Preventative and Treatment Effects of Whey Protein Hydrolysates and Arginine on Fatty Liver Condition Induced in Mink by Dietary Orotic Acid

by:

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Dedicated

To

my Thaththa, Amma, Malli and Niluni,

who have always encouraged me to pursue my dreams and

make me able to succeed.

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ABSTRACT

Fatty liver disease is one of the most severe health concerns for mink and believed to occur through the same mechanism as nursing sickness. Orotic acid (OA) plays a critical role in lipid metabolism and can increase hepatic lipids by regulation of different lipid metabolic pathways. This study was conducted to identify particular pathways and regulatory control points involved in fatty liver development, and evaluate the effectiveness of arginine and whey protein hydrolysates (WPH) for prevention and treatment of fatty liver disease in mink. A total of 45 mink were used in 9 treatments. The experimental diets consisted of 1% OA, 2% L-arginine and 5% of whey protein hydrolysates. At the end of the experimental period, the mink were anaesthetized, sampled for histological, biochemical and molecular assays. OA feeding was attributed to significant increases in plasma glucose and plasma cholesterol levels. NASH Activity Index levels shows clear liver fat progressed with 1% OA in the diet for 10 days' treatment and a return to baseline health was possible with a mink diet composition of 2% L-arginine or 5% WPH or a combination of the two. Feeding of OA resulted in an increase in liver inflammation, which may be explained by the elevated mRNA expression in HMGCS2 and GRP78 in liver. Based on results, it is evident that 1% orotic acid induced simple steatohepatitis can be reversible within 10 days of diet containing 2% arginine or 5% WPH or a combination.

LIST OF ABBREVIATIONS USED

ACE	Angiotensin I converting enzyme
acyl-CoA	Acetyl coenzyme A
AMPK	Adenosine monophosphate-activated protein kinase
ANOVA	Analysis of variance
Arg	Arginine
ATP	Adenosine triphosphate
BMI	Body Mass Index
CCAC	Canadian Council of Animal Care
cDNA	Complementary DNA
ChREBP	Carbohydrate-responsive element-binding protein
CO_2	Carbon dioxide
CPT	Carnitine palmitoyl transferase
CPT1A	Carnitine palmitoyl transferase1A
Ct	Cycle threshold
CTRL	Control
CV	Coefficient of variation
CYP	Cytochrome P450
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ER	Endoplasmic reticulum
FA	Fatty acid
$FADH_2$	Flavin adenine dinucleotide
FFA	Free fatty acids
FLD	Fatty liver disease
FOXA2	Forkhead Box A2
FoxO1	Forkhead box O1
G6PC	Glucose-6-Phosphatase, Catalytic Subunit
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase

GRP-78	78 kDa glucose-regulated protein
H$_2$O$_2$	Hydrogen peroxide
HMGCS2	3-Hydroxy-3-Methylglutaryl-CoA Synthase 2
HPRT1	Hypoxanthine Phosphoribosyltransferase 1
hr	hour
HSK	Housekeeping genes
IL-6	Interleukin-6
KCl	Potassium chloride
LCFAs	Long chain fatty acids
MgCl$_2$	Magnesium Chloride
min	Minutes
mRNA	Messenger ribonucleic acid
mRNA	Messenger RNA
MTTP	Microsomal Triglyceride Transfer Protein
NADH	Nicotinamide adenine dinucleotide
NAFLD	Non-alcoholic fatty liver disease
NAI	Non-alcoholic steatohepatitis activity index
NASH	Nonalcoholic steato hepatitis
NHANES	National Health and Nutritional Examination Survey
O^{2-}	Superoxide anions
OA	Orotic acid
OH$^\bullet$	Hydroxyl radicals
PEPCK	Phosphoenolpyruvate carboxykinase
PI3K	Phosphoinositide 3-kinase
PUFA	Polyunsaturated fatty acids
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
RNAse	Ribonuclease
ROS	Reactive oxygen species

RP2	Retinitis pigmentosa 2
s	Second
SREBP-1c	Sterol regulatory element binding protein-1c
TGs	Triacylglycerols
TNF-α	Tumor necrosis factor-alpha
UPR	Unfolded protein response
VLDL	Very low density lipoprotein
WPH	Whey protein hydrolysate

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CHAPTER 1. INTRODUCTION

1.1 Introduction

The increasing prevalence of metabolic diseases has become a serious international concern. In Canada, 59% of the human population is considered overweight (Body Mass Index (BMI)> 25 kg/m^2), while 23% or nearly a quarter of the population is obese (BMI > 30 kg/m^2) (Lau et al. 2007). Obese individuals are at an increased risk of developing chronic and progressive metabolic disorders including non-alcoholic fatty liver disease (NAFLD) and cardiovascular diseases. Currently, liver related complications are one of the leading causes of mortality in Canada and the western world, while NAFLD is the most prevalent liver disease in North America (Vernon et al. 2011).

Fatty liver disease and nursing sickness are among the three most severe nutritional concerns for the mink industry. Nursing sickness is the largest cause of death in female lactating mink and is accompanied with the development of hepatic lipidosis, which is associated with seasonal body weight fluctuations (Nieminen et al. 2001; Rouvinen-Watt 2003a). In North America, nursing sickness affects about 45% of mink farms with an average incident rate of 22.5% (Rouvinen-Watt and Hynes 2004). In Canada, it is responsible for an annual commercial loss of about $1,000,000 for the industry nationwide due to the high mortality rate of female mink. In addition, loss of female mink in the farms causes a decline in reproductive longevity of breeder females, loss of the best genetic lineage in the mink due to culling of offspring of affected females, or the loss of productivity in young growing animals.

NAFLD is primarily associated with the development of insulin resistance as a part of many metabolic changes that occur during the progression of obesity, type II diabetes, hypertension and dyslipidemia (Dowman et al. 2010; Lomonaco et al. 2013). Anstee et al. (2013) suggested that, as a result of impaired liver function in the regulation of glucose and lipid metabolism, fatty liver should be considered as the hepatic trigger of the metabolic syndrome, rather than its target. The liver *de novo* lipid synthesis is involved in the development of fatty liver (Rouvinen-Watt et al. 2012; Rouvinen-Watt et al. 2014). Development of fatty liver in mink is influenced and promoted by several biochemical pathways, including up-regulation of hepatic gluconeogenesis, β-oxidation and ketogenesis (Rouvinen-Watt et al. 2010; Rouvinen-Watt et al. 2014). Over-nutrition or unhealthy eating styles are the primary driving forces of the metabolic diseases in humans. Diet-based animal models provide a source for fat accumulation and consequent damage to the liver that mimics the pathology of NAFLD (Anstee and Goldin 2006). Orotic acid (OA) can be used to induce fatty liver in a rat model (Wang et al. 2011b). Dietary admission of 1% of OA resulted in the development of fatty liver in rat model within 10 days, and the resulting fatty liver disease is expected to exhibit similar biochemical and molecular characteristics with the human NAFLD (Paola et al. 2013). Similarly, it was expected that dietary OA administration will result in the development of liver steatosis in mink within 5-10 days. Furthermore, whey protein-derived peptides can be antioxidative (Hernandez-Ledesma et al. 2005; Pena-Ramos and Xiong 2001) , and their bioactivity can be the basis of physiological effects in the prevention or treatment of OA-induced changes in the mink liver.

CHAPTER 2: LITERATURE REVIEW

2.1 Morphology and Functions of Liver

2.1.1 Liver Anatomy

Liver plays an important role in such vital tasks as molecule synthesis, detoxification, waste product disposal and general metabolism, which makes the liver a complex multifunctional organ. The main functions performed by the liver include:

(i) Vascular functions for storage and filtration of blood;

(ii) Metabolic functions concerned with the majority of metabolic systems in the body;

(iii) Secretory and excretory functions responsible for formation of bile flow through bile ducts into the gastrointestinal tract.

The liver performs functions that are essential to life. The liver lobule is formed by parenchymal and nonparenchymal cells. Hepatocytes and cholangiocytes are two major components of liver epithelia. Hepatocytes, parenchymal cells, form almost 80% of the total liver volume and 60% of the total number of liver cells. The typical hepatocyte forms a cubical cell of 15 μm sides. Mammalian hepatocytes are normally characterized by a polyploidy, and 30-40% of hepatocytes in human adults are polyploidy (tetrapolyploid) with dispersed chromatin and prominent nucleoli (Celton-Morizur and Desdouets 2010). In addition, hepatocytes contain thousands of enzymes essential to perform vital metabolic functions, e.g. maintenance of glucose, amino acids, ammonia and bicarbonate homeostasis, the synthesis of plasma proteins, the storage and processing of signal molecules, and bile salt excretion (Campbell 2006).

Non-parenchymal liver cells, which contribute to only 6.5% to the liver volume but 40% of total liver cells, are localized in the sinusoidal compartment of the tissue. Liver consist of a variety of cells called, cholangiocytes, Kupffer cells, hepatic stellate cells, and natural killer lymphocytes. Endothelial cells, fat-storing cells, and parenchymal cells are immobile cells (Malarkey et al. 2005).

Cholangiocytes are epithelial cells that line the intra- and extra-hepatic ducts of the biliary tree. The primary physiological function of cholangiocytes is the modification of bile of canalicular origin and drainage of bile from the liver (Tabibian et al. 2013). Cholangiocytes are also targets in several human diseases including primary biliary cirrhosis, IgG4 autoimmune cholangitis, and may lead to cholangiocarcinoma. Hepatic sinusoidal lining cells have a huge endocytic capacity for many ligands including glycoproteins, components of the extracellular matrix and immune complexes (Saile et al. 1999).They are also active in the secretion of cytokines, endothelin-1 and nitric oxide (Kmiec 2001).

Kupffer cells are resident macrophages, which adhere to the endothelial lining, and are preferentially located in the periportal sinusoids. Structurally, Kupffer cells mostly show an abundant clear cytoplasm which often contains lysosomes. They present as irregular cell coat, wormlike structures, and vacuoles. Hepatic macrophages secrete potent mediators of the inflammatory response, nitric oxide, carbon monoxide, tumor necrosis factor-alpha (TNF-α), and other cytokines, and thus control the early phase of liver inflammation, playing an important role in the innate immune defense. Apart from typical macrophage activities, Kupffer cells also play an important role in the clearance of senescent and damaged erythrocytes (Kmiec 2001).

2.1.2 Hepatic Functions and Lipid Metabolism

2.1.2.1 The Role of Fatty Acids in the Liver

Fatty acids play an important role as an energy source for the liver as well as a storage form (stored in adipose tissue as triglycerides) of fuel both within and outside the liver. In contrast to the glucose pathway, the oxidation of fatty acids to CO_2 and water has the highest ATP production and thus is the most efficient long-term storage form of energy. Synthesis and metabolism of fatty acids are regulated by various factors and the liver plays a central role in the regulation of the body's total fatty acid needs (Nguyen et al. 2008).

2.1.2.2 Oxidative Metabolism: Electron Transport Chain

The electron transport chain is located in the inner mitochondrial membrane and is composed of electron transport complexes (Vassiletz et al. 1967). The electron transport chain transports electrons from NADH or $FADH_2$ to molecular oxygen and produces a transmembranous proton gradient used for the generation of ATP and for other reactions. Therefore, a failure in the production of the transmembranous proton gradient impairs not only ATP synthesis, but also leads to a reduced uptake of substrates for mitochondrial metabolism. Most importantly, it may also inhibit the uptake and processing of newly synthesized proteins. An adequate mitochondrial function is very essential for the survival of a given cell and also for provision of functionally intact mitochondria to new cells.

2.1.2.3 Mitochondrial ß-oxidation

Beta-oxidation of fatty acids provides an essential fuel source for multiple organs, including the liver. In contrast to fatty acid synthesis, which occurs in the cytosol of the cell, the ß-oxidation of fatty acids takes place in the mitochondrion. Selective mitochondrial transport systems allow for shuttling of substrates required for mitochondrial

metabolism such as ß-oxidation. Long chain fatty acids cross the plasma membrane of target cells and are activated to acyl-CoAs in the cytoplasm by an ATP-utilizing acyl-CoA synthase. The acyl-CoAs, which cannot traverse the mitochondrial membranes without modification, are esterified to acyl-camitines by carnitine palmitoyl transferase I (CPT1) and transported across the inner mitochondrial membrane, in exchange for carnitine, by the carnitine translocase (Gusdon et al. 2014). The ß-oxidation cycle comprises a series of four enzyme-catalysed reactions, which cleave a two-carbon acetyl-CoA part at each complete cycle. Two oxidation steps release the reducing equivalents $FADH_2$ and NADH, which transfer their electrons to the electron transport chain. Acetyl-CoA, the product of ß-oxidation, can be transported out of the mitochondria by the citrate carrier and can then be used as a substrate for lipogenesis. It can be further oxidized to CO_2 by the reactions of the Krebs cycle in the mitochondrial matrix or can be converted into the ketone bodies acetoacetate or ß-hydroxybutyrate by the enzymes of the HMG-CoA cycle (Quant 1994).

