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THE EFFECT OF EXPOSED DIET TYPE ON MONDO FAMILY TRANSCRIPTION FACTORS IN SPRAGUE DAWLEY RAT LIVER

MASTER THESIS

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30.11.2022

Mustapha Abou Rached

BEYAN

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LIST OF SYMBOLS AND ABBREVIATIONS

TF: transcription factor

TBP: tata-binding protein

PIC: pre-initiation complex

MLX: max-like protein X

GSM: glucose sensing module

GRACE: glucose-response activation conserved element

LID: low-glucose inhibitory domain

G6P: glucose-6-phosphate

Xu5P: xylulose 5-phosphate

F2,6-BP: fructose-2,6-biphosphate

L-PK: liver-type pyruvate kinase

ACC: acetyl-CoA carboxylase

FAS: fatty acid synthase

ACLY: ATP citrate lyase

SCD1: stearoyl-CoA desaturase-1

GPDH: glycerol-3-phosphate dehydrogenase

GLUT: glucose transporter type

WAT: white adipose tissue

BAT: brown adipose tissue

UCP1: uncoupling protein 1

ARRDC1: arrestin domain containing 4

TXNIP: thioredoxin interacting protein thioredoxin interacting protein

PPP1R3A: phosphoprotein phosphatase 1 regulatory subunit 3A

PP1: protein phosphatase 1

PPARG: peroxisome proliferator-activated receptor gamma

SIRT1: sirtuin 1

HFCS: high fructose corn syrup

FGF21:fibroblast growth factor 21

HFD: high-fat diet

NAFLD: non-alcoholic fatty liver disease

NASH: non-alcoholic steatohepatitis

TAG: triglyceride

HCD: high carbohydrate diet

SD: standard diet

ABSTRACT

Abou Rached, M. (2022). The Effect of Exposed Diet Type on Mondo Family Transcription Factors in Sprague Dawley Rat Liver. Yeditepe University, Institute of Health Science, Department of Physiology, MSc Thesis, İstanbul.

Excessive weight gain and related diseases due to high-calorie intake are one of the most important health problems of today. High-calorie intake is usually due to a high carbohydrate diet. As with ketogenic diets, high-fat diets remain popular today. It is known that consumed diets which are rich in carbohydrates and fats trigger obesity and insulin resistance. In recent years, it is known that carbohydrates taken in appropriate amounts are necessary for longevity, but excessive carbohydrate restriction or excess carbohydrate intake (especially sugar) paves the way for metabolic diseases, and this is done by changing gene expressions related to metabolism. For this reason, it is important to know the change in transcription factors responsible for the modification of metabolism-related genes according to the diet.

In our study, we planned to reveal the change of MONDO A and CHREBP, transcription factors which are known to directly modulate the metabolism, according to the exposed diet, Sprague Dawley rats were fed with standard, high-fat and high-carbohydrate diets during maternal and maturation periods. Afterwards, liver morphology was assessed by H&E staining, the fat accumulation of the livers was determined biochemically, and the expression levels of the transcription factors MONDO A and CHREBP were determined by immunofluorescence staining.

According to the results obtained from our study, both the high-carbohydrate diet and high-fat diet caused more lipid accumulation in the liver and increased expression levels of MONDO A and CHREPB transcription factors compared to the standard diet.

Keywords; HFD, HCD, MONDO A, CHREBP (MONDO B)

ÖZET

Abou Rached, M. (2022). Maruz Kalan Diyet Tipinin Sprague Dawley Sıçan Karaciğerinde Mondo Ailesi Transkripsiyon Faktörleri Üzerindeki Etkisi. Yeditepe Üniversitesi Sağlık Bilimleri Enstitüsü, Fizyoloji AD. Yüksek Lisans Tezi. İstanbul.

Yüksek kalori alımına bağlı olarak gelişen aşırı kilo alımı ve ilişkili hastalıklar günümüzün en önemli sağlık sorunlarından biridir. Yüksek kalori alımı genellikle yüksek karbohidrat içerikli beslenme sebebiyle olmaktadır. Ketojenik diyetlerde olduğu gibi yüksek yağ içerikli diyetler günümüzde populerliğini korumaktadır. Tüketilen diyetlerin karbonhidrat ve yağdan zengin olması diyetlerin obezite ve insülin direncini tetiklediği bilinmektedir. Son yıllarda yapılan çalışmalar uygun miktarda alınan karbohidratın uzun ömür için gerekli olduğunu ancak aşırı karbonhidrat kısıtlaması veya fazladan karbohidrat alımının (özellikle şeker) ise metabolik hastalıkların önünü açtığı, bunu da metabolizma ile ilişkili gen ekspresyonlarını değiştirerek yaptığı bilinmektedir. Bu sebeple maruz kalınan diyete göre metabolizma ile ilişkili genlerin modifikasyonundan sorumlu transkripsiyon faktörlerinde meydana gelen değişimi bilmek önemlidir. Maruz kalınan diyete göre, metabolizma ile direk ilişkili olduğu bilinen hepatik transkripsiyon faktörlerinden MONDO A ve CHREBP'in değişimini ortaya koymayı planladığımız çalışmamızda, Sprague Dawley sıçanlar maternal ve maturasyon dönemlerinde, standart, yüksek yağlı ve yüksek karbohidratlı diyetler ile beslenmişlerdir. Sonrasında hayvanların karaciğerleri H&E boyaması ile morfolojik olarak değerlendirilmiş, karaciğer yağlanması, biyokimyasal olarak tayin edilmiş, MONDOA ve CHREBP transkripsiyon faktörlerinin ekspresyon seviyeleri immunfloresan boyama ile belirlenmiştir.

Çalışmamızdan elde edilen sonuçlara göre, yüksek karbohidratlı beslenme ve yüksek yağlı beslenmenin her ikisi de standart beslenmeye göre karaciğere daha fazla yağlanmaya ve MONDO A ve CHREPB ekpresyon seviyelerinde artışa sebep olmuştur.

Anahtar kelimeler; HFD, HCD, MONDO A, CHREBP (MONDO B)

1. INTRODUCTION and PURPOSE

Our purpose in this study is to show the effect of exposed diet type on Mondo family transcription factors in Sprague Dawley rats. It is known that carbohydrates and lipds levels regulate gene expression. In other words, some molecules sense the level of those macronutrients and work as transcription factors. Transcription factors named Mondo A and Mondo B (CHREBP) are one of the main players in nutrient-dependent gene regulation. These transcription factors play key roles in metabolic homeostasis, especially in glucose and lipid metabolism and also in the adaptive response to non-physiological situations, such as overeating. So, it is important to determine the effect of diet on these transcription factors.

2. GENERAL INFORMATION

2. 1. The Overview of The Liver

2. 1. 1. Anatomy and Histology of Liver

The liver is the largest organ in the body weighing approximately 1200 to 1500 kg in adults. Its located in the upper right abdominal quadrant and extends to the left upper quadrant. The liver is surrounded by a fibrous membrane known as Glisson's capsule which gives it a fairly definite shape. The liver suspends from the diaphragm and the anterior abdominal wall by various ligaments. It suspends superiorly from the diaphragm by a ligament called the coronary ligament which in turn extends outwards to form the left and right triangular ligaments. Anteriorly it suspends by the falciform ligament, while at the porta hepatis it suspends by the hepatoduodenal and gastrohepatic ligaments. (Juza and Pauli 2014).

