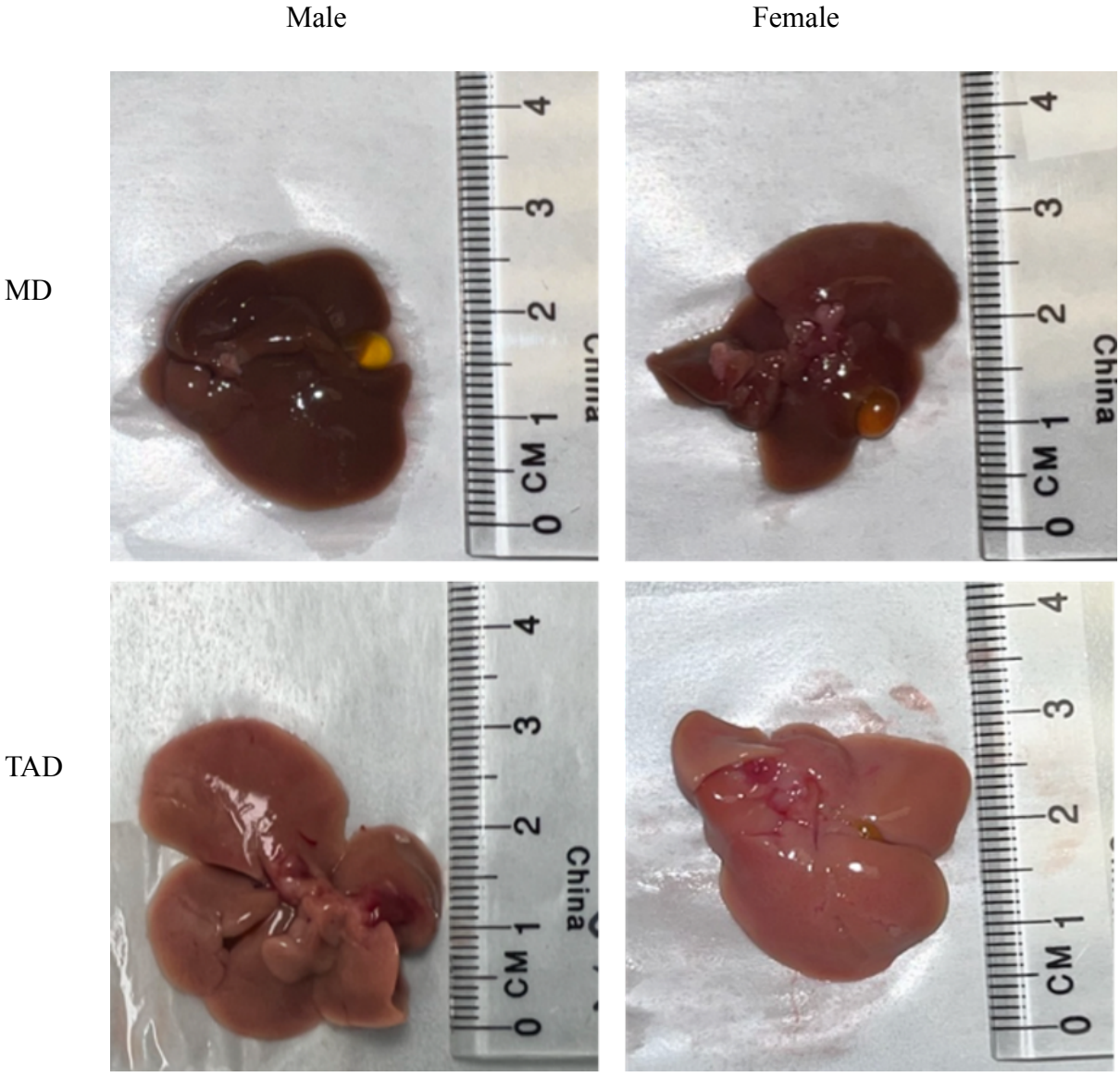

Figure 1. Whole livers isolated from mice after 6 months of diet administration at 7 months of age.

After the livers were initially removed and photographed, they were weighed. The organ weight graphs below depict the difference in weight between mice on the MD and TAD. The male mice on the TAD have significantly heavier livers compared to those on the MD. Although the female TAD mice did not have significantly heavier livers, the difference approached statistical significance. This weight increase seen in the TAD livers is presumably due to the increased fat deposition in these livers from the high levels of saturated fatty acids found in the TAD.