2.1.2.4 Ketogenesis

Ketogenesis is a metabolic process that occurs exclusively in the mitochondrial compartment of certain cells in most species, and produces ketone bodies and ß-hydroxybutyrate, from fatty acids. Liver mitochondria are the main producers of ketone bodies, but kidney, small intestine and white adipose tissue mitochondria also synthesize ketone bodies in some metabolic states or during certain stages of development. Ketone bodies serve as oxidizable chemical fuels for the brain and peripheral tissues in some metabolic (prolonged starvation), nutritional (high-fat diet) and developmental (during suckling) states (Quant 1994). In the transition from the fed to the fasted condition, carbohydrate utilization and fatty acid synthesis in the liver cease and are replaced by the

oxidation of fatty acids and the induction of ketogenesis. The influence of fatty acids on ketogenesis depends on the metabolic state of the organism (fasted, lactating or diabetic states) (Hegardt 1999).

2.1.2.4.1 Regulation of Ketogenesis

Hepatic ketogenesis is controlled internally by the liver as well as externally by other systems. External controls may include those reactions involved in generating and releasing fatty acids from adipose tissue, regulation of blood flow, secretion of pancreatic hormones, and nervous inhibition. It is also controlled by those reactions producing ketone bodies in the kidney or intestine, and those in the brain and peripheral tissues that oxidize ketone bodies or use them as substrates for lipogenesis and further into gluconeogenesis (J D McGarry and Foster 1980).

Intrahepatic control reactions include activation of long-chain fatty acids by acyl-CoA synthase, transport of acyl-CoAs into mitochondria involving the CPT1/2 system and the carnitine/acylcarnitine translocase, the enzymes of ß-oxidation, the HMG-CoA pathway and/or the Krebs cycle and efflux of ketone bodies from the mitochondria via pyruvate exchange. The physiological rate of ketogenesis varies. However, it is elevated under conditions where the glucagon-to-insulin ratio is high: during high fat diet, during late pregnancy, during lactation, and during prolonged starvation when ketones provide an alternative fuel to spare glucose levels. Moreover, ketogenesis is also controlled within the mitochondria. Such internal controls include mainly two systems: entry of fatty acyl-CoA into mitochondria catalysed by the CPT system (Hoppel 2003); and enzymatic activity of mitochondrial HMG-CoA synthase (Hegardt 1999).

2.2 Non-Alcoholic Fatty Liver Disease

Fatty liver disease (FLD), also called non-alcoholic fatty liver disease (NAFLD), is characterized by fat accumulation and oxidation, which induces an inflammatory response in the human liver. It is strongly associated with metabolic syndrome and is the leading cause of chronic liver disease worldwide (Anstee and Day 2013). NAFLD is a clinico-pathological entity that integrates simple hepatic steatosis with varying stages of fibrosis, known as nonalcoholic steatohepatitis (NASH), and cirrhosis. NAFLD is characterized by simple steatosis and a relatively benign condition with little risk of progression. In contrast, NASH presents a distinct histological panel of concurrent lesions, such as lobular inflammation. In clinical settings, both NAFLD and NASH coexist with a cluster of obesity-related cardio-metabolic complications, including insulin resistance, type 2 diabetes (Tziomalos et al. 2012), dyslipidemia and atherosclerosis (Pacifico et al. 2008), and are considered as the hepatic manifestation of metabolic syndrome.

It has been shown that there is an increased relative risk for NALFD of 75.8% in obese persons, with a body-mass index (BMI) of least 30 kg/m^2 (Bellentani et al. 2000a). Truncal obesity is also an important risk factor for NAFLD, in patients with a normal BMI (Ruderman et al. 1998). The most common underlying risk factor for the development of NASH is the presence of insulin resistance. Furthermore, patients with a history of type 2 diabetes are at an increased risk to develop NASH (Leite et al. 2014; Stepanova et al. 2015).

In spite of the various pathophysiological conditions that might determine NAFLD – such as certain drugs (estrogens, glucocorticoids, methotrexate, tamoxifen, diltiazem, amiodarone), parenteral nutrition (Tillman 2013), gastrointestinal surgical interventions (Mummadi et al. 2008), and inflammatory intestinal disease (Canbay et al. 2006), the term

of NAFLD is currently reserved for its use within metabolic pathologies with undefined histological modifications (Wakai et al. 2011). Hypertriglyceridemia, but not hypercholesterolemia, is also an important risk factor for NAFLD (Perry et al. 2013). Ekstedt et al. (2006) showed that most patients with NAFLD develop diabetes or impaired glucose tolerance in the long term and progression of liver fibrosis is associated with more severe insulin resistance and weight gain.

Approximately, 25-37% of the US population has NAFLD and 2-5% has NASH. These estimates are based on the National Health and Nutritional Examination Survey (NHANES) conducted from 1976 to 2004 (study examined data from NHANES conducted at 5 time periods), where identical procedures for conducting anthropometry were used throughout NHANES surveys to ensure comparability of anthropometric measures over time (Skelton et al. 2009). The prevalence has been estimated to be at between one-fifth and one-third of the adult population, depending on the country, and diagnostic criteria used. Additionally, NAFLD affects not only adults, but its prevalence is also increasing in children: ~3% overall, ~50% in obese children, and its prevalence in morbidly obese subjects is ~37% (Ogden et al. 2006).

2.2.1 Pathophysiology of NAFLD

The mechanisms involved in the pathogenesis of NAFLD are not fully understood and are complex. However, due to NAFLD being common and associated with other common disorders such as metabolic syndrome, type 2 diabetes and obesity, considerable progress has been made in understanding the mechanisms involved. Alcoholic liver disease and NAFLD share similar pathogenesis; however, they are differentiated by the amount of alcohol consumed (Toshikuni et al. 2014).

In the past decade considerable progress has been made in understanding the pathogenesis involved in the initiation and progression of NAFLD. A "two-hit" theory suggested that the initiation of steatosis known as the "first-hit" and the progression to its more severe stages (NASH) known as the "second hit" are caused by different sets of molecular mechanisms and environmental factors (Day and James 1998). Day and James (1998) suggested that some mechanisms may be involved in the first hit as well as the second hit. However, with ongoing studies on NAFLD in recent years, the initiation and progression of NAFLD is thought to be multi-factorial with combined mechanisms and environmental factors participating in disease initiation and progression. Obesity, type 2 diabetes and components of the metabolic syndrome are common risk factors causing NAFLD and therefore, disease-related pathogenesis such as abnormal lipid metabolism, insulin resistance and oxidative stress are thought to play an integral role in the pathogenesis of NAFLD. A main mechanism addressed in this thesis is the involvement of oxidative stress; however, other mechanisms such as lipid metabolism and insulin resistance will be covered briefly.

2.2.2 Lipid Transportation and Lipid Accumulation in Liver

About 95% of the lipids in the human diet are triacylglycerols (TGs). Hydrolysis of dietary lipids in humans is catalyzed by lipases from gastric and pancreatic origins, with pancreatic colipase dependent lipase being functionally the most predominant enzyme under physiological conditions. The micelles are aggregates of amphiphilic lipid molecules that orient themselves with the hydrophobic regions on the inside of the micelles and the polar groups exposed to the aqueous environment. This macromolecular structure has a

high water solubility. The main lipoproteins responsible for transporting dietary fat from the intestine are the chylomicrons.

According to Westergaard and Dietschy, the brush border membrane of the enterocytes is separated from the bulk fluid phase in the intestinal lumen by an unstirred water layer, which is relatively impermeable to lipolytic products, especially the long-chain fatty acids. Bile salts remain in the lumen of the small intestine to facilitate further digestion (Westergaard and Dietschy 1976). It is known that the unsaturated and less hydrophobic structure of unsaturated fatty acids such as essential fatty acids relies less upon bile for solubilization in the intestine compared with saturated long chain fatty acids. As lipolysis of medium-chain triacylglycerols is more complete than that of long-chain triacylglycerols, the medium-chain fatty acids (unlike long-chain fatty acids) are absorbed mainly as free fatty acids, and only rarely as mono- and diacylglycerols (You et al. 2008).

After absorption into the enterocyte, dietary lipids migrate to the endoplasmic reticulum and are packaged into lipoprotein particles called chylomicrons. Fatty acids are predominantly reacylated into TGs inside the enterocyte and assembled into chylomicrons to be excreted and transported into the lymph (Dixon 2010). Before being transported into the lymph, TGs are assembled together with phospholipids and apolipoproteins to form a lipoprotein particle. Apolipoproteins provide a system of receptor identification that governs which cells in the body receive and metabolize these lipoproteins. Lipid is transferred to apolipoproteins during its translation by the actions of microsomal transfer protein. Hepatic apolipoprotein content in itself is also regulated, not by inducing changes in apolipoprotein mRNA levels, but by modulating its degradation (Dixon and Ginsberg 1992). High intake of dietary fat and at the peak of absorption, the chylomicrons tend to be

larger and contain more TGs than when the rate of lipid transport is lower. The chylomicrons are released into the bloodstream via the lymph system for delivery of triacylglycerols to the tissues.

Free fatty acids (FFA) are derived both from ingested meals, as well as generated from triglyceride hydrolysis. A third source of liver FFA are the triglycerides stored within the liver. Post-prandially, FFA are mainly stored in the liver as triglycerides, whereas during fasting, they are mainly oxidized (Day 2006). The exact pathogenesis of hepatic lipid accumulation seems to be very complex and only partially understood. Because of the intricacy of the mechanisms involved, it is difficult to identify synergetic effects of genetics and the environment (Attar and Van Thiel 2013; Bellentani et al. 2000b). Day and James (1998) proposed the "two-hit" hypothesis that suggested that the development of NASH required two hits, which were steatosis (the first hit) and oxidative stress (the second hit). Most recently, a "multi-hit" hypothesis was developed to describe a new theory for the pathogenesis of FLD (Tilg and Moschen 2010). Multiple parallel hits, such as adipose tissue derived factors, as well as endoplasmic reticulum (ER) stress, together with steatosis and oxidative stress, all promote hepatic inflammatory response play important roles in the pathogenesis and progression of FLD (Takaki et al. 2013).

Anstee et al. (2006) conducted research using a mouse model, and their findings indicated that intrahepatic lipid accumulation can occur through several mechanisms such as: increased delivery and uptake of long chain fatty acids into hepatocytes, due to excess dietary intake or release of lipids from adipose tissue stores; increased *de novo* hepatic LCFAs and TG synthesis, through activation of sterol regulatory element binding protein-1c (SREBP-1c) or carbohydrate-responsive element-binding protein (ChREBP), and;

failure of very low density lipoprotein (VLDL) and TG export from the liver or failure of elimination of excessive fatty acid, caused by impaired hepatic mitochondrial β-oxidation (Anstee and Goldin 2006). A fatty liver is one in which there is an accumulation of more than 5-10% lipid content (Reddy and Rao 2006).

2.2.3 Liver Inflammation

Inflammation in the liver, with concurrent lipid accumulation, can lead to an advanced form of FLD. It is characterized by hepatic steatosis, hepatocellular injury and lobular inflammation (Schwimmer et al. 2005). Inflammatory cells within the lobules, including lymphocytes, eosinophils, occasionally polymorph nuclear leukocytes, and activated Kupffer cells, are the common features of steatohepatitis (Tiniakos et al. 2010). Inflammatory mediators, such as inflammatory cytokines, have been implicated in the pathogenesis of FLD. The elevated production of inflammatory cytokines, such as interleukin-6 (IL-6) or tumor necrosis factor–alpha (TNF-α), together with decreased secretion of adiponectin or resistin (anti-inflammatory factors), can lead to the recruitment of inflammatory cells and initiation of inflammatory response in the liver (Baranova et al. 2006).