At a cellular level, the liver is composed of five types of cells which can be classified as parenchymal and nonparenchymal types. Parenchymal cells are hepatocytes, while non-parenchymal are stellate cells, Kupffer cells, sinusoidal endothelial cells and Cholangiocytes. Each one of these cell types has a specific role to play in the various functions of the Liver. Hepatocytes dominate most of the liver mass and form cords of cells that surrounds individual sinusoidal capillaries. They have a wide range of functions including synthesis, storage and filtration of the portal venous blood. Kupffer cells function as liver specific macrophages where they perform immunological and phagocytic funtions.Sinusoidal endothelial cells form a fenestrated plexus which allows the communication of portal blood with hepatocytes. Stellate cells play an integral part in the liver remodelling process associated with hepatic fibrosis. Cholangiocytes functions as the trasporter of bile, and secrete both bicarbonate and water. (Juza and Pauli 2014)

The lobule is considered to be the functional structural unit of the liver, where all liver cells are found to be organized around it. (Juza and Pauli 2014)

2. 1. 2. General Functions of the Liver

The Liver is a major contributor to several physiological processes. These processes include macronutrient metabolism, endocrine control of growth

signalling pathways, immune system support, blood volume regulation, lipid and cholesterol homeostasis, and the breakdown of xenobiotic compounds, including many current drugs. Digestion and metabolism of macronutrients provide the energy needed for the previously mentioned processes, and here is where the most critical function of the liver lies. The macronutrients that we will discuss are glucose, Lipids and cholesterol, protein and amino acids. (Trefts, Gannon et al. 2017)

When discussing glucose metabolism we should keep in mind that depending on the feeding state the liver activates different metabolic processes. In the state of feeding, the liver transitions from net output to net input. This means the level of glucagon drops with the opposite increase in insulin leading to the decrease of glucose output form the liver which is usually caused by glycogen breakdown and gluconeogenesis, while on the other hand promoting glycolysis and glycogen deposition in hepatocytes. On the other hand, when the organism switches to the fasting state, the liver response is the absolute opposite. In this case, the insulin levels drop with a significant increase in glucagon leading the liver to shift into a net glucose output by breaking down glycogen and gluconeogenesis. A point worth mentioning here is the pathological concerns about an inadequate liver response in the case of overfeeding. The key factor here is insulin resistance which usually leads to multiple diseases like Diabetes Type 2, nonalcoholic fatty liver and cardiovascular diseases. (Trefts, Gannon et al. 2017).

The liver plays a critical role as well in the utilization and metabolism of lipids and lipoproteins. In the process of chylomicron formation, the liver extracts fatty acids from chylomicron remnants with the help of lipoprotein lipase. Those are used as an internal energy source for the liver and they are utilized to supply energy to other organs. The liver forms triglycerides by assembling bothe fatty acids and glycerol which in turn are packaged in low-density lipoproteins and secreted in the bloodstream. These triglycerides reach organs such as the adipose tissues where they can be stored, or the muscles where they can be used as an energy source.(Trefts, Gannon et al. 2017).

Cholesterol is also absorbed from the intestines and is synthesized de novo in the liver. Improper utilization of cholesterol, where low cholesterol is damaging while high levels of it can be detrimental since it can lead to cardiovascular diseases such as atherosclerosis due to its function in the assembly of the cellular membrane. (Trefts, Gannon et al. 2017).

Lastly, when it comes to proteins and amino acids, the liver contribute to 85-90% of proteins circulating in the body. The most abundant secreted protein is albumin which is responsible for the maintenance of blood volume and works as a transporter for lots of essential molecules in the bloodstream (hormones, lipids, etc.). The liver also possesses the ability to break down proteins into amino acids to utilize them as an energy source.(Trefts, Gannon et al. 2017).

2. 1. 3 Transcription Factors

The human genome has 3 billion base pairs and almost 22000 genes. Out of them, only 3 % of the DNA is a protein coding sequence, The majority rest of the remaining DNA is used for other purposes. After extensive research, it turns out that a huge component of the remaining DNA function as regulatory sequences, which control the protein-coding region's function and activity.

Understanding Transcription factors (TF) starts with understanding the process that these factors are involved in. Transcription is the process of producing an mRNA from a DNA sequence to produce tissue-specific proteins. This process is so specific in a way that even though there are thousands of DNA sequences in the genome, only specific regions are activated and are actively transcribed in specific regions in the body. Transcription factors are the major contributors in the process of activation of the transcription process. It all starts with the assembly of the PIC (pre-initiation complex). PIC includes RNA polymerase II and 6 general transcription factors (TFIIA, TFIIB, TFIID, TFIIE, TFIIF, TFIIH), which bind up in a step-by-step cycle. TATA-binding protein (TBP) binds to the promoter (the TATA box), which is an A/T-rich sequence, creating a sharp bend in the promoter

DNA. TBP then recruits TFIIA and TFIIB to the promoter. TFIIB recruits RNA polymerase II and TFIIF. TFIIE joins the growing complex and recruits TFIIH which has both a protein kinase activity and DNA helicase activity. All these steps lead to the formation of the transcription bubble which initiates RNA formation (Warren 2002).

2. 1. 3. 1. Mondo Family Transcription Factors

The basic helix-loop-helix leucine zipper family includes the Mondo Family Transcription Factors, which include Mondo A (also known as MLXIP) and carbohydrate response element binding protein (ChREBP, also known as Mondo B and MLXIPL). Mondo and MLX translocate to the nucleus after interacting with MLX (Max-like protein X), where they bind to carbohydrate response elements on target gene promoters to activate a transcriptional response to nutrition.(Ke, Luan et al. 2021).

It is important to mention the structural domains of both ChREBP and Mondo A to make up an understanding of the process of activation of these transcription factors. Both transcription factors contain a nutrient-sensing domain which plays a major role in the activation process called the glucose sensing module (GSM). GSM consists of a glucose-response activation conserved element (GRACE) which is usually repressed by the low-glucose inhibitory domain (LID) under normal conditions. In the case of alteration of nutrient balance, such as glucose elevation, GRACE is activated leading to the activation of both Mondo A and ChREBP TFs which with the help of importing into the nucleus regulate the organ's response to the increased glucose level. MONDO A and ChREBP, even though they share a similar structure they tend to be more abundant in different regions of the human body. Mondo A is more localized in the skeletal muscles, while ChREBP is found mainly in the liver and adipose tissues. (Ke, Luan et al. 2021).

Numerous food molecules, mostly glucose and its metabolites, control ChREBP. When blood glucose levels are high, G6P binds to Mondo B's GRACE domain, activating this transcription factor. Fructose-2,6-bisphosphate (FDP) and

xylulose 5-phosphate (Xu5P) are additional ChREBP activators (F2,6-BP). Fatty acids and branched chain ketoamino acids, however, are discovered to decrease ChREBP activity in the condition of low blood glucose levels. Through its important control over several core enzymes, including fatty acid synthase (FAS), acetyl-CoA carboxylase (ACC), liver-type pyruvate kinase (L-PK), ATP-citrate lyase (ACLY), stearoyl-CoA desaturase-1 (SCD1), and glycerol-3-phosphate dehydrogenase(GPDH), ChREBP is thought to play a major role in the regulation of lipogenesis in the Liver. (Ke, Luan et al. 2021).