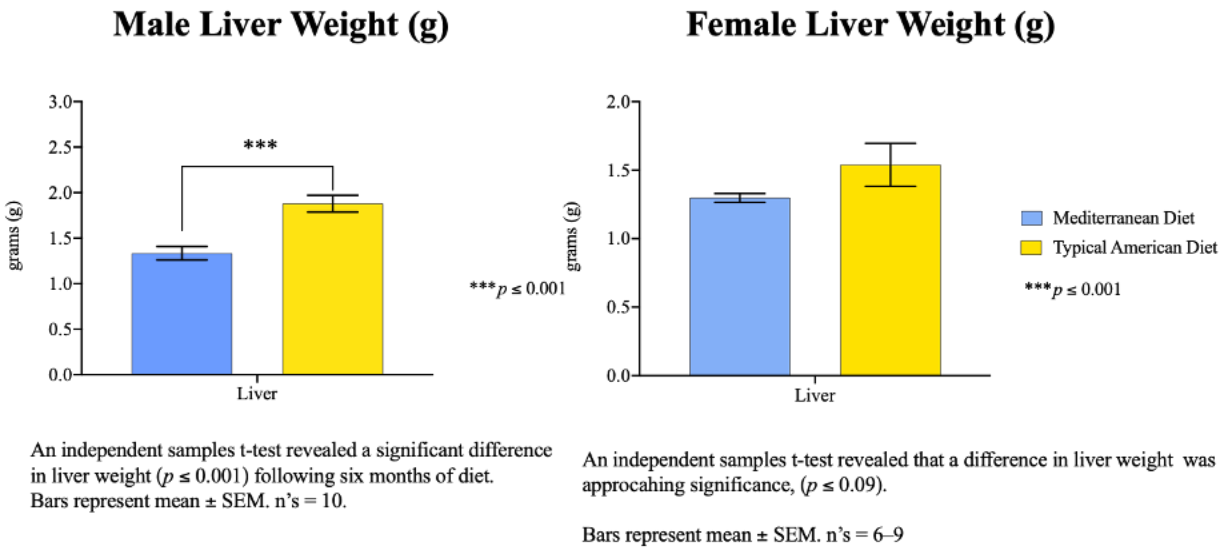

Figure 2. Liver weight results for both male and female mice administered MD or TAD.

Liver tissue sectioned using a microtome was then stained using Hematoxylin and Eosin (H&E). Below (Figure 3 and 4) are images of both male and female livers on the MD and TAD at 4X and 20X magnification. The white space in the MD livers can be identified as arteries, veins, or bile ducts. However, during the H&E staining process, fat is removed. Therefore, much of the white space is where lipids originally were in the liver tissue. It can be concluded that the increased white space in the TAD livers is due to the increased fat deposition in these livers.