2.2.4 Oxidative Stress in NAFLD

Oxidative stress plays an important role in the transition of benign hepatic steatosis to steatohepatitis. Oxidative stress is defined as an imbalance of free radical (reactive oxygen species and reactive nitrogen species) production and the ability of antioxidants to detoxify the reactive intermediates in the body. Under physiological conditions, β-oxidation of fatty acids in the liver takes place in the mitochondria (Reddy and Rao 2006). During FLD, the elevation of free fatty acids in the liver results in a surge of reactive

oxygen species (ROS) production. Consequently, ROS cause oxidative stress and activates a series of inflammatory pathways, leading to mitochondrial dysfunction (Evans et al. 2003). It has been found that hepatic mitochondrial dysfunction in patients and in experimental animals is associated with NASH (Sanyal 2005). The above process can be involved in the excessive production of ROS. Increased amounts of ROS can also trigger alterations in the production of cytokines, lipid peroxidation of cell membranes, as well as insulin resistance (Sanyal et al. 2001).

Antioxidants are considered stable molecules that are able to donate an electron to free radical, thereby scavenging and stopping the free radical from causing further damage. Antioxidants are found naturally in a variety of whole foods, specifically in fruits and vegetables; for instance, the most common vitamins for scavenging free radicals are vitamin E (alpha-tocopherol), vitamin C (ascorbic acid), and beta-carotene. These vitamins are not produced by the body and must be supplied through the diet (Lobo et al. 2010). All hepatic lipids originate from dietary intakes of carbohydrates or lipids (Grundy and Denke 1990). Furthermore, dietary contents have been shown to be powerful inducers and suppressors of the pathways central to hepatic fatty acid metabolism. Dietary trends in NAFLD patients appear to involve increased intakes of simple sugars, and sugar-sweetened drinks, as well as reduced intakes of polyunsaturated fatty acids (PUFA), in particular n-3 PUFAs (Di Minno et al. 2012).

2.2.5 Reactive Oxygen Species

ROS are produced from oxygen during normal cell function, and they include superoxide anions ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($^{\bullet}OH$). ROS in the liver can be generated by the mitochondria, peroxisomes, microsomes, reduced nicotinamide

adenine dinucleotide oxidase, cyclooxygenase, lipoxygenase, and xanthine oxidase (Koek et al. 2011). Mitochondria are the main ROS generating organelle (Poyton et al. 2009). Excessive production of ROS can lead to oxidative damage of macromolecules (DNA, lipids and proteins) in the cells. In animal models, increased production of ROS has been shown to be linked to liver diseases including NAFLD (Wei et al. 2008).

2.2.7 Stages of Fatty Liver Disease

In terms of potential for progression, NAFLD patients could be categorized into NASH and non-NASH. Approximately one third of NAFLD patients develop NASH, which is usually defined as steatosis with inflammation, cellular ballooning and fibrosis (Jou et al. 2008; Lackner 2011). Non-NASH fatty liver is characterized by simple-steatosis or steatosis with minimal inflammation. The association of multiple comorbidities in NAFLD population is to be expected, since NAFLD is closely associated with insulin resistance and metabolic syndrome (Gastaldelli et al. 2009; Schwimmer et al. 2003).

In order to progress from fatty liver to NASH, there needs to be a secondary trigger that increases inflammation. Fibrosis begins to develop during the NASH phase of NAFLD (Alkhouri and McCullough 2012). Fibrosis is the buildup of collagen in the interstitial space as a defense mechanism against further cell damage, this interferes with normal cell function by making it more difficult to transport molecules from the cell to the blood and vice versa. Fibrosis develops into cirrhosis when not treated. Cirrhosis disturbs normal function of the liver by blocking blood from entering the liver, inhibiting normal filtration and metabolic functions (Kravetz et al. 1989).

2.3 Fatty Liver Disease in Mink

Nursing sickness was reported to cause up to 14% total mortality among 1774 lactating female mink in Denmark (Clausen et al. 1992). In a sample of 48 farms in Ontario that experienced mink mortality rates of 2-10% during the lactation period, nursing sickness was diagnosed in 56% of the mortalities (Schneider and Hunter 1993). The incidence of nursing sickness varies among farms, but may reach as high as 15% (Rouvinen-Watt 2003a). The development of fatty liver disease may occur occasionally and is associated with a genetic tendency for the development of nursing sickness (Rouvinen-Watt 2003a). Bjornvad et al. (2004) found that short-term fasting of mink resulted in accumulation of intra-hepatic lipid vacuoles, resulting in liver weight remaining the same, while body weight decreased. Rouvinen-Watt et al. (2010) reported that fasting mink for 0-7 days resulted in an increase in intra-hepatic TGs, an increase in liver fat percent, and a decrease in n-3 PUFA, while re-feeding reversed these changes. Persson et al. (2013) found that seasonal body weight fluctuations in wild male mink correlated with an increase in liver weight, which was associated with an increase in liver fat. Fatty liver disease not only affects mink, but also other species. Another Mustelid, the European polecat, has been documented to possess a similar fatty acid (FA) profile to that of humans when hepatic lipidosis is induced through food deprivation (Nieminen et al. 2006b).

Mink nursing sickness is one of the largest causes of morbidity, and 8% of premature death in adult mink females annually on most Canadian mink farms (Clausen et al. 1992; Rouvinen-Watt 2003a). Fatty liver is often a consequence of rapid body weight loss due to lack of appetite or restricted feeding managements (Rouvinen-Watt et al. 2012; Rouvinen-Watt et al. 2010). Fatty liver disease has been induced in mink (Mustonen et al. 2005b;

Nieminen et al. 2006a; Rouvinen-Watt et al. 2010) and is believed to occur through the same mechanism as nursing sickness, through mobilization of body fat reserves to meet energy demand (Nieminen et al. 2009). The signs of mink nursing sickness are similar to those seen in cats with idiopathic feline hepatic lipidosis. These include weight loss, depression, anorexia, and dehydration (Wamberg et al. 1992). Upon necropsy, the livers of animals suffering from fatty liver appear greasy in texture, yellow in color, and enlarged (Mustonen et al. 2005c). However, liver weight increases over the same time period (Mustonen et al. 2005c), indicating lipid accumulation in the liver as a result of mobilization of body fat reserves. Clinical signs of the disease include anemia, hypo-proteinaemia, azotemia, hypocalcaemia, hyper-bilirubinaemia, and high liver enzyme activities.

Nursing sickness can cause high mortality in females with large litters and becomes visible after one month of lactation (Rouvinen-Watt 2003b). Affected females are usually observed in late June, but symptoms may appear after the kits have been taken away. Sick mothers have significantly larger litters than healthy mothers. However, age, litter size, and female weight loss were all major determinants of the risk of nursing sickness. In the last 2 weeks of lactation, healthy females lost about 14% of their body weight, whereas sick females lost about 31% (Clausen et al. 1992). Sick females exhibited signs of advanced dehydration, emaciation and other indicators of progressive catabolism (Clausen et al. 1992). In the advanced stage of the disease, coma and death appear to be the inevitable outcome, because of the strain of continuing milk production (Wamberg et al. 1992).

2.4 Fatty Liver Disease in Domestic and Farm Animals

Fatty liver disease is also commonly observed in different species of domestic animals including cattle (Gerloff 2000), cats (Armstrong and Blanchard 2009), dogs (Xenoulis and Steiner 2010), mink (Rouvinen-Watt et al. 2012), poultry (Bannister et al. 1985), and sheep (Helman et al. 1995). Hepatic lipidosis is the most common liver disease in domestic cats (Hall et al. 1997; Mazaki-Tovi et al. 2013) and the cause remains unknown, but involvement of protein and lipid metabolism pathways could be a possibility.

Symptoms in high producing dairy cattle in early lactation may include retained placenta, mastitis, uterine infection, displaced abomasum and metritis (Stengarde et al. 2011). Ruminant species with high milk production are more susceptible to develop fatty livers (Pullen et al. 1990). Clinical and subclinical problems occur in obese dairy cattle within 3 weeks of calving and potentially compromise health, production and reproduction in cattle (Jorritsma et al. 2000). Dairy cattle are fed high-energy diets to meet the energy demand for high production. Regardless of the energy density and protein content of the peripartal diets, feed intake decreases by about 30% during the 3 weeks before parturition and about 90% of that during the last prepartal week (Doepel et al. 2002; Hayirli et al. 2002). Several factors may be causing the peripartal DMI decrease. The increasing size of the gravid uterus can physically limit the rumen size, thereby causing decreased feed intake. Moreover, since the diet fed during the dry period is usually high in roughage, the rumen is not adapted to the high carbohydrate diets fed peripartum and consequently indigestion or ruminal acidosis may result that could limit feed intake. The endogenous β-endorphins and enkephalins that are secreted by the pituitary gland and the hypothalamus to soothe the parturition discomfort may further exacerbate the decrease of decreasing the gut motility

(Grummer 1993). The clinical symptoms may relate to associated production diseases that may aggravate the fatty liver situation (Ingvartsen and Andersen 2000), depression, lack of appetite, weight loss, acidosis, hypocalcemia and may affect the nervous system, with symptoms such as somnolence, coma, and recumbence (Bobe et al. 2004).

Several breeds of animals have been used in a variety of studies to provide a clarification of the difference in metabolic rates and in adipose tissue size. Variations in fat distribution have been observed among several breeds of sheep and goats (Kempster 1981). They are likely related to genetic differences in the metabolic regulators, resulting in different lipogenic activity (Belk et al. 1993). Meat-type breeds and dairy breeds have different genetic background, which changes the nutrient utilization and turnover (Pfuhl et al. 2007). However, both subcutaneous and perirenal adipose tissue from beef cattle had higher rate of fatty acid synthesis than the same type of adipose tissue from dairy cattle (Vernon 2005).

2.4 Orotic Acid

OA (1,2,3,6-tetrahydro-2,6-di-oxo-4-pyrimidine carboxylic acid) is an organic compound synthesized in the body *via* pyrimidine nucleotide biosynthesis (Smith and Baker 1959). It is normally found in the milk of many species and most significant amounts are found in cow's milk. As an intermediate in the pyrimidine pathway, OA plays a critical role in the synthesis of pyrimidine nucleotides and their associated involvement in cellular growth, function, regulation and other various physiological processes (Brosnan and Brosnan 2007). OA has been connected with increasing lipid concentration in liver by enhancing the expression SREBP-1, which has a target gene involved in fatty acid biosynthesis (Wang et al., 2011). OA has also been shown to inhibit the phosphorylation of adenosine monophosphate-activated protein kinase (AMPK) (Jung et al. 2011a). This inhibition

amplifies the expression of sterol regulatory element-binding proteins-1 (SREBP-1), which stimulates fatty acid biosynthesis. SREBP regulates the expression of genes involved in synthesis and uptake of fatty acids and triglycerides. SREBP-1c is a major gene in insulin-mediated homeostasis in the liver, where it acts as a transcriptional regulator of lipogenic genes. OA has been shown to cause a decrease in the hepatic CPT1 activity and expression, which is critical for fatty acid β-oxidation (Wang et al. 2011b). This inhibition of CPT1 has been shown to increase hepatic triglyceride stores (Rasmussen et al. 2002).

2.4.1 Urea Cycle and Orotic Acid Biosynthesis

The urinary excretion of OA, an intermediate in the pyrimidine biosynthesis pathway. Any inborn errors in the urea cycle and transportation of molecules in the mitochondrial cytoplasm for urea synthesis can result in an overproduction of OA and uridine nucleotides (Brusilow and Maestri 1996). Genetic and metabolic disorders may pose an increased level of OA (Becroft et al. 1969). Increased excretion of OA has been reported in children with hyper-ammonemia due to ornithine transcarbamoylase deficiency (Maestri et al. 1996; Schuchmann et al. 1980); as well as in animal models (Matsuda et al. 1971).

Diets lacking in ornithine, citrulline or arginine have also been shown to result in the elevated excretion of OA. Orotic acid has been shown to be metabolically converted to uridine nucleotides (Bennett and Allan 1976); and exposure to OA results in increase of hepatic uridine nucleotides (Marchetti and Puddu 1964). Several biochemical studies indicated that OA needs to be metabolized to uridine nucleotides to utilize its biological and biochemical effects (Le et al. 2013). These studies suggest that creation of an imbalance in nucleotide pools is critical for OA to exert its adverse biological effects.