One of the major roles of ChREBP functions worth mentioning is its role in fructose metabolism. ChREBP stimulates the production of GLUT2 and GLUT5 in enterocytes as well as KHK, an enzyme required for fructose metabolism, in the liver. During this process, ChREBP can be considered a promoter of the negative effects of fructose since when fructose is sensed it is immediately transformed into glucose, which in turn with the help of ChREBP gets transferred into the liver where it gets transformed into lipids where it can get stored, which leads to hepatic steatosis and hyperglycemia. (Ruiz & Medina & Bovida, 2021)

Another core function of ChREBP is that it plays in adipose tissues. In White adipose tissues, ChREBP promotes de novo lipogenesis through the increasing uptake of glucose through GLUT4. In the case of hypothermia white adipose tissues (WAT) go through a process called browning where those tissues transform into brown, nutrient consuming and heat-producing brown and Beige adipose tissues. This browning process is usually promoted by UCP1. Overexpression of ChREBP especially the beta version has proven to downgrade the function of UCP1 leading to the inhibition of the browning process. While instead promoting lipogenesis and lipid deposition in adipose tissues.(Ke, Luan et al. 2021).

Mondo A is the 2nd member of the mondo family. But unlike ChREBP, it is more abundant in skeletal muscles rather than the liver and adipose tissues. Mondo A function lies in the fact that it controls the uptake of glucose by skeletal muscles. It has been demonstrated that MondoA promotes transcription of the arrestin

domain containing 4 (ARRDC4) and thioredoxin interacting protein (TXNIP) under conditions of high fructose and glucose ingestion, preventing glucose absorption. Through the inhibition of GLUT, TXNIP prevents skeletal muscles from absorbing glucose. Additionally, Mondo A increases glycogen production by increasing the transcription of the genes producing the protein phosphatase 1 (PP1) (glycogen targeting subunits) and phosphoprotein phosphatase 1 regulatory subunits 3A (PPP1R3A) and 3B (PPP1R3B). Mondo A is the 2nd member of the mondo family. But unlike ChREBP, it is more abundant in skeletal muscles rather than the liver and adipose tissues. Mondo A function lies in the fact that it controls the uptake of glucose by skeletal muscles. It has been demonstrated that MondoA promotes transcription of the arrestin domain containing 4 (ARRDC4) and thioredoxin interacting protein (TXNIP) under conditions of high fructose and glucose ingestion, preventing glucose absorption. Through the inhibition of GLUT, TXNIP prevents skeletal muscles from absorbing glucose. Additionally, Mondo A increases glycogen production by increasing the transcription of the genes producing the protein phosphatase 1 (PP1) (glycogen targeting subunits) and phosphoprotein phosphatase 1 regulatory subunits 3A (PPP1R3A) and 3B (PPP1R3B).(Ke, Luan et al. 2021).

2.1.3.2. Brown and Beige Adipocytes and CHREBP Function

Obesity is considered to be a public health issue since it affects a wide number of people around the globe, and it has raised the attention of medical researchers to find the proper ways to tackle it and prevent its detrimental effects on human health. Obesity is defined as an imbalance between energy intake and consumption, where intake overwhelms the body's organs and keeps getting stored in adipose and liver tissues leading to weight gain. When it comes to adipose tissues, there are three types, White which mainly stores energy, and beige and brown which contribute to energy consumption through thermogenesis. To increase energy consumption in adipose tissues, White adipose tissues (WAT) undergo a process called browning, in which they are transformed into beige and brown tissues. Browing process is usually influenced through directly or indirectly stimulating UCP-1 s, including peroxisome proliferator-activated receptor g

(PPARg), PPARg coactivator-1a (PGC-1a), silent information regulator type 1 (SIRT1) and FGF21 by multiple transcription and endocrine factors. ChREBP tends to promote de novo lipogenesis in the case of high glucose and sucrose intake. Lipogenesis is usually induced through GLUT_4 glucose intake leading to inducing the overexpression of ChREBP leading to the promotion of *denovo* lipogenesis. Multiple studies have suggested that there is a correlation between ChREBP expression and energy metabolism in adipose tissues. Overexpression of active ChREBP in adipocytes indirectly promotes UCP-1 leading to the browning of WAT which usually leads to increased energy consumption. Yet, on the other hand, the other isoform of ChREBP, ChREBPb overexpression in brown adipocytes impaired thermogenesis and WAT browning, which is understood as a compensation process in cases of acute cold exposure.(Ke, Luan et al. 2021).

2.1.3.3. Mondo Family Targeting in Metabolic Disorders

As mentioned earlier, Mondo family transcription factors play a major role in adjusting and influencing the body's response to different types of diet by controlling the metabolic processes performed by different body organs, mainly the liver muscles and adipose tissues. Mondo A was shown to have a major effect on skeletal muscles where it impairs the intake of glucose by the muscles while promoting muscle triglyceride accumulation and insulin resistance. Moreover, Mondo B was shown to influence the browning process of WAT promoting energy consumption through thermogenesis, while the ChREBPb isoform performed a counter effect. Therefore, manipulating the levels of Mondo A and B, such as blocking the effect of Mondo A in muscle tissues leading to increase consumption of glucose in skeletal muscle, can help as a treatment regimen for obese patients leading to decreasing their chances of suffering from chronic diseases such as diabetes. (Ke, Luan et al. 2021).

2.1.3.4. ChREBP Response to Carbohydrates Consumption

Carbohydrates are a major source of energy that we consume daily. The amount and type of carbohydrates consumed can indicate mortality since some types can have detrimental effects. High fructose diets, such as those containing high fructose corn syrup (HFCS), are positively associated with type 2 diabetes, heart disease, and obesity. High fructose levels associated with high glucose levels can lead to weight gain which in turn can be pre-exposure for multiple comorbidities. Understanding how ChREBP responds to this type of diet helps us understand how influencing it can help us control the side effects of high fructose diets. Contrary to glucose, sucrose and fructose were shown to be readily absorbed in the intestines, and their metabolites can increase ChREBP overexpression, which then encourages de novo lipogenesis in the liver and adipose tissues and results in weight gain and hepatic steatosis. At the same time, ChREBP was shown to have a positive influence on FGF21, a hepatocyte, which has shown that at high levels it can lower sugar-eating behaviour. Thus, we can draw the conclusion that sucrose-glucose activation of ChREBP can be both advantageous and detrimental at the same time, where it can result in weight gain, liver steatosis, and insulin resistance

through de novo lipogenesis, while simultaneously reducing the sugar-eating behavior through its effect on FGF21 hepatokine. (Iizuka 2021).

2. 2. The Overview of the Relationship Between Diet and Liver

2. 2. 1. Consumption of High Fat Diet (HFD) o High Carbohydrate Diet (HCD) causes hepatic dysfunction

Obesity is a major health crisis that usually leads to detrimental diseases such as insulin resistance and type 2 diabetes. The complete mechanism of how a low-quality diet leads to hepatic dysfunction is not understood yet, but multiple studies have shown a great correlation between HFD and Hepatic dysfunction. HFD has been shown to perturb hepatic architecture, disrupt bile acid handling and enhance hepatocyte apoptosis. Since the liver is the major contributor to glycemic regulation, any disturbances in the insulin response will have a great effect on the liver's ability to perform its basic functions such as gluconeogenesis. So putting to the fact that HFD promotes insulin resistance, it will strongly contribute to multiple structural and functional deformities in the liver leading to impaired glucose homeostasis and Hepatic dysfunction.(Soltis, Kennedy et al. 2017).