4X Magnification

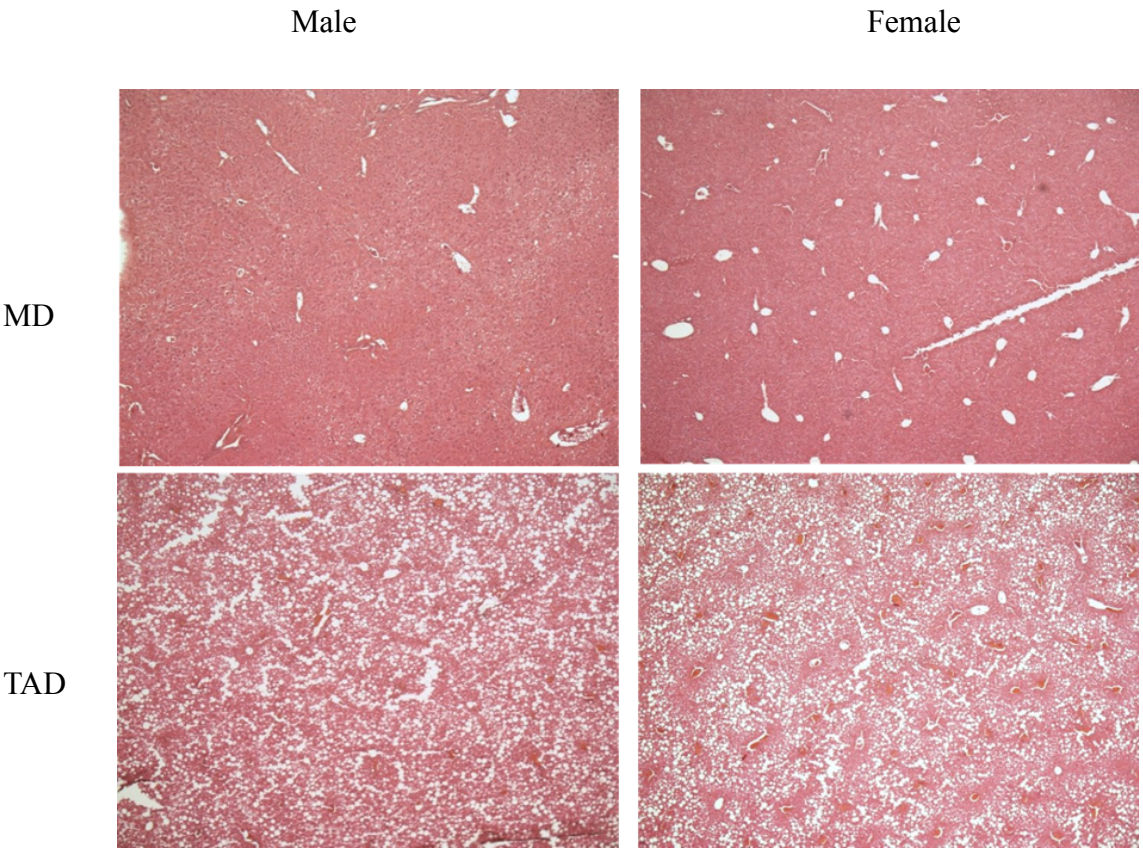

Figure 3. Bright field images of H&E stained male and female mouse livers administered MD and TAD. Images taken at 4X magnification using Nikon Eclipse 90i microscope using NIS Elements Software (Nikon Instruments Inc., Melville, NY).