2.5 Whey Protein

Consumers are becoming aware of the nutritional and physiological advantages of whey proteins. During cheese production, globular whey proteins are not coagulated by acid and are resistant to the action of rennet enzyme. Whey protein is a high quality, easily digestible protein, as it has a protein efficiency ratio, and typically a mixture of beta-lactoglobulin (65%), alpha-lactalbumin (25%), bovine serum albumin (8%), and immunoglobulins (Haug et al. 2007). Additionally, whey protein is a rich source of sulfur-containing amino acids, such as methionine and cysteine (Alexander et al. 1980). These amino acids are important in maintaining antioxidant levels and enhancing immune function (immunomodulatory properties) in the body, and can be used to produce glutathione, an intracellular antioxidant that protects the body against free radical damage (Grimble 2006; Li et al. 2007). Whey protein and peptides are especially beneficial for promoting muscle anabolism (Tipton et al. 2004), weight management (Frestedt et al. 2008), satiety (Luhovyy et al. 2007), and other physiological benefits (McGregor and Poppitt 2013).

2.5.1 Bioactive Peptides and Physiological Effects

Whey protein possesses a number of physiological benefits due to embedded bioactive peptide sequences. The bioactive peptide sequences, also known as cryptides, are active only upon the release from the polypeptide chain of the intact protein (Udenigwe 2014; Udenigwe and Aluko 2012). Release of bioactive peptides often occurs upon *in vivo* or *in vitro* hydrolysis of the parent protein. Reported biological activities of whey peptides include gastrointestinal functions, anticarcinogenic activity, antimicrobial activity, growth promoting activity, immunoactivity, and antihypertensive activity (Meisel et al., 1989; Walzem, 1999; Shah, 2000; Ha and Zemel, 2003; Smithers, 2008).

Antihypertensive activity is one of the most studied properties of whey-derived bioactive peptides as hypertension is a prominent health issue in developed countries. Roughly 1 in 3 adults in the U.S. has hypertension, and less than half of those with hypertension have it under control (CDC, 2012). Hypertension can cause damage to blood vessels and heart, increasing risk for coronary heart disease and stroke, which are two of the leading causes of death for Americans (Kochanek et al., 2011). Overall, treatment of hypertension costs Americans over $47.5 billion each year (Heidenreich et al., 2011). This makes bioactive peptides with antihypertensive (and other bioactive) properties all the more important to the food and health industry.

2.5.2 Whey Protein Hydrolysate (WPH)

Whey protein hydrolysate (WPH) is produced by enzymatic or chemical hydrolysis to cleave peptide bonds and generate smaller peptide fractions and free amino acids, resulting in whey protein with improved functionality, digestibility and enhanced bioactivity, among other properties (Foegeding et al. 2002; Sinha et al. 2007). However, enzymatic hydrolysis is preferred over chemical hydrolysis to achieve specific cleavage, to avoid the loss of essential amino acids such as tryptophan, and to avoid the formation of toxic substances such as lysino-alanine. There are a wide variety of enzymes that can be used to hydrolyze whey proteins, including digestive enzymes trypsin, pepsin and chymotrypsin, as well as plant-derived enzymes such as papain, and bacterial and fungal enzymes, such as subtilisin (Klompong et al. 2007). Apart from the type and specificity of the enzyme used, the degree of hydrolysis, the percentage of peptides bonds cleaved by an enzyme, depends on multiple factors, such as pH, temperature and the nature of the whey protein.

Hydrolysis of proteins has often been used to enhance the solubility, heat stability, viscosity and emulsifying and foaming properties of the protein. Whey protein solubility can be improved by 10-20% over a wide range of pH. Hydrolysis decreases molecular size and changes the conformation and strength of inter- and intra-molecular interactions within the protein (Frestedt et al. 2008). The loss of secondary structure prevents heat induced structural changes, thus increasing solubility and thermal stability of the protein over a wider temperature and pH range. Furthermore, the loss of secondary structure allows for even distribution of hydrophobic and hydrophilic regions in the protein, increasing gelation, foaming and emulsification properties. Studies have also shown that the amino acids and peptides in WPH are absorbed into the bloodstream, enhancing the recovery process of athletes (Buckley et al. 2010). Athletes recovered their full peak power and eliminated muscle soreness after just six hours of consuming WPH, compared to several days when intact whey protein was consumed (Buckley et al. 2010). Another benefit of WPH is the presence of bioactive peptides that are released during enzymatic hydrolysis. As a result, WPH has promising health benefits attributed to several bioactivities such as antihypertensive (Saito 2008), antioxidant (Pena-Ramos and Xiong 2001), antibacterial, anticancer (Gill and Cross 2000), and hypolipidaemic properties (Howard and Udenigwe 2013; Udenigwe and Rouvinen-Watt 2015).

2.6 L-Arginine

Arginine can be found in considerably high amounts in sea food, nuts, seeds, algae, meat, and soy protein isolate (Wells et al. 2005). There are two isomers of arginine, namely the D and L isomers. L-arginine is the isomeric form that is naturally available and considered the active form in the human body. Arginine was first discovered by Schultze in 1886 and

is considered to be a nutritionally semi-essential amino acid (Cylwik et al. 2005). Essential amino acids must be supplied in the diet as the body cannot synthesize adequate amounts to support growth in children or to maintain regular physiological functions in adults.

Arginine is an endogenous amino acid that can be synthesized by most mammals, except for cats and ferrets, but is not produced at sufficient rates to support the growth of children. Arginine is involved in many physiological and nutritional processes such as the transport, storage and excretion of nitrogen, as an intermediate in the elimination of urea, in the ammonia detoxification and the formation of creatinine, and as a structural amino acid in proteins. Most importantly, arginine is the precursor to the endothelial-derived vasodilator, NO (Creager et al. 1992).

2.6.1 Metabolism of L-Arginine

Arginine (Arg) synthesis can occur in a variety of sites such as the intestines, kidneys and the liver, but the majority of synthesis, in adults, occurs through an intestinal-renal axis (Dhanakoti et al. 1990; Wakabayashi et al. 1991). The kidneys are responsible for approximately 60% of arginine synthesis. Enterocytes of the small intestine synthesize citrulline from glutamate, glutamine and proline. In infants, synthesized citrulline is converted to arginine locally within the enterocytes. However, in adults, citrulline synthesized by the enterocytes, is transported to the kidneys and then converted to arginine (Wu and Morris 1998). In the proximal tubules of the kidney, arginosuccinate synthase and arginosuccinate lyase are responsible for the conversion of citrulline to arginine. The highest rates of arginine synthesis do occur within the hepatic urea cycle, a cycle that needs to be continuously replenished in order to sustain further urea synthesis (Wu and Morris, 1998). However, net production of arginine from the liver is small compared with the inter-

organ pathway. This is due to high arginase activity and the constringent channeling of intermediates within the urea cycle (Huynh and Chin-Dusting 2006).

Orotic acid synthesis is abnormally high with hereditary deficiencies of urea-cycle enzymes or and also elevated during feeding of diets high in protein or deficient in arginine. Impaired endogenous arginine synthesis resulted deficiencies in arginosuccinate synthase or arginosuccinate lyase and induce orotic aciduria (Choi et al. 2015). Paddon-Jones et al. (2004) reported that arginine decrease the availability of long-chain fatty acyl-CoA for triglyceride synthesis and of acetyl-CoA for fatty acid synthesis result in an overall increase in the oxidation of both glucose and fatty acids, while decreasing fat deposition. Several researchers have reported that an arginine free diet leads to orotic acidemia. Prior and Gross (1995) demonstrated that, orotic acidemia developed in piglets fed an arginine-free diet and dietary ornithine can partially correct the arginine deficiency. Similarly, Milner et al. (1974) growing rats fed an arginine deficient diet had growth retardation, orotic aciduria and fatty liver. Urinary orotic acid has been used as an indicator of arginine deficiency and non-invasive indicator of liver health in mink (Damgaard 1998). Lanteri et al. (2006) and Tousoulis et al. (2002) have also confirmed that oral arginine has a protective function against reactive oxygen species, thus decreases intensity of obesity related changes in heart, pancreas and liver.

Orotic acid has recently been used to induce fatty liver condition in rat model, and dietary administration of OA has resulted in the development of severe hepatic lipidosis in the course of 10 days as documented by increase hepatic expression of lipogenesis, impaired mitochondrial β-oxidation, and reduced assembly and secretion of VLDL particles from the liver (Wang et al. 2011b). In our study, we anticipate that dietary administration of OA

would result in the development of liver steatosis in mink within 5 to 10 days, similar to findings reported by Wang et al. (2011b), and that the resulting fatty liver disease would exhibit similar biochemical and molecular characteristics with the human NAFLD (Adams et al. 2005; Dowman et al. 2010). Orotic acid accumulates during arginine deficiency due to inability of carbomoyl phosphate to enter the urea cycle in the absence of ornithine, which is derived from arginine. In mink, increased accumulation of OA as a result of arginine deficiency during prolonged food deprivation (Brosnan and Brosnan 2007; Mustonen et al. 2005a) and/or activity of MTTP reduce the liver's ability to cope with the elevated supply of TG. It is likely that the catabolic state during fasting result in a loss of amino acids essential for liver function, such as arginine, which can predispose animals to accelerated hepatic fatty infiltration. Huang et al. (2009) reported that 2% of L-arginine supplementation reduced the oxidative damage to and inflammatory response in the liver of young rats. Whey protein has exhibited antioxidant activities, which are likely due to its high content of free sulfhydryl group of cysteine residues. Hamad et al. (2011) reported that oral administration of whey protein hydrolysates (WPH) reduced the final body weight of rats and activities of liver enzymes, ALT and AST. These results indicate a possible role of oral administration of WPH in the regulation of liver biochemistry in rat model of NAFLD.

2.7 Research Objectives

2.7.1 Overall Objectives

The overall objectives of this research are to develop a new experimental animal model of OA-induced fatty liver and to study the effects of whey peptides for prevention and treatment of the resulting hepatic manifestation of metabolic syndrome associated with obesity and diabetes.

2.7.2 Specific Objectives

1. To identify and characterize OA-induced fatty liver in mink;

2. To identify the effectiveness of whey peptides for the prevention and treatment of OA-induced fatty liver in mink;

The project outcomes are expected to help the mink industry to address and lower mortality due to hepatic lipidosis and related nursing sickness. The mink model can also be used for translational research in human medicine to test potential therapies for fatty liver prevention and treatment.

CHAPTER 3: MATERIAL and METHODS

3.1 Hydrolysis of Isolated Proteins

Whey protein isolate was purchased from the Bulk Barn Food Limited (Truro, NS, Canada). As stated by the supplier, the isolate contained 90% protein, 0% fat and 0.1% sugars. The whey protein powder was suspended in deionized water at 5% (w/v), and then hydrolyzed with Flavourzyme (Protease from *Aspergillus oryzae*) at enzyme-to-substrate ratio of 1:100 (w/w). The reaction was conducted in a beaker for 5 h at the optimum temperature of 37°C and pH 7.0 respectively. The hydrolysis was terminated by adding 0.5 M HCl to decrease the pH to 4.0 and inactivate the protease. The reaction mixture was cooled to room temperature followed by centrifugation at 15,000 × g for 15 minutes to remove all insoluble matter, and freeze drying to obtain the WPH powder for use in the animal feeding experiment.

3.2 Animals and Experimental Treatments

A total of 60 female mink were used in two animal trials. All the mink that have not been pregnant were 8-10 months old and black colour, and were donated by Willowdale Farms Inc. (Berwick, NS). At the time of delivery, the mink were in good health and kept 5 days minimum before commencing the experiment diets to adapt new environment. The mink were individually housed in standard size cages (38 × 20 cm) at the Canadian Centre for Fur Animal Research located at the Dalhousie University, Faculty of Agriculture. The cages for each treatment were kept together and clusters of different treatments were kept apart to avoid cross contact of feeds. All procedures and husbandry practices were in accordance with the Canadian Council of Animal Care (CCAC 1993), and were approved by the Animal Care and Use Committee of the Dalhousie University, Faculty of

Agriculture. For both experiments, experimental diets were fed for the assigned duration and the mink were monitored for health status twice daily. The experimental diets were administered with three supplements: OA monohydrate (Sigma, Aldrich, Cat: 08402-500G), L-arginine (Sigma, Aldrich, Cat: 776408) and WPH (peptide).