2. 3. Non-Alcoholic Fatty Liver Diseases

Approximately 25% of adult populations worldwide today suffer from NAFLD, the most prevalent liver condition). Prevalence rates are now highest in the Middle East (32%), followed by South America (30%), and Africa (13%), although they are significantly higher in some subpopulations, such as those who are very obese (90%) and those who have type 2 diabetes (76%). The phrase "NAFLD" refers to a buildup of fat in the liver that is not brought on by drinking alcohol or by other primary or secondary causes including viruses, drugs, or endocrine disorders.

The creation of lipid droplets inside hepatocytes as a result of intracellular lipid buildup is the distinguishing feature of NAFLD. An imbalance between lipid production and oxidation is what is responsible for this buildup (Bellanti, Villani et al. 2017). Day and James proposed the two-hit theory in 1998 to explain the development of NAFLD and its progression to non-alcoholic steatohepatitis despite

the fact that the aetiology of NAFLD is poorly known (NASH). According to this theory, insulin resistance or poor fatty acid -oxidation constitute the first strike, both of which contribute to the buildup of hepatic lipids. The second blow consists of oxidative stress or inflammation, which might make the steatosis worse and speed up the development of (NASH).

Currently, a multiple-hit model can better describe the pathophysiology of NAFLD or the development of the two hits model Numerous genetic and environmental variables (hits) interact with one another on an individual basis in this regard. A variety of diverse lifetime infections have dynamically influenced each NAFLD patient, posing both diagnostic and treatment hurdles. These elements include, but are not limited to: food (e.g., exposure to excessive fat and sugar), epigenetic alterations (Eslam, Valenti et al. 2018) diet (e.g., exposure to excessive fat and fructose) and lack of exercise (Mahady and George 2016) obesity and insulin resistance (IR) (Polyzos, Kountouras et al. 2017) lipotoxicity (Mota, Banini et al. 2016) stress endoplasmic reticulum stress (Buzzetti, Pinzani et al. 2016) dysregulation of adipokines (Polyzos, Kountouras et al. 2016) dysbiosis of intestinal microbiota (Koukias, Buzzetti et al. 2017) and endocrine disorders (Polyzos, Kountouras et al. 2012) Under the combined influence of some of these factors, which also dynamically cross each other, fat is accumulated in the liver cells leading to simple steatosis (SS). If it is not intervened on time, the liver is penetrated by immune cells, thus adding an inflammatory process, a condition characterized as NASH (Mazzoccoli, De Cosmo et al. 2018).

It is possible to use insulin resistance and the release of free fatty acids (FFAs) from adipose tissue to demonstrate the pathophysiology of NAFLD. Obesity, which causes lipolysis, the breakdown of fatty acids in adipose tissue, is typically linked to insulin resistance. The liver absorbs FFAs, which triggers the start of fatty acid production. Furthermore, mitochondrial dysfunction decreases fatty acid oxidation by upregulating acetyl CoA carboxylase (ACC) and fatty acid synthase (FAS) and down-regulates the expression of carnitine palmitoyltransferase I (CPT-1). Fatty acids therefore boost the production of lipid droplets, cholesterol, and triglycerides

in the liver. In order to raise the levels of circulating low-density lipoprotein LDL, the liver secretes triglycerides and cholesterol together with apolipoprotein B (Apo B) in the form of very low-density lipoprotein (VLDL) (Brumbaugh and Friedman 2014).). Infants born to obese moms exhibit greater intrahepatocellular fat accumulation, according to human research (Rector, Thyfault et al. 2010). These kids have a higher chance of developing obesity, NAFLD, cardiovascular disease, and hepatic cancer later in life. (Rinella and Sanyal 2016).

Recent research has revealed that a maternal diet rich in fat may contribute to the first "hit, insulin resistance or reduced -oxidation of fatty acids" occurring in utero. Triacylglyceride (TAG) levels in the liver of offspring with this disorder have been reported to rise by three times immediately after delivery. The presence of hepatocyte ballooning and lobular inflammation histologically characterizes NASH, the more severe type of NAFLD. Despite the high frequency of NAFLD, only some NAFLD patients also develop NASH. Studies have shown that those with NASH are more likely to develop cirrhosis, severe liver disease, and hepatocellular cancer (HCC)(Wong, Adams et al. 2018).

2. 3. 1. Hepatic Lipid Accumulation

The buildup of lipids is the primary characteristic of NAFLD. Therefore, fatty acid buildup occurs when fatty acid absorption and production surpass the liver cells' ability to oxidize them. In a research utilizing stable isotopes, Donnelly et al. found that de novo lipogenesis is the second-most important source of fat accumulating in the liver after serum-free fatty acids (Donnelly, Smith et al. 2005). In general, lipolysis in white adipose tissue (WAT) is a crucial factor in the amounts of serum-free fatty acids.(Fabbrini, Sullivan et al. 2010).

2. 3. 2. De Novo Lipogenesis and Mondo Family Transcription Factors

As the primary regulator of lipid homeostasis, the liver is a crucial organ in lipid metabolism. The liver also oversees the synthesis of fresh fatty acids, their export and subsequent redistribution to other tissues, as well as their use as energy substrates. (Younossi, Koenig et al. 2016).

Acetyl-CoA is transformed to malonyl-CoA by the enzyme acetyl-CoA carboxylase (ACC), and then passes through several cycles of metabolic processes to generate one molecule of palmitate, which is then used by the liver to manufacture fatty acids denovo. The nuclear transcription factors sterol regulatory element-binding proteins (SREBPs), carbohydrate responsive element-binding protein (ChREBP), liver X receptor (LXR), farnesoid X receptor (FXR), and peroxisome proliferator-activated receptors(PPARs), as well as the fatty acid synthase (FAS) complex, ACC 1 and 2, diacylglycerol acyltransferase (DGAT) 1 and 2 regulate the rate of de novo lipogenesis. Insulin and glucose independently control hepatic DNL through the transcriptional activation of virtually all DNL-related genes by SREBP-1c and ChREBP. Data from research using animal models reveal that hyperinsulinemia or hepatic overexpression of SREBP-1c increases lipogenesis and results in hepatic steatosis. (Asrih and Jornayvaz 2015).

2. 4. Hypothesis of The Study

The exposed type of diet can alter the expression levels of the hepatic mondo family transcription factors.

3. MATERIALS & METHODS

3. 1. Experimental Procedure

Laboratory animals were used and maintained by the Yeditepe University Animal Research Ethics Committee. Housing, feeding and euthanasia of Sprague Dawley rats were carried out at Yeditepe University Experimental Animal Research Center. Sprague Dawley rats were allowed to access ad libitum chow and tap water. Their accommodation was at 22 °C temperature and 12 h light: 12 h dark cycle.

The study is planned in 2 stages. In the first stage, adult female Sprague-Dawley rats (mothers) were exposed to standard or high-fat diets or high carbohydrate diets during pregnancy (21days) and lactation (21 days) periods. Then, in the second part of the study, male offspring from these three main groups were exposed to SD, HFD and HCD for 120 days.