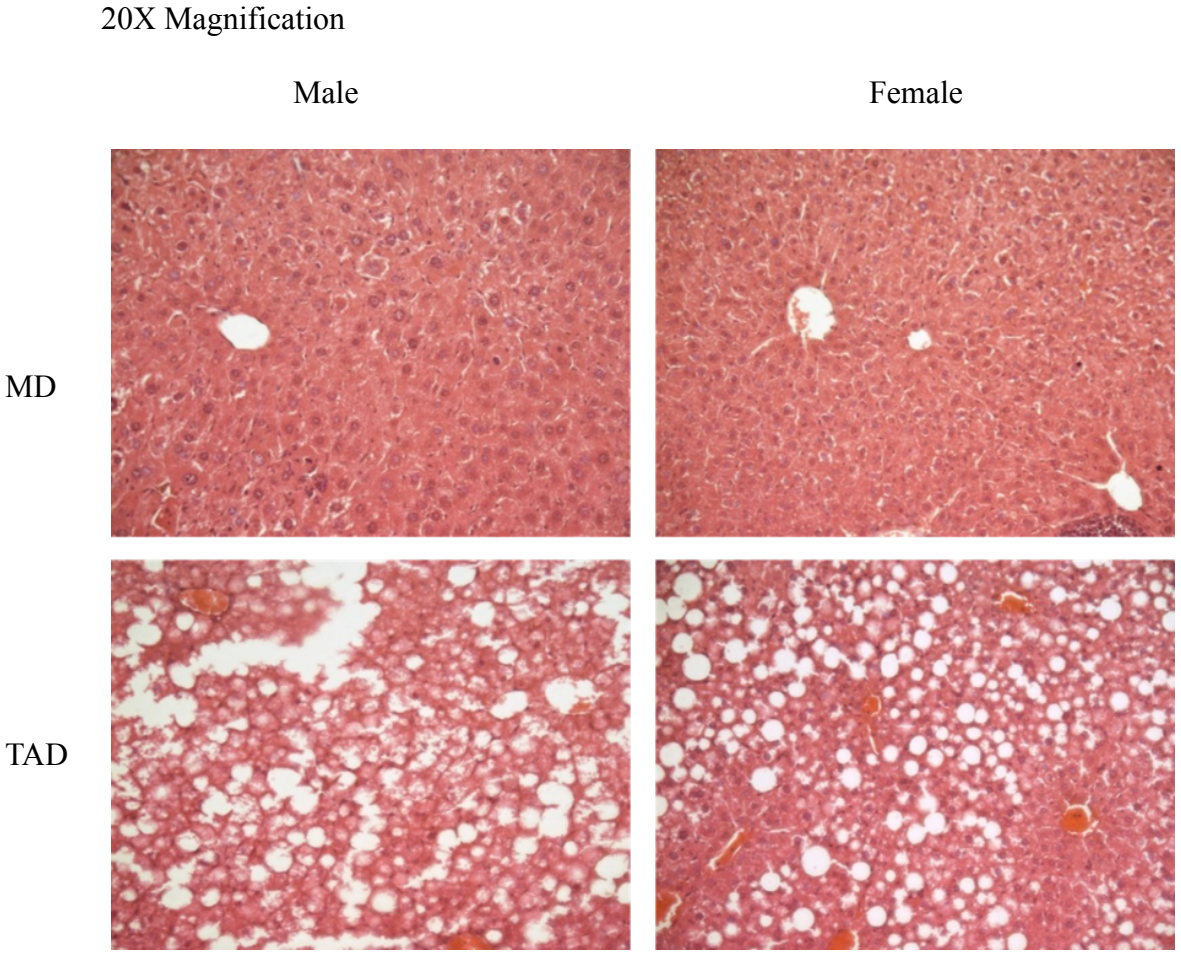

Figure 4. Bright field images of H&E stained male and female mouse livers administered MD and TAD. Images taken at 20X magnification using Nikon Eclipse 90i microscope using NIS Elements Software (Nikon Instruments Inc., Melville, NY).

Liver tissue from animals fed the MD were stained using Oil Red O which stains lipids. The red droplets seen in the images below are lipids, whereas the purple/blue spots are nuclei stained with hematoxylin.

MD Male MD Female

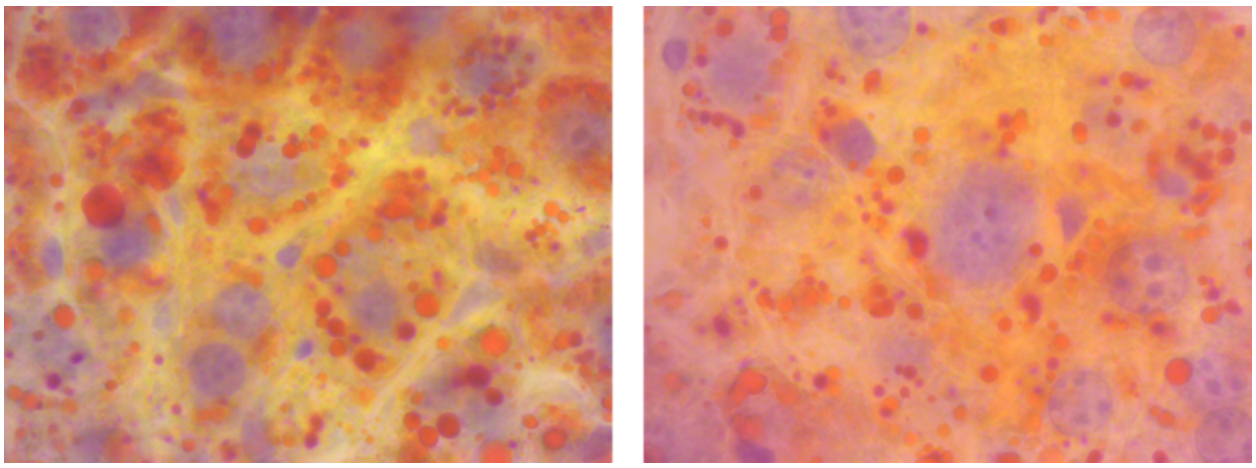

Figure 5. Bright field images of male and female mouse livers administered MD. Images taken at 100X magnification using Nikon Eclipse 90i microscope using NIS Elements Software (Nikon Instruments Inc., Melville, NY).