3.2.1 Experiment 1: Pilot Study, Development and Characterization of OA-induced Fatty Liver Disease in Mink

The primary objective of the 10-day pilot study was to establish and identify the best period of time for the development and progression of fatty liver in mink. This was to ensure that no acute toxicity would result from the planned exposure of 1% OA in a 5 days or 10-day period. The pilot study was conducted from 28th November to 5th December, 2014. Three treatment groups of female mink (total of 15 female mink with n=5 in each treatment group) were used to induce fatty liver disease. The animals were fed with the experimental diet as follows: (1) Control (CTRL), fed regular mink diet for 10 days; (2) Orotic Acid 5 (OA5), fed a mink diet containing 1% of orotic acid for 5 days; and (3) Orotic Acid 10 (OA10), fed a mink diet containing 1% of orotic acid for 10 days.

3.2.2 Experiment 2: Testing of Whey Protein Hydrolysate and Arginine for Their Effectiveness in the Prevention and Treatment of OA-induced Fatty Liver Disease in Mink

Nine treatment groups of female mink (total number of 45 female mink with n=5 in each treatment group) were used for testing of the Arg and WPH for their effectiveness in treatment and prevention of OA-induced fatty liver in mink. The experiment was conducted from 6th February to 27th February, 2015. Five female mink were used as a baseline control (0 day) group and were anaesthetized at 0 day. The experimental diets were fed to the mink as follows: (1) Baseline control (CTRL0), 0 day; (2) Control (CTRL), fed regular mink diet

for 10 days; (3) OA, fed a diet containing 1% orotic acid for 10 days; (4) OA+Arg, fed a diet containing 1% orotic acid supplemented with 2% L-arginine, for 10 days; (5) OA+WPH, fed a diet containing 1% orotic acid supplemented with 5% Whey Protein Hydrolysates, for 10 days; (6) OA+Arg+WPH, fed a diet containing 1% orotic acid supplemented with 2% L-arginine and 5% Whey Protein Hydrolysates, for 10 days; (7) OA-CTRL+Arg, fed a diet containing 1% orotic acid for 10 days followed by control diet supplemented with 2% L-arginine for 10 days; (8) OA-CTRL+WPH, fed a diet containing 1% orotic acid for 10 days followed by control diet supplemented with 5% Whey Protein Hydrolysates for 10 days; (9) OA-CTRL+Arg+WPH, fed a diet containing 1% orotic acid for 10 days followed by control diet supplemented with 2% L-arginine and 5% Whey Protein Hydrolysates for 10 days. The nutrient compositions of the treatment diets are shown in Table 3.1. The recommended dietary allowance guidelines were used for sex and month of the year (Rouvinen-Watt et al. 2005), and further adjusted for the individual metabolic body weight of the mink. The mink were fed once a day for the study period. After being fed the diet regimen, each treatment group were selected to undergo an overnight fast of 14-16 hours prior to sampling.

3.3 Tissue Sample Collection and Analysis

The mink were anesthetized using an intra-muscular injection of xylazine (3.4 mg/kg BW) and ketamine hydrochloride (8.5 mg/kg BW) for blood sampling. Blood samples were collected into 4 mL EDTA Vacutainer® tubes using cardiac puncture. One set of tubes from the mink were used for haematological analysis. The other set was spun for 15 minutes at $1000 \times g$ at 4°C, and the isolated plasma samples were stored at -80°C for analysis. Immediately following the cardiac puncture, the mink were euthanized with an

intracardiac injection of pentobarbital (106 mg/kg BW). To determine Body Mass Index (BMI), the length of the mink were measured on a measuring board with a V-shaped slot from the base of the tail to the tip of the nose. BMI was calculated as: body weight (g)/body length^2 (cm^2). Hepato-somatic index (HSI) was calculated as follows: (liver weight, (g)/body weight, (g)) × 100. The livers were collected from the mink and weighed. A slice of the left median lobe from each liver was taken and preserved in 10% buffered formalin solution and the remaining liver flash frozen in liquid nitrogen and stored at -80°C. Flash frozen liver samples were later used for lipid extraction using the Folch method (Folch et al. 1957).

Table 3. 1: Nutrient compositions of experimental diets

Treatment	Dry Matter (%)	Crude Protein (%)		Crude Fat (%)		Ash (%)	
		As Fed	Dry	As Fed	Dry	As Fed	Dry
CTRL0/CTRL	40.34	12.85	31.86	10.38	25.73	3.03	7.52
CTRL+OA	40.93	13.97	34.12	9.77	23.86	3.14	7.68
OA+Arg	41.53	15.22	36.65	10.37	24.97	2.63	6.34
OA+WPH	41.96	14.87	35.45	10.19	24.28	2.97	7.08
OA+Arg+WPH	42.19	16.53	39.17	9.97	23.63	2.78	6.59
OA-CTRL+Arg	40.93	14.30	34.94	9.91	24.21	3.00	7.33
OA-CTRL+WPH	41.15	14.14	34.35	9.92	24.11	3.02	7.35
OA-CTRL+Arg+WPH	41.81	15.40	36.83	10.04	24.02	3.01	7.19

3.4 Blood Analysis

Haematological analyses were performed on whole blood at Fundy Veterinary Ltd. (Murray Siding, N.S., Canada). The SCIL Vet abc Animal Blood Counter was used to perform red blood cell count, white blood cell count, haemoglobin, haematocrit, platelet count, mean corpuscular volume, mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration, red cell distribution width, mean platelet volume, lymphocyte count, monocyte count, and granulocyte count. Clinical chemistry was performed on previously frozen plasma (stored at -80°C) at the Atlantic Veterinary College (Charlottetown, P.E.I., Canada) for urea, glucose, alanine aminotransferase, alkaline phosphatase, total protein, albumin, globulin, and albumin-to-globulin ratio.

3.5 Histological Analysis

The preserved liver samples were processed for histological evaluation and were stained with Hematoxylin and Eosin stain at the histology laboratory of the Veterinary Services of the Nova Scotia Department of Agriculture and Fisheries, Quality Evaluation Branch (Truro, NS). NASH Activity Index (NAI) was used to determine the presence and severity (the grade) of NAFLD. The four variables measured by NAI are steatosis, hepatocyte ballooning, lobular inflammation, and portal inflammation (Table 3.2). NAI gives an indication of hepatocyte damage and presence of inflammation. This scoring system also analyzes NASH in the same manner as the Brunt et al. (1999) method. The presence of fibrosis indicates risk of disease progression from simple fat accumulation to more severe and potentially irreversible liver pathology, which can progress to cirrhosis and then liver necrosis. This scoring method, developed by Merat et al. (2010), assigns values for the

grade and stage of disease progression and allows for quantitative analysis of liver

inflammation in mink (Rouvinen-Watt et al. 2014).

Table 3. 2: NAI scoring system as proposed by Merat et al. (2010) used to evaluate mink liver

Variable	Score	Description
1. Steatosis	0	None
	1	Up to 33% of acini, mainly macrovesicular
	2	34-66% of acini, commonly mixed steatosis
	3	Over 66% of acini, commonly mixed steatosis
2. Hepatocyte ballooning	0	None
	1	Occasional in zone III
	2	Obvious in zone III
	3	Marked, predominantly in zone III
3. Lobular inflammation	0	None
	1	Scattered neutrophils, occasional mononuclear cells, 1 or 2 foci per 25 × objective
	2	Neutrophils associated with ballooned hepatocyte, mild chronic inflammation, 3 or 4 foci per 25 × objective
	3	Acute and chronic inflammation, neutrophils may concentrate in zone III, over 4 foci per 25 × objective
4. Portal inflammation	0	None
	1	Mild, some portal areas
	2	Mild to moderate, most portal areas
	3	Moderate to severe, most portal areas
5. Stage	0	No fibrosis
	1	Zone III peri-venular peri-sinusoidal fibrosis
	2	Stage I changes and peri-portal fibrosis
	3	Bridging fribosis
	4	Cirrhosis

3.6 Molecular Biology Assays

3.6.1 RNA Extraction and Purification

Total RNA content from the liver tissue were extracted using the RNeasyMini Kit with QIAshredder homogenisers following the manufacturer's protocol (QIAgen, Valencia, CA, USA). Liver samples were removed from storage and placed in liquid nitrogen, and 20-30 mg of tissue was separated. Buffer RLT with 2-mercaptoethonol was added to the tissue to prevent RNA degradation. A micro grinder (Kimble, Vineland, NJ, USA) was used to mechanically homogenize the liver tissue. The high molecular weight cellular components were then homogenized with a QIAshredder (QIAgen, Valencia, CA, USA). RNA was then isolated from the homogenized lysate using the kit. RNA quality and quantity were assessed as the ratio of absorbance at 260/230 nm (salt and ethanol contamination) and 260/280 nm (protein contamination) using a spectrophotometer. RNA quality was also assessed by 1% agarose gel electrophoresis based on intact 28S and 18S ribosomal RNA bands.

3.6.2 Gene Sequencing and Quantitative PCR (qPCR)

In order to take mink specific sequences, published mink sequence data is available for GRP78 (Rouvinen-Watt et al. 2014), and sequences are available in GenBank databases for G6PC, HMGCS2, CPT1A, PEPCK and MTTP. Three reference genes (GAPDH, RP2 and HPRT1) were used in data normalization. Quantification of the mRNA was carried out by qPCR according to our previously described method (Rouvinen-Watt et al. 2014).

3.6.3 Complementary Deoxyribonucleic Acid Synthesis

RNA was reverse transcribed using a High Capacity (cDNA) Reverse Transcription Kit (ABI, Carlsbad, CA, USA) including a MultiScribeTM Reverse Transcriptase according

to the manufacturer's protocol. A 2 × Mastermix was prepared with an equal volume of 1.0 μg RNA in 20 μL aliquots and was pipetted into 8-well strips. The 8-well strips were run in the Dyad® Peltier Thermal Cycler (Bio-Rad, Mississauga, ON, Canada) for 10 minutes at 25°C, 120 minutes at 37°C, 5 seconds at 85°C, and held at 10°C until termination of the program. cDNAs were aliquoted in 0.1× and 0.01× dilutions using 10 mM Tris-HCl buffer (pH 7.4) and then stored at -30°C until further analysis.

3.6.4 Quantitative Real Time Polymerase Chain Reaction

Real time assays were assembled using the epMotion 5070 liquid handling robot (Eppendorf, Mississauga, ON, CAN) and quantified by the LightCycler® 480 Real-Time PCR System (Roche Applied Science, Laval, QUE, CAN). All samples were run on a single 384-well plate (Roche Applied Science, Laval, QC, Canada) to remove plate-to-plate variation with triplicate technical replicates to reduce sampling error. Real time assays were run with a 9-point standard curve, which consisted of a 5-fold dilution series of the copy RNA standards. Melt curves were examined to eliminate the possibility of undetected false positives by ensuring that all samples and standards had consistent melting peaks indicating amplification of a single gene product.

3.6.4.1 qRT-PCR Plate Preparation

The liquid handling robot completed all pipetting, thus reagents were prepared in stock solutions consisting of a mastermix, standards, samples, water, and Tris-HCl. A real-time mastermix was assembled in an Eppendorf epMotion 5075 mastermix reservoir consisting of Ultrapure^{TM} DNase/RNase-free distilled water, 1X PCR buffer containing 200 mM Tris-HCl (pH 8.4) and 500 mM KCl, 2.5 mM MgCl$_2$, 0.2 mM GeneAmpdNTP mix, 0.4 μM forward primer, 0.4 μM reverse primer, 0.8 U GoTaq® HS DNA Polymerase (Promega,

Madison, WI, USA), and 1 × Evagreen® (Biotium, Hayward, CA, USA). 45 µL of mastermix was pipetted into a 96-well plate followed by 5µL of each sample cDNA or 5 µL of DNase/RNase-free distilled water for the blank, and mixed twice by pipetting. 10 µL of each mixture was then pipetted onto the 384-well plate in triplicate. Standards were prepared by adding 90 µL of 10 mM Tris-HCl (pH 7.4) to 10 µL of a 0.01× concentration of copy RNA standard and was aliquoted into a 1.5 mL centrifuge tube. 10 mM of Tris-Cl, pH 7.4 was aliquoted into a 1.5 mL centrifuge tube from which the liquid handling robot pipetted 80 µL of the Tris-HCl into 9 wells on a 96-well plate. The stock standard (60 µL) was pipetted onto the 96-well plate, from which subsequently 20 µL was mixed continually into the next well containing 80 µL of Tris-HCl until a 5-fold dilution series was created. Each standard (5 µL) was then mixed with 45 µL mastermix, and 10 µL was aliquoted onto the 384-well plate in triplicate, as carried out for the samples and blanks above. The plate with all components loaded was run on the LightCycler® LC 480 II (Eppendorf, Mississauga, ON, Canada).