Facts of Diets	Standard Diet (SD)	High Fat Diet (HFD)	High Carbohydrate Diet (HCD)
Total Calorie (kcal/g)	3,3	5,2	3,4
Fat (% kJ)	13	60	3
Protein(% kJ)	23	20	23
Carbohydrate(% kJ)	64	20	74

Table 3.1 The macronutrient percentages of experimental diets.

Briefly; 250-300 gr female rats (mothers) were allowed to access the standard fat diet (SD), high-fat diet (HFD) and high carbohydrate (HCD) during two weeks as an adaptation period for the diets. After the food adaptation period, one male rat and two female rats were placed together in a cage for the mating

period. This period was approximately 8 days which means two oestrous cycles and at the end of the mating period, male rats were removed from the cage. During of mating period, vaginal smears were checked daily to determine the mating day and smear-positive animals were assumed as the first day of gestation. At approximately the 21st day of gestation, the offspring were born and mother animals continued feeding with the same experimental diet in groups during the lactation period. After the lactation period (21st day), the mothers and female pups were taken from cages and the remaining 18 male animals were fed with SD, HFD and HCD for the maturation period. On the 120th day, the experiment was terminated, and liver tissues were collected.

A total of 18 Sprague Dawley rats in three groups were used in this study. After the sacrification, the liver was dissected and a part of the liver tissue was stored at -80°C to determine lipid accumulation and the other part was stored in formalin for histological parameters. Experimental groups are available in Table 3.2.

Groups	**Exposed Diet**
Group 1 (n=6)	Standard Diet
Group 2 (n=6)	High Fat Diet
Group 3 (n=6)	High Carbohydrate Diet

Table 3.2 Experimental groups.

3. 2. Parameters

3. 2. 1. Paraffin Embedment and Microtomy of Paraffin-Embedded Section

The Materials:

- Floatation (water) bath (Leica RM2245, Germany)

- Microtome (Leica RM2245, Germany)
- Tissue following system (Leica 1020, Germany)
- Paraffin embedding system (Leica EG1160, Germany)
- %10 Neutral Formaline (Sigma Aldrich #, USA)
- Clean Slides (ISOLAB)

Method: Tissue following the protocol for Paraffin Embedment has been performed by the given steps below (Table 3.3).

Stations	Number of Stations	Duration
Tap Water	3	3 x 10 minutes
70% Alcohol	1	3 hours
80% Alcohol	1	3 hours
90% Alcohol	1	3 hours
100% Alcohol	2	2+3 hours
Xylene	2	1 +2 hours
Paraffin	2	> 10 hours

Table 3. 3. Tissue following protocol.

Microtomy allows tissue to be sectioned and fixed to a surface for further histological examinations. Embedding the tissue blocks into paraffin wax is effective in maintaining tissue integrity and allows the cutting of very thin sections. Following the procedure given in Table 3.3 to perform paraffin section microtomy, the liver was excised and applied to the tissue following device. All procedures except fixation were performed on a tissue-following device. The liver tissues were

embedded in paraffin and the paraffin blocks were kept at room temperature until the cutting. Paraffin blocks were placed at the appropriate location for serial sectioning and 5 μm thick sections were obtained with a microtome (Leica RM2245, Germany). The obtained sections were then transferred to the float bath (Leica RM2245, Germany) at 37°C with forceps and a brush to prevent tissue folding. After 60-90 seconds, tissues were transferred from water onto slides. The slides were allowed to dry on filter paper and placed in the slide box for storage at room temperature.

3. 2. 2. Hematoxylin-Eosin Staining

Principle: In the hematoxylin-eosin staining method used for histological purposes, it acts as an acidic and basic compound. On the one hand, hematoxylin binds to basophilic sections of tissues containing nucleic acid fragments as an acidic dye. At the end of the reaction, hematoxylin gives a blue-black colour at the nucleus of the cell. On the other hand, cytoplasm, RBCs, muscle and collagen found in the basic elements of the regions are painted pink, orange and red colours by acidic eosin.

Reagents:

- Hematoxylin (Merck # HX68297074, USA)
- Eosin Y Alcoholic Solution (Bio Optica # 05-10003/L)
- Absolute Ethanol (Sigma Aldrich #24102, USA)
- Mounting Medium (Bio Mount HM) (Bio Optica #05-BMHM500)
- Xylene (Sigma Aldrich # 16446, USA)
- Slide Staining System (Leica Autostainer XL, Germany)
- Light Microscope (Leica CTR6000, Germany)

Method: Hematoxylin-Eosin staining protocol for paraffin embedment has been performed by the given steps below (Table 3.4).

Stations	Number of Station	Duration
Oven	1	3 minutes
Xylene	3	3+3+5 minutes
%100 Alcohol	1	2 minutes
%96 Alcohol	2	2+2 minutes
Tap Water	1	2 minutes
dH_2O	1	1 minute
Hematoxylin	1	2,1 minutes
Tap Water	5	1x5 minutes
%96 Alcohol	1	30 seconds
Eosin Y	1	4,1 minutes
%96 Alcohol	3	30+30+60 seconds
%100 Alcohol	1	1 minute
Xylene	3	1x2 minutes

Table 3.4. Hematoxylin-Eosin Staining protocol.

3. 2. 3. Immunofluorescent (IF) Staining

Immunostaining is conventionally identified as a method for detecting certain antigens in cellular structures using certain fluorescent-labelled antibodies in tissues or cells. Immunofluorescence authorizes researchers to determine whether a particular sample represents the antigen in question.

In this procedure, to decide which slides will be applied to the IFC procedure, the sections were examined under the microscope and the selected sections were kept in the etuve at 37°C, overnight. The next day the slides were incubated at 60 ° C for 1 hour for deparaffinization and then the slides were kept in xylene for 20 minutes, twice. Then, for the dehydration of the liver, the slides were passed through an alcohol (ethanol) bath in decreasing concentrations for 10 minutes each (100%, 90%, 80%, and 70%, respectively). Then the sections were

kept in dH20 for 5 minutes 2 times each. Then the sections were washed in PBS for 5 minutes, 2 times, placed in EDTA buffer, boiled for 4 minutes at the "med-high" setting of the microwave oven, and cooled at room temperature for 20 minutes to eliminate antigenic masking in the tissue. Then again, slides were washed with PBS-T for 5 minutes, 3 times each and the periphery of the samples was drawn with a hydrophobic pen. The sections were blocked in 3% bovine serum albumin at room temperature for 1 hour, primary rabbit Mondo A antibody (MyBioSource-MBS249916, 1: 100 dilution) was dropped into positive groups and PBS was dropped into negative groups and the sections were incubated overnight in a refrigerator at +4°C. The sections were washed in PBS-T for 5 minutes, 3 times each on the next morning, then the secondary anti-rabbit antibody (diluted 1: 1000) was applied for 90 minutes, washed in PBS-T for 5 minutes 3 times each, and DAPI capping solution was applied for the determination of cell nuclei. The staining has been completed and displayed under the confocal microscope and the expression level and localization of Mondo A were shown. The same procedure was used to image Mondo B (MONDO B-CHREBP Antibody, Novus Biologicals, NB400–135).