DISCUSSION

In this study, male and female mice were assigned an MD or TAD diet for six months. After six months on their assigned diet, livers were isolated, fixed, processed, embedded, sectioned, stained, and histologically analyzed under a microscope. The composition and structure of the livers were analyzed. It was determined that the mice on the TAD had higher levels of fat deposition in the liver compared to their MD counterparts, as seen in the images obtained from isolated livers, liver weight, and micrographs employing H&E and Oil Red O staining. Similar findings, such as increased fat deposition as a result of high fat diet consumption has been shown in previous studies (Tsuchida, 2018).

When the livers were initially isolated from the mice, a color difference could be noticed between the MD and TAD mice. The livers from the TAD mice were lighter in color and slightly larger, presumably from the increased fat deposition in these livers. The liver weight also showed

a significant difference in weight between the male mice where the TAD livers weighed more. Although the female TAD livers did not show a significant weight difference, it was approaching significance. This shows that mice on the TAD have increased liver weight which is likely due to the increased fat deposition in these livers. Due to the balanced macronutrients in both the TAD and MD, an extremely high fat content was not present in the TAD. We hypothesize that the lower percentage of fat in the TAD compared to previous studies looking at the WD is contributing to the diminished liver weight difference between mice that were on the TAD.

Although the difference was obvious to even the naked eye, the increased lipid composition was most apparent when looking at the H&E stained livers. In the TAD livers, many holes were observed indicating an increase in the presence of fat in these mice (Tsuchida, 2018). As previously mentioned, the process of embedding tissue in paraffin removes all fatty acids, resulting in empty spaces within the tissues. Due to the high percentage of saturated fatty acids in the TAD, these fats are not as readily metabolized and used which causes them to be deposited and stored in organs, such as the liver. Conversely, the MD contains high percentages of monounsaturated fatty acids and polyunsaturated fatty acids which are more readily metabolized and used as energy (Castellanos-Tapia, 2020). The results indicating a higher fat deposition in the TAD livers show that the source and type of fat has a substantial impact on whether it is deposited into the liver or not. It is apparent from our findings that the saturated fatty acids contained in the TAD are deposited in high amounts. The fat macronutrient percentage was the same in each diet while the sources of fat was the only variable component. This is important because the source of fat and type of fatty acid, saturated or unsaturated, contributed to drastically different liver compositions. Although these results were expected based on the

primary literature that was reviewed prior to this study, more livers need to be analyzed in order to strengthen the results found in this experiment.

Further analysis and staining must ensue to obtain better analysis of lipids. Due to the thickness of the liver tissue sectioned using the vibratome, multiple cell layers are seen in the images taken of the Oil Red O stained tissue. In order to perform a better analysis of this tissue, thinner sections should be cut to provide a single cell layer. Although lipids were seen in the MD mice that were stained, liver tissue from TAD mice should also be sectioned and stained in order to compare MD and TAD lipid composition. More lipids would be expected in the TAD mice based on previous studies and the results from the H&E stained livers that showed increased fat deposition in the TAD livers (Zheng, 2019).

The results of this study have shown that mice fed the TAD have increased fat in their livers compared to the mice fed the MD, as shown in other studies (Shively, 2019). These data suggest that the mice fed the TAD developed fatty liver disease which has been shown to lead to an increase in proinflammatory cytokines and amyloid beta plaques – both of which are common in AD pathology (Więckowska-Gacek, 2021). Notably, paralell data from our laboratory demonstrated increased proinflammatory cytokines in the periphery and increased Ab protein in the brains of the mice fed the TAD. Therefore, these data collectively suggest that consumption of the TAD leads to a fatty liver which is correlated with AD pathology.

Future directions for this study could include the use of Oil Red O staining to highlight and identify adipose cells within the liver. Immunohistochemistry could also be used to identify the presence of Ab in the liver. The liver is important for the clearance of Ab from the periphery of the body, however, this function is disrupted due to fat deposition in the liver. Pathologies like NAFLD impair the liver's function of Ab clearance from the periphery which can lead to the

damage of tight junctions that make up the BBB. This damage of the BBB can then increase in deposition of Ab in the brain which is common in neurodegenerative diseases, such as AD (Vegas-Suárez, 2022). Further, immunohistochemistry could be used in future studies to identify Aß in the liver using microscopy.

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