3.6.4.2 Data Normalization

The reference gene used for normalization include GAPDH, RP2 and HPRT1. GAPDH was chosen as the internal standard since the gene has been demonstrated to possess a constant level of gene expression among all cells in the liver tissue enabling correction for experimental variation. The real-time assay was prepared identically to GAPDH, RP2 and HPRT1 using the liquid handling robot. The mastermix contained Ultrapure™ DNase/RNase-free distilled water, 1× PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM GeneAmpdNTP mix, 0.4 µM forward primer, 0.4 µM reverse primer, 0.8 U GoTaq DNA polymerase, and 1× Evagreen® (Biotium, Hayward, CA, USA). Ratios were calculated by

dividing the concentration of each gene (calculated as the geometric mean of the triplicate samples, by the LightCycler® LC 480 II software) by the reference gene resulting in a normalized gene expression ratio.

3.6.4.3 Controls

No reverse transcriptase (no RT) controls were run to check for DNA contamination of the RNA. RNA from each sample was pipette into its own tube of a labeled 8-well strip. RNAse free dd H_2O was added to each tube. A 1/10 dilution using dd H_2O as the diluent was made of these controls into another 8-well strip. The no RT controls were run as a separate assay. The housekeeping gene, GAPDH mRNA, was used as the normalizing factor and as confirmation that the RNA was of high quality resulting in good cDNA. Salt contamination control was achieved by assessing the ratio of the A260/A230 readings and the concentration of the RNA was quantified as absorbance at 260 nm using the Nanodrop ND1000 spectrophotometer (Thermo Fisher Scientific, Delaware, USA). A ratio greater than 1.8 indicated lack of salt contamination.

3.7 Statistical Analyses

Data were analyzed for the effects of the dietary treatments on the measured parameters in mink using an analysis of variance (ANOVA) for a completely randomized design. Model assumptions of normality and constant variance were check using Minitab 17.2.1 (Minitab Inc., USA). Differences between different treatments were assessed using Turkey test comparing means across treatment at each means and statistical significant was set at $P = 0.05$. The results are presented as means ± standard error of the mean (SE).

CHAPTER 4: RESULTS

4.1 Experiment 1: Pilot Study, Development and Characterization of OA-induced Fatty Liver Disease in Mink

Table 4. 1: Variables of plasma clinical chemistry in mink in response to 1% OA for 5 days and 1% OA for 10 days.

Treatments	CTRL	OA5	OA10
Urea (mmol L^{-1})	4.50±0.83	4.85±0.97	5.17±0.93
Glucose (mmol L^{-1})	8.40±0.91^a	11.14±0.98^b	10.80±0.95^b
Cholest (mmol L^{-1})	4.98±0.80^a	5.78±0.85^a	7.33±1.01^b
Alk. Phos (U L^{-1})	56.01±1.34	52.75±1.44	64.80±1.45
ALT (U L^{-1})	145.0±2.12	78.3±1.95	154.5±2.24
Total Protien (g L^{-1})	60.6±1.12	59.1±1.23	59.2±1.21
Albumin (g L^{-1})	30.75±1.14	28.80±1.12	30.81±1.11
Globulin (g L^{-1})	27.75±1.05	29.01±1.23	31.25±1.05

CTRL, Control; OA5, 1% OA for 5 days; OA10, 1% OA for 10 days, OA: Orotic acid Values are means ± SE. Labeled means with no common letters differ within the row ($P \leq 0.05$). Alkaline phosphatase, Alk. Phos; Alanine Aminotransferase, ALT; Cholesterol, Cholest;

Table 4. 2: Total lipid content in mink liver in response to 1% OA for 5 days and 1% OA for 10 days.

Treatment	CTRL	OA5	OA10
Total Lipid Content	6.94 ± 0.99	6.18 ± 1.07	6.68 ± 1.03

CTRL, Control; OA5, 1% OA for 5 days; OA10, 1% OA for 10 days, OA: Orotic acid
Labeled means of the groups are not statistically significant (P>0.05).

Table 4. 3: NASH Activity Index (NAI) in the liver of mink that received the control diet, or diet supplemented with 1% OA for 5 days and 1% OA for 10 days.

	CTRL	OA5	OA10
NAI	0.76 ± 0.80	1.42 ± 0.87	1.23 ± 0.88

Means ± SE shown for the effect of different treatment on liver NAI in mink. Means of 0=no fatty liver, 1-4= mild fatty liver, 5-8=moderate, 9-12= severe.

Table 4. 4: Hematological variables in mink in response to control, 1% OA for 5 days and 1% OA for 10 days.

Parameters	CTRL	OA 5	OA 10
White blood cells (10^9 L^{-1})	4.32±1.04	4.72±1.11	5.46±1.16
Red blood cells (10^{12} L^{-1})	8.59±0.84	8.85±0.79	8.34±0.97
Hemoglobin (g dL^{-1})	16.75±0.97	17.62±0.87	16.27±0.98
Hematocrit (%)	57.7±1.22	59.3±1.14	56.1±1.29
Platelets (10^9 L^{-1})	698.4±2.64	812.0±2.28	810.3±2.53
Mean corpuscular volume (μm^3)	66.0±1.33	67.2±1.05	65.6±1.39
Mean corpuscular hemoglobin (pg)	19.14±1.07	19.90±0.80	20.07±0.82
Lymphocytes (%)	1.62±0.88	1.20±0.86	1.75±0.92
Monocytes (%)	0.30±0.65	0.24±0.60	0.30±0.67
Granulocytes (%)	2.40±0.94	3.62±1.07	3.28±1.03

Values are means ± SE. Labeled means of the groups are not statistically significant (P>0.05). Control, CTRL; Orotic acid 1% for 5days, OA5; Orotic acid 1% for 10 days, OA10
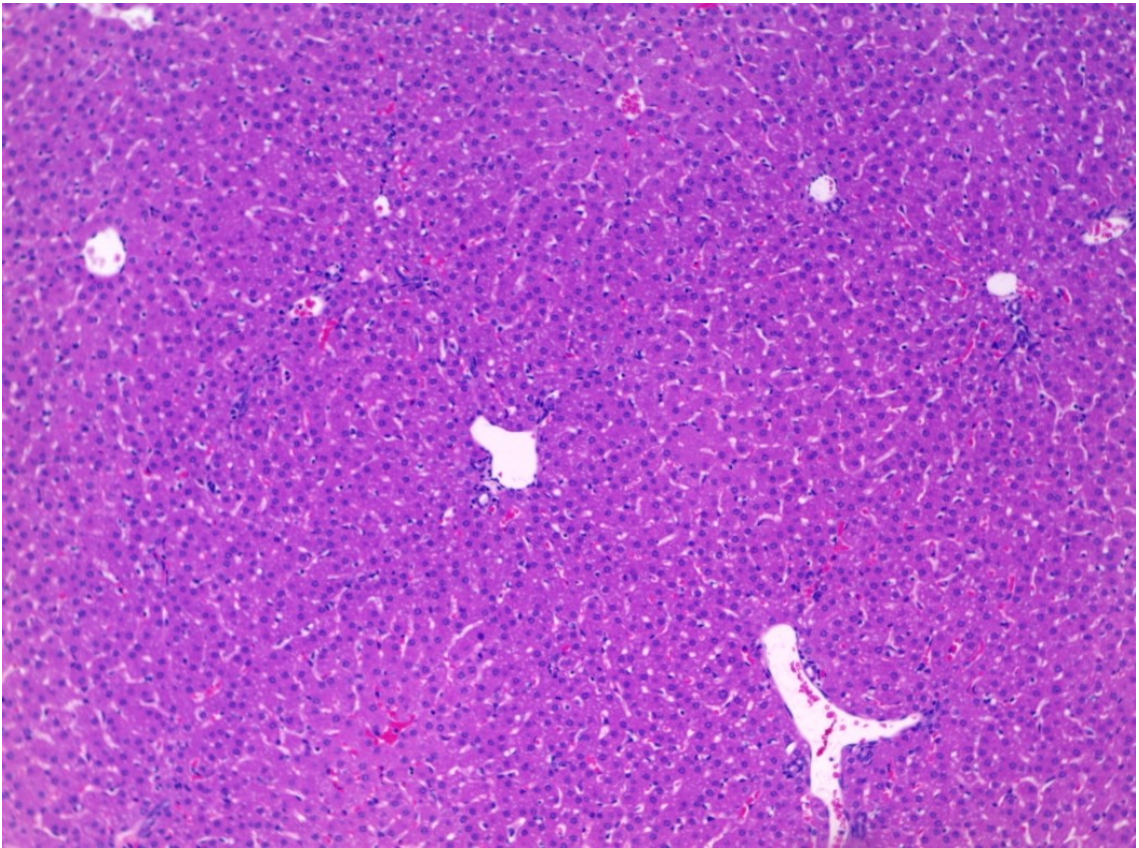
Figure 4. 1: Liver morphology in control mink. Liver morphology appears normal. Plates of hepatocytes are arranged in an orderly and radiating outward from the central vein to the lobule periphery. Magnification 100×.
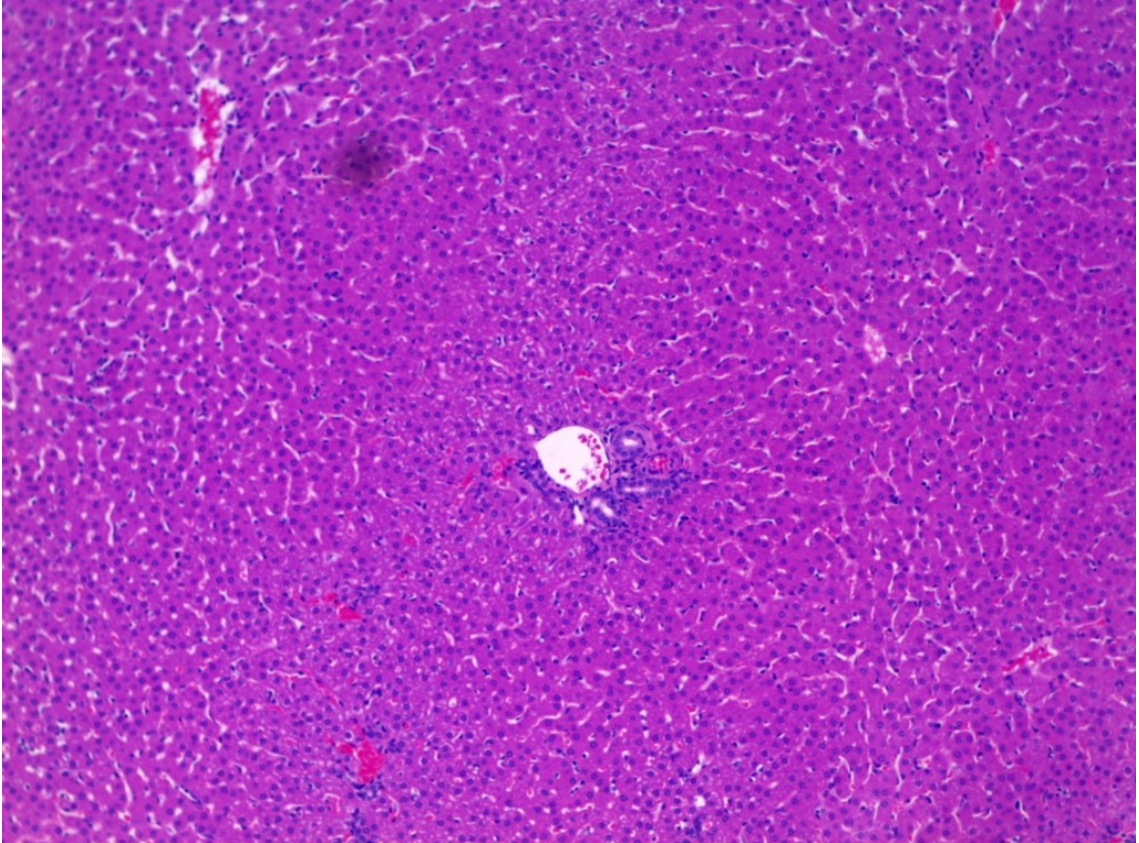
Figure 4. 2: Liver morphology in OA5 mink. Liver morphology appears normal. Magnification 100×
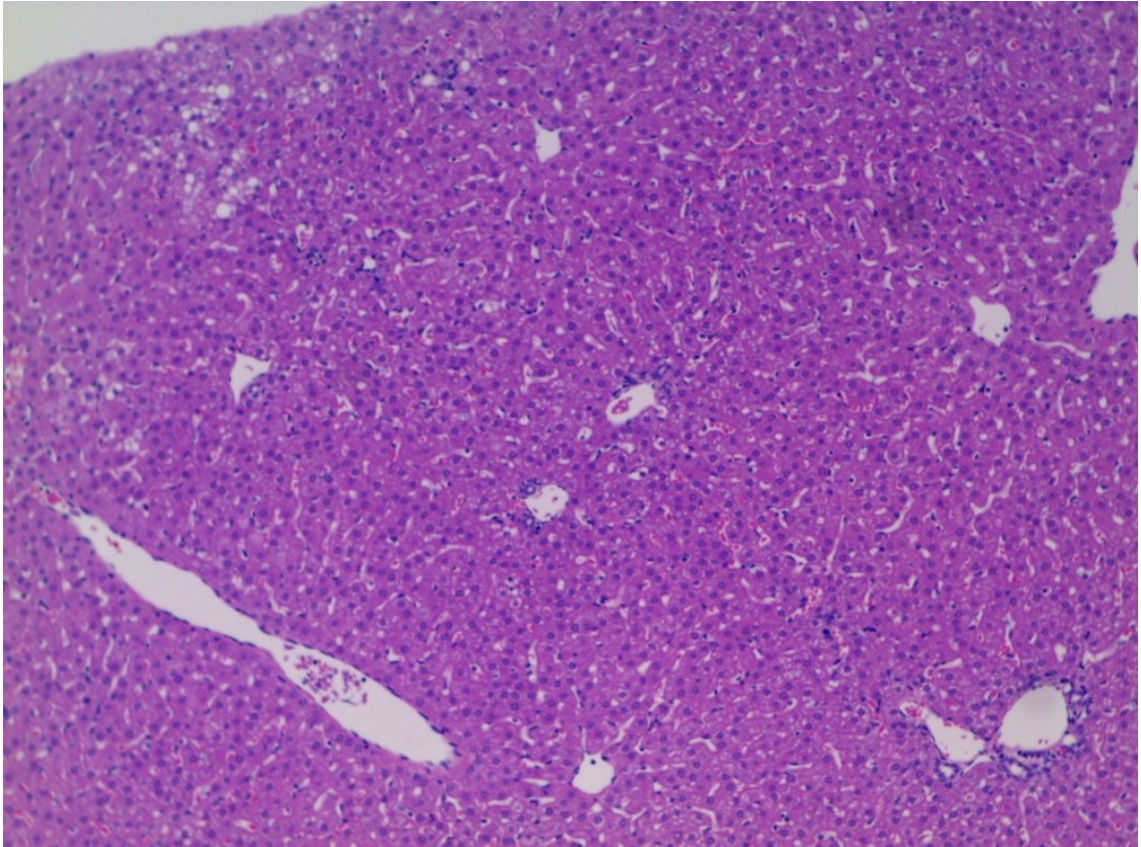
Figure 4. 3: Liver morphology in OA10 mink. Liver morphology appears normal. Small periportal lipid droplets and moderate lipid accumulation in hepatocytes are evident. Magnification 100×