3. 2. 4. Liver Lipid Assay

Principle: One of the most widely used techniques for separating a variety of neutral lipids and phospholipids from biological materials is the Folch extraction procedure. It makes use of the water, methanol, and chloroform biphasic solvent system. The fundamental idea behind this extraction method was to homogenize a tissue sample in a 2:1 volumetric combination of chloroform and methanol. the outcome of the initial extraction phase. A fresh aqueous phase is created when water (or a buffer) is introduced to the chloroform/methanol combination. Hydrophilic elements and salts are enhanced in the upper phase, which is mostly made of water and methanol, while lipids are retained in the bottom phase, which is primarily made of chloroform. The crude lipid-containing phase is next washed with water (or a buffer) to remove as few salts, non-lipids, and hydrophilic components as possible. One of the most crucial procedures when working with a

tissue sample is homogenization since it can guarantee that all fractions have the same makeup.

Reagents:

- Liver Tissue (Sprague Dawley Rat)
- Methanol (J.T Baker #9830-03, Poland)
- Chloroform (LabKim, C.0288.18.21, Turkey)
- -80 C Freezer (New Brunswick Scientific, USA)
- Sonicator
- Microbalance (Shimadzu, Japan)
- Homogenizer (Dia X 900 Heidolph, Germany)
- Centrifuge (Rotina 38R, Hettich Centrifuges, Germany)
- Sample condensator
- Microfilter
- Glass tubes

Method: Weighed 150 mg of liver tissue and empty glass tubes for the study. For each liver sample, a 10 mL chloroform-methanol mixture (2:1 ratio) was added into the tubes. The samples were homogenized in 2 minutes and sonicated in 30 seconds for tissue disruption. The samples were placed on the shaker for 12 hours. 4 mL of deionized H_2O was added to each tube. Tubes were centrifuged for 20 minutes at 2000 rpm. The methanol-H_2O layer was discarded from the tubes. The sample was filtered and rinsed three times. The first time with 2 mL of chloroform, the second time with 2 mL of chloroform and the third time with 4 mL of chloroform. The sample then was left to dry under nitrogen gas for 6 hours at 50 ^{0}C. Weighed the glass tubes which include lipid remnant. Calculated the percentage of total lipid in liver tissue.

3.3. Statistics

The conformity of the data to the normal distribution was evaluated with Kolmogorov-Smirnov and Shapiro-Wilk tests. Comparison of the data, non-parametrically, multiple comparisons were made with the Kruskal-Wallis test, and

pairwise comparisons were made with the Mann-Whitney U test. Values were expressed as the mean ± standard error of the mean (SEM). A value of $p<0.05$ was considered statistically significant.

4. RESULTS

4. 1. Morphologic Assessment of The Liver Tissues with Hematoxylin-Eosin Staining

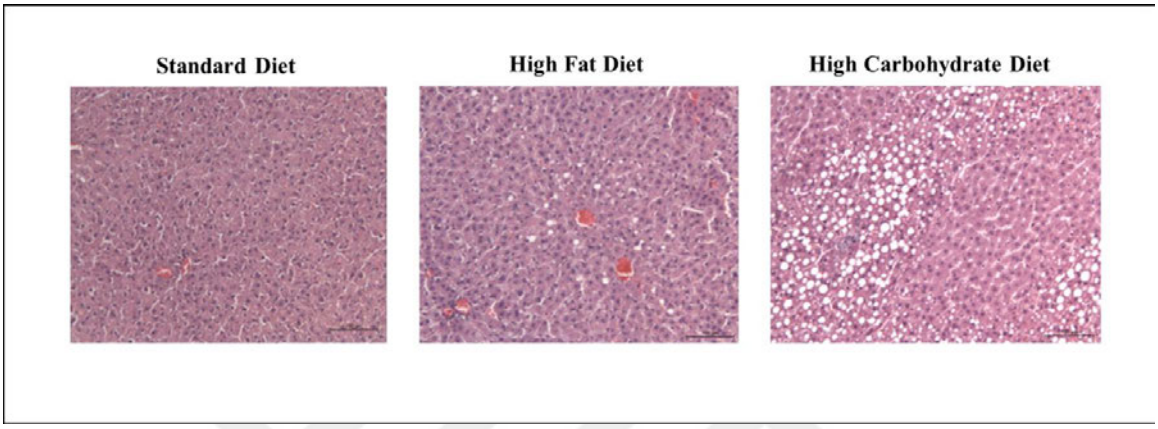

Figure 4. 1. Hematoxylin & Eosin (H&E) staining of liver sections. H&E staining was performed on paraffin sections of SD, HFD and HCD-exposed rats. 20X Magni. (n=4-6)

We collected livers from SD, HFD and HCD fasted rats to examine fat content by both histological and biochemical assessments. As seen in Figure 4.1, A normal liver architecture with sinusoidal cords of hepatocytes has been seen in the standard diet group of rats. Fatty liver has been observed both though the rats fed with HFD and HCD diets. Microvesicular steatosis is denoted by a black arrow in HFD and macrovesicular steatosis is denoted by a black arrow in the HCD group.

4. 2. Determination of Hepatic Total Lipid Level

As shown in figure 4.2 and table 4.1, the total lipid percentage was found 8.2 ± 1.7 in SD group and 10.1 ± 2.7 in HFD group. The increased lipid percentage was found in HCD group 12.8 ± 3.4 ($p<0.05$).

Groups	Total Lipid Percentage (%)
SD	6.2 ± 1.7
HFD	10.1 ± 2.7*
HCD	12.8 ± 3.4*

Table 4. 1. Total lipid percentage in livers of experimental groups (means ± SD).

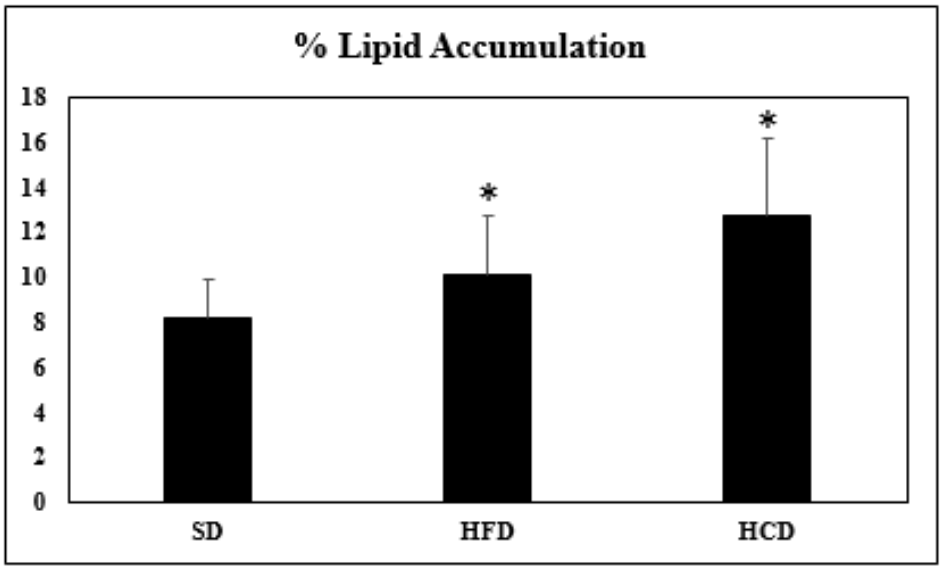

Figure 4.2. The effect of SD, HFD and HCD on the total percentage of lipid level in liver tissue. (mean ± standard deviation) (n=4-6) (* $p<0.05$).

4. 3. Determination of Hepatic Mondo-A Protein Expression with Immunofluorescent (IF) Staining

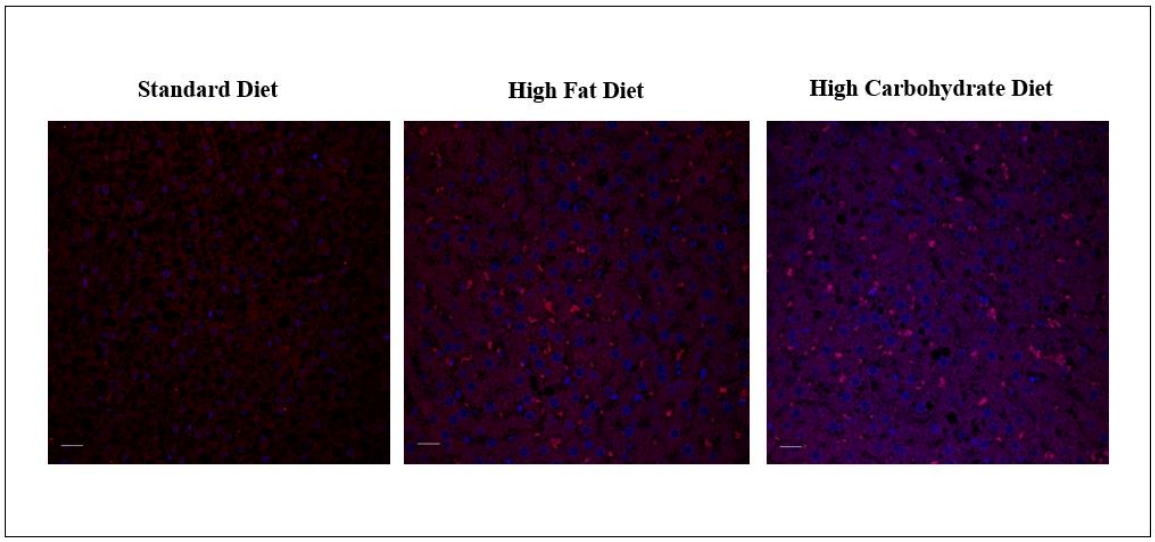

Figure 4.3.a. The effect of SD, HFD and HCD on the expression of hepatic MONDO A level

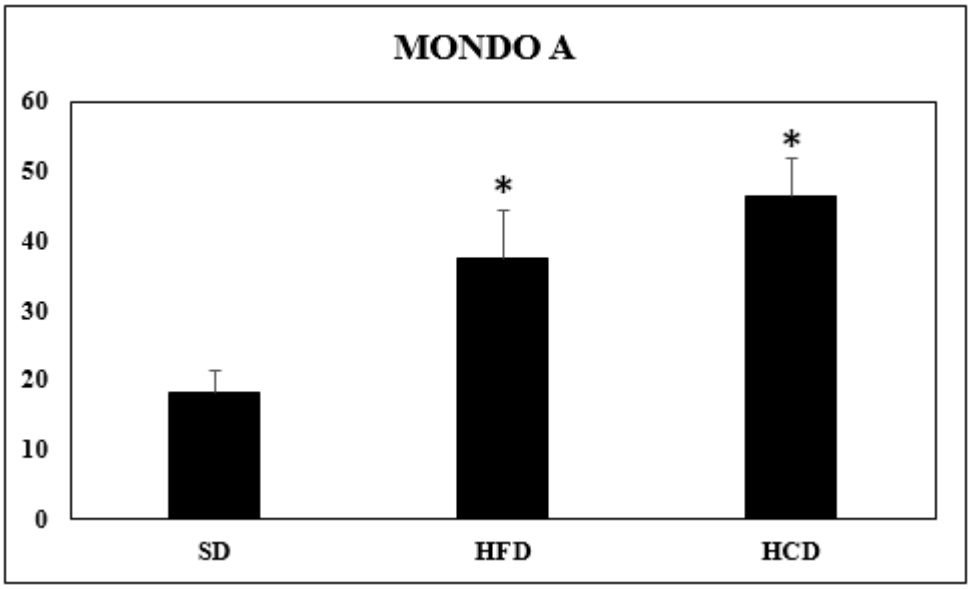

Figure 4. 3. b The effect of SD, HFD and HCD on the expression of hepatic MONDO A level (n=4-6) (* $p<0.05$).

As seen in figure 4.3. a and 4.3. b, hepatic MONDO A expressions were higher in HFD and HCD groups when compared to the SD group.

4. 4. Determination of Hepatic CHREPB (MONDO B) Protein Expression with Immunofluorescent (IF) Staining

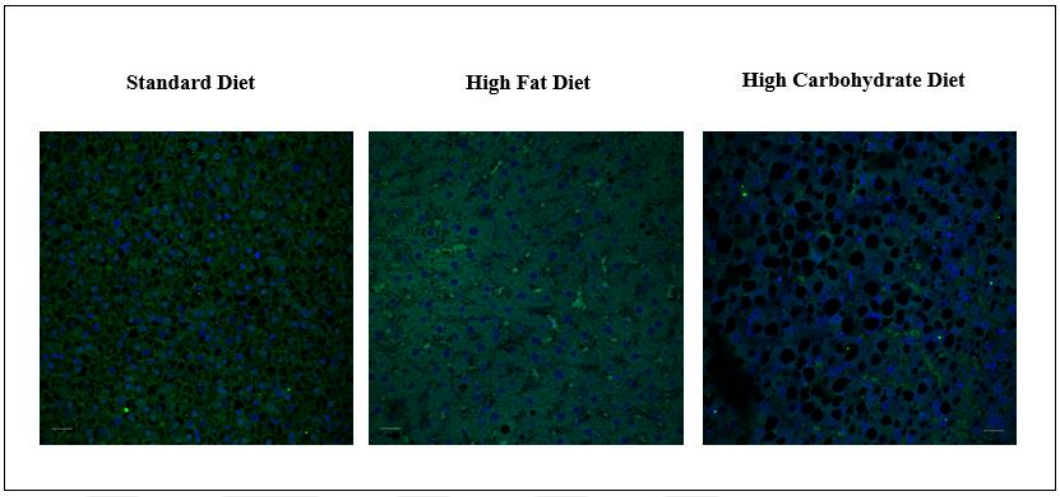

Figure 4.4.a. The effect of SD, HFD and HCD on the expression of hepatic CHREBP level.