4.2 Experiment 2: Testing of Whey Protein Hydrolysate (WPH) and Arginine for Their Effectiveness in the Prevention and Treatment of OA-induced Non-alcoholic Steatohepatitis (NASH) Condition in Mink

Table 4. 5: Effect of different diet groups on NASH Activity Index (NAI) in the liver of mink.

	CTRL0	CTRL	OA+CTRL	OA+Arg	OA+WPH	OA+Arg+WPH	OA-CTRL+Arg	OA-CTRL+WPH	OA-CTRL+Arg+WPH
NAI	0.82±0.0.84[a]	0.76±0.82[a]	4.21±0.85[b]	1.1±0.86[a]	0.96±0.76[a]	0.88±0.83[a]	0.84±0.85[a]	1.01±0.80[a]	0.79±0.73[a]

Means ± SE shown for the effect of different treatment on liver NAI in mink. Letter grouping indicates significant differences for different treatment effects. Means of 0=no fatty liver, 1-4= mild fatty liver, 5-8=moderate, 9-12= severe. Different letters indicate significant differences between groups ($P<0.05$).
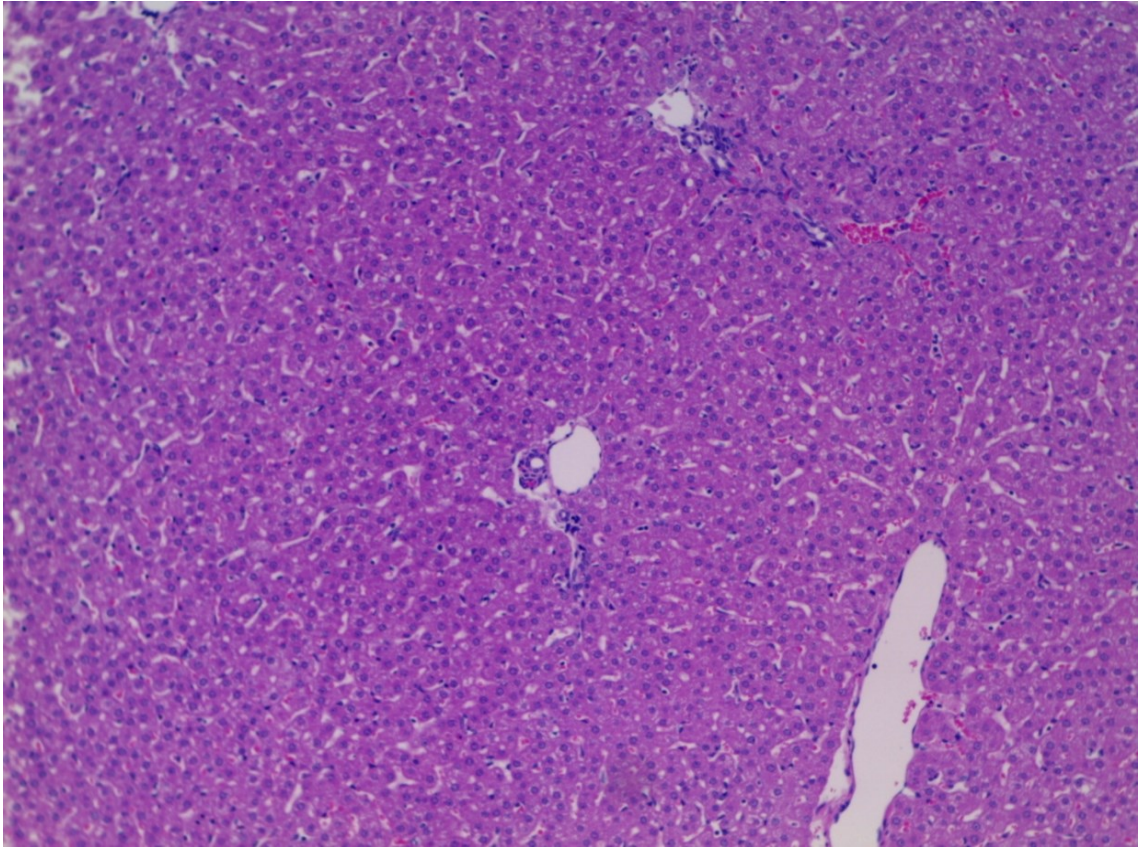
Figure 4. 4: Liver morphology in control (CTRL) mink. Liver morphology appears normal. Plates of hepatocytes are orderly and radiating outward from the central vein to the lobule periphery. Magnification 100×
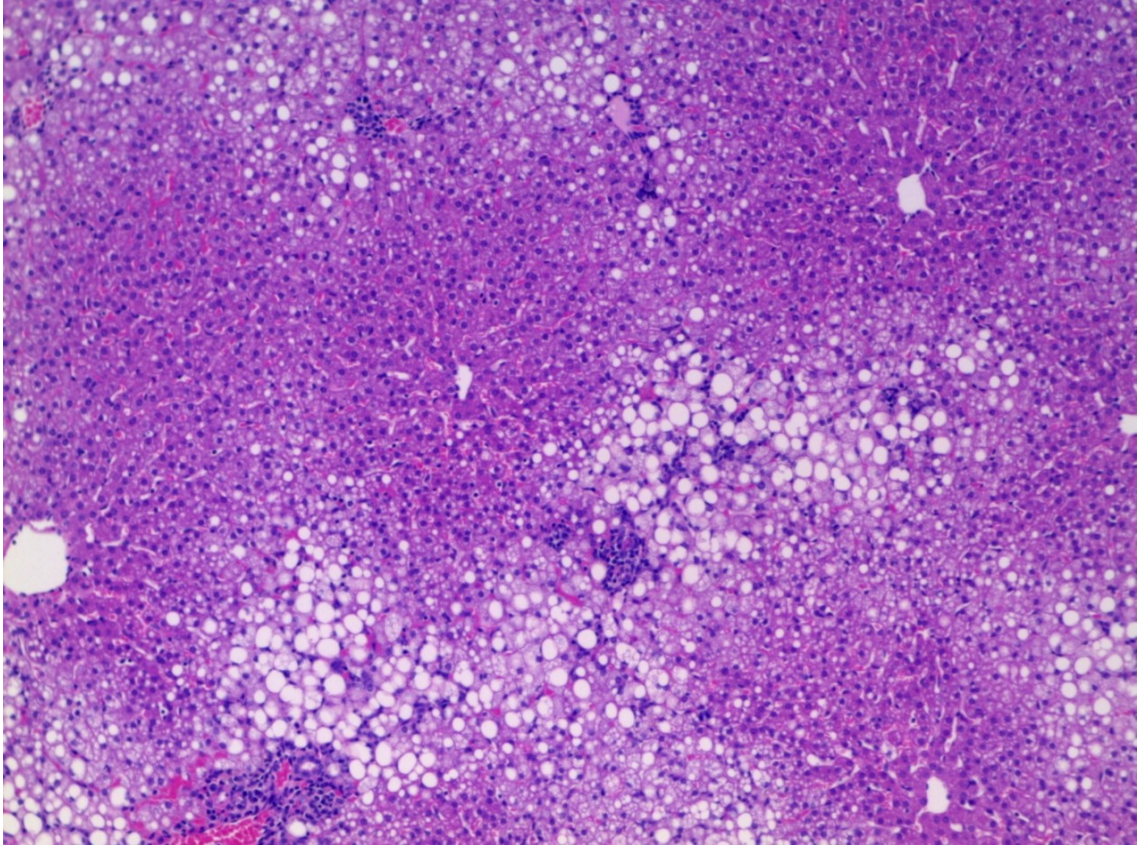