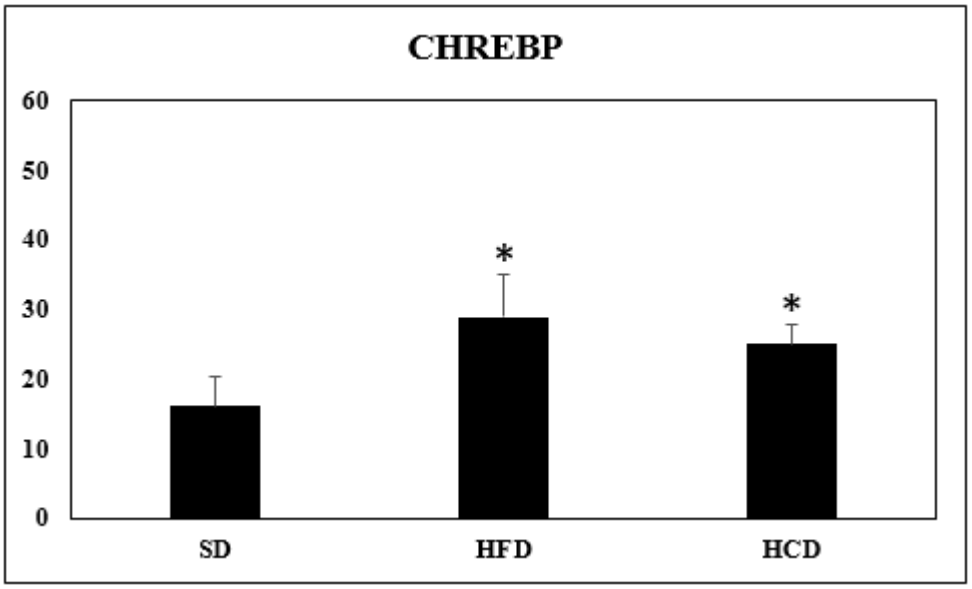

Figure 4. 4. b. The effect of SD, HFD and HCD on the expression of hepatic CHREBP level. (n=4-6) (* $p<0.05$).

As seen in figure 4.4. a and 4.4. b, hepatic CHREBP expressions were higher in HFD and HCD groups when compared to the SD group.

5. DISCUSSION and CONCLUSION

Because of their smell, taste, flavor, and textural qualities, fats and carbohydrates, which are considered macronutrients, have the largest energy content in the diet (Passilly-Degrace, Gaillard et al. 2009). However, it is believed that eating a high-fat diet or being exposed to high levels of carbohydrates is linked to the diagnosis of several chronic illnesses, including obesity, type II diabetes, cardiovascular disease, and nonalcoholic fatty liver disease (NAFLD), in adulthood. One of the most significant health issues in the world is particularly fatty liver. It is the first and most typical reaction to a diet that is too heavy in fat and sugar. It is also shown that excessive food intake (overfeeding) changes gene expressions which is related to lipid and carbohydrate metabolisms (Bravo-Ruiz, Medina et al. 2021). For this reason, it is important to know the change in transcription factors responsible for the modification of metabolism-related genes according to the consumed diet. For this purpose, we designed a two-stage study. In the first stage, adult female Sprague-Dawley rats (mothers) were exposed to standard or high-fat diets or high-carbohydrate diets during pregnancy and lactation periods. Then, in the second part of the study, male offspring from these three main groups were exposed to three different diets SD, HFD and HCD for 120 days. We evaluated the liver morphology, hepatic lipid accumulation, and the change in the carbohydrate and lipid metabolism-related transcription factors, Mondo A and CHREBP (Mondo B).

Both HFD and HCD-fed rats showed increased hepatic fat deposition with evidence of both micro and macrovesicular steatosis as compared to their respective controls, according to the histopathological analyses of the formalin-fixed H&E stained liver sections (Simoes, Janikiewicz et al. 2019). Overconsumption of a high-fat or high-sugar diet can result in fatty liver, which has a similar pattern of hepatic lipid buildup. As anticipated, liver lipid accumulation was higher in HFD and HCD-fed rats compared to SD-fed animals, which taught us that enhanced fat synthesis and/or decreased fat catabolism are both possible explanations for body fat gain, depending on whether an individual is overeating or not.

It is known that Mondo A and ChREBP regulate the response to the increased glucose level. Mondo A is more localized in the skeletal muscles, while ChREBP is found mainly in the liver and adipose tissues.(Ke, Luan et al. 2021). According to the evaluation of hepatic MONDO A and CHREBP expressions, both transcription factors' expression levels were higher in HFD and HCD-fed animals compared to SD animals. We were expecting higher expression levels of CHREBP in HCD-fed animals compared to HFD-fed animals. Although there was no statistically significant difference in CHREBP levels between HFD and HCD-fed animals, interestingly MONDO A levels were higher in both HCD and HFD-exposed animals.

According to the results obtained from our study, both the high-carbohydrate and high-fat diet consumption caused increased lipid accumulation in the liver and increased expression levels of MONDO A and CHREPB transcription factors compared to the standard diet. So we can see the direct correlation between the type of diet and the influence it has on the level of Mondo family TF leading to an increase in fat accumulation in liver tissues.

To clarify and discuss better the effecting mechanism of consumed diet type on hepatic lipid accumulation, and disrupted liver morphology, we have to determine the changes exerted by MONDO A and ChREBP on their targets such as the activity of lipid synthesis-related enzymes. Only then can we make up a great foundation to start more extensive research aiming at finding a way to control these TFs to influence a more healthy way of combating the long-term comorbidities caused by HFD and HCD.

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7. APPENDICES

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(YÜDHEK)

ETİK KURUL KARARI

Toplantı Tarihi	Karar No	İlgi	Proje Yürütücüsü
23.12.2016	575	07.12.2016	Yrd.Doç.Dr. Burcu GEMİCİ BAŞOL

'**Sprague Dawley Sıçanlarda Maternal Beslenme Durumuyla Yavrunun Yağ Tadı Algısı ve Yağlı Besin Tercihi Arasındaki İlişkinin İncelenmesi**' adlı bilimsel çalışma etik kurulumuzda görüşülmüş olup, çalışmanın etik kurallara uygun olduğuna oy birliğiyle karar verilmiştir.

Etik Onay Geçerlilik Süresi: 3 Yıl	Hayvan Türü ve cinsiyeti: Rat ♂	Hayvan Sayısı: 84

GÖREVİ	ADI SOYADI	İMZA
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Başkan Yardımcısı	Prof. Dr. Erdem YEŞİLADA	
Raportör	Vet. Hekim Engin SÜMER	
Üye	Prof. Dr. M. Ece GENÇ	
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Üye	Doç. Dr. Soner DOĞAN	
Üye	Doç. Dr. Ediz DENİZ	
Üye	Prof. Dr. Gamze TORUN KÖSE	
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MEDICAL DEGREE (MD) – Yeditepe University

02/11/2020 – 11/11/2020 – Istanbul, Turkey
CERTIFICATE OF ANIMAL USE IN EXPERIMENTAL RESEARCH – Yeditepe University Animal Experiments Local Ethics Committee (HADYEK) Completed Animal Lab training and qualified to receive a Category A certificate

LANGUAGE SKILLS:
Mother tongue(s): ARABIC, ENGLISH
Other language(s): TURKISH, GERMAN

CONFERENCES AND SEMINARS:
ACP (American College of Physicians) annual Internal Medicine Meeting **11-13 April 2019**

2 EMBS (Engineering in Medicine and Biology Society) **6-7 May 2017**

11. National Medical Student Conference 'Neuropsychiatry and Cognitive sciences' **22-24 April 2017**

HONOURS AND AWARDS:

- A full scholarship between the years 2004 and 2013 was granted by my school for my high performance and being the top student.
- A full Scholarship for my entire Medical School was given to me by the university for my high grades and Great performance.
- A full Scholarship covering my Master's Program.

ORGANISATIONAL SKILLS:
Injaz Project in Lebanon where I attained a certificate of achievement for attending a 10-month management and leadership program **27-11-2011**

CERTIFICATIONS:

Clerkship Program of Transition to Clinical Settings' **September 9 2017**
UNRWA Summer Camp as an English Teacher Volunteer for 4 weeks **07-08-2013**