Figure 4. 5: Liver morphology in CTRL+OA mink. Lipid accumulation in hepatocytes is clearly visible. Lipid accumulation is pushing the nuclei and sinusoids are compressed. Magnification 100×
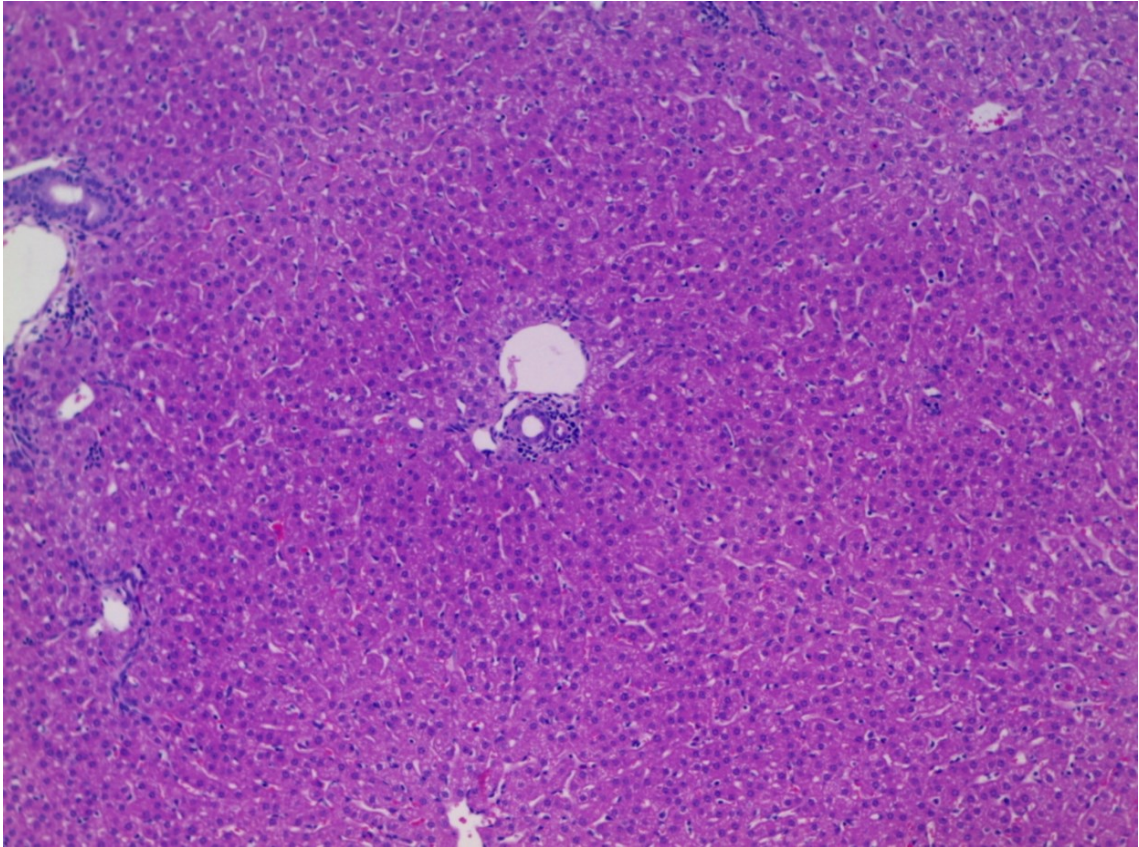
Figure 4. 6: Liver morphology in OA+Arg mink. Liver morphology appears normal. Plates of hepatocytes are orderly and radiating outward from the central vein to the lobule periphery. Magnification 100×
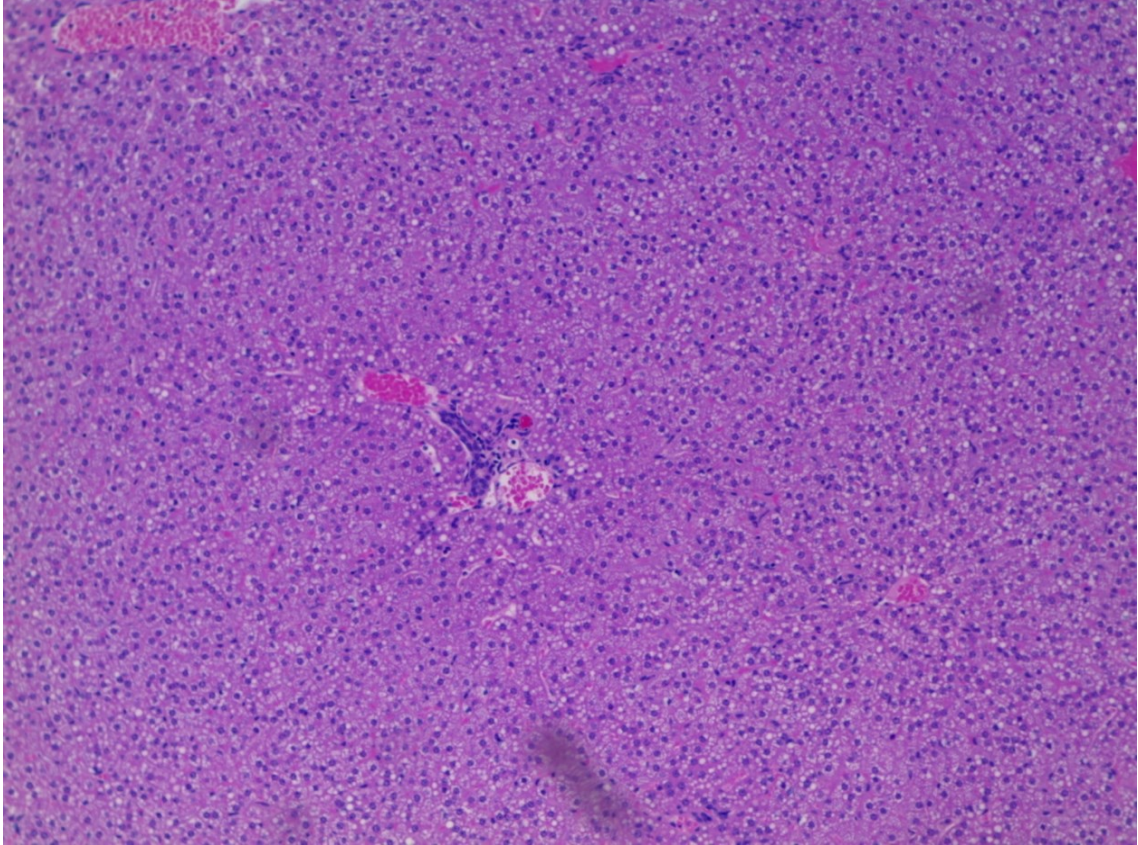
Figure 4. 7: Liver morphology in OA+WPH mink. Liver morphology appears normal. Plates of hepatocytes are orderly and radiating outward from the central vein to the lobule periphery. Magnification 100×
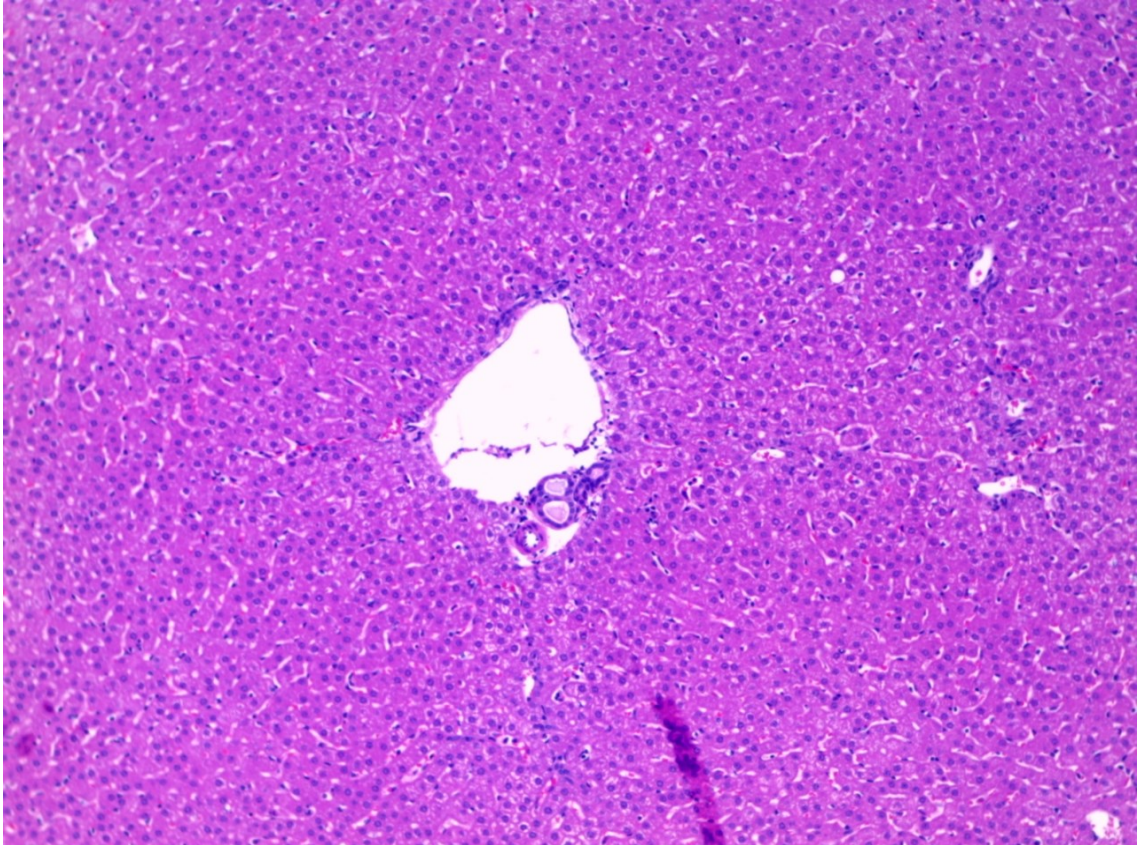
Figure 4. 8: Liver morphology in OA+Arg+WPH mink. Liver morphology appears normal. Plates of hepatocytes are orderly and radiating outward from the central vein to the lobule periphery. Magnification 100×
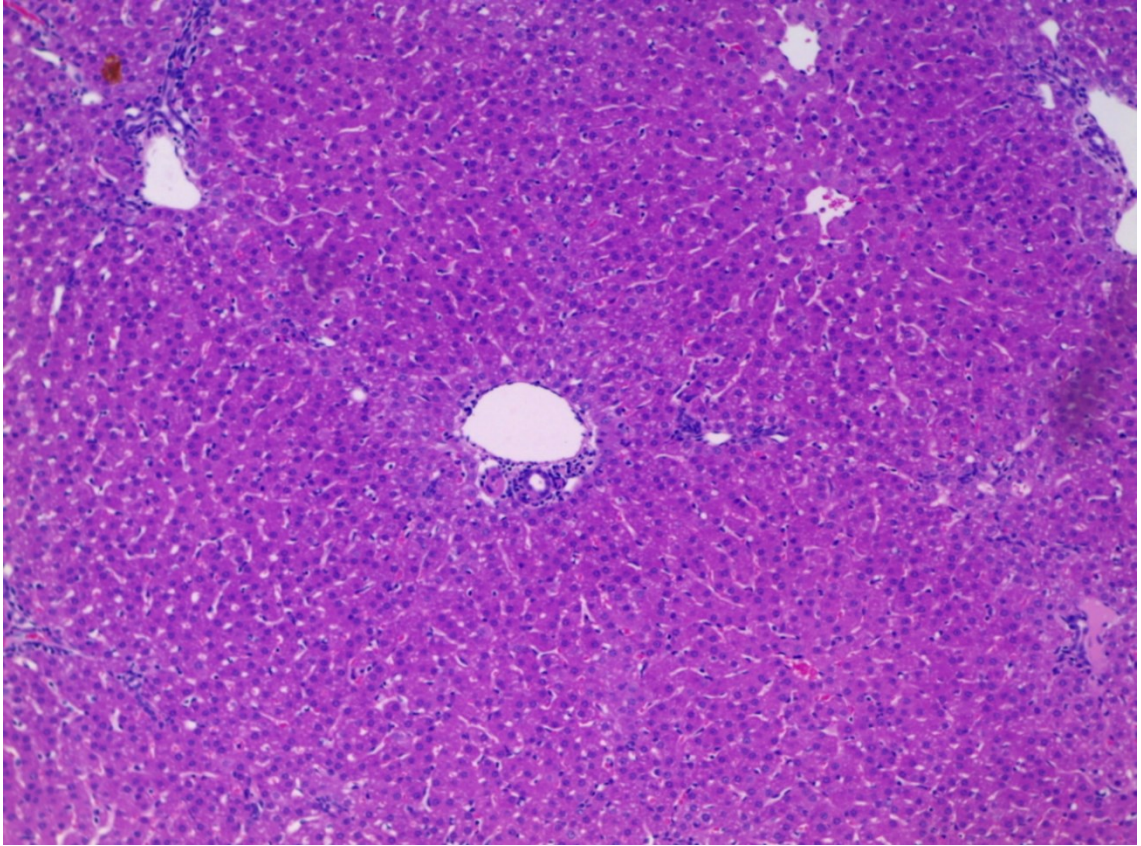
Figure 4. 9: Liver morphology in OA-CTRL+Arg mink. Liver morphology returns to normal. Plates of hepatocytes are orderly and radiating outward from the central vein to the lobule periphery. Magnification 100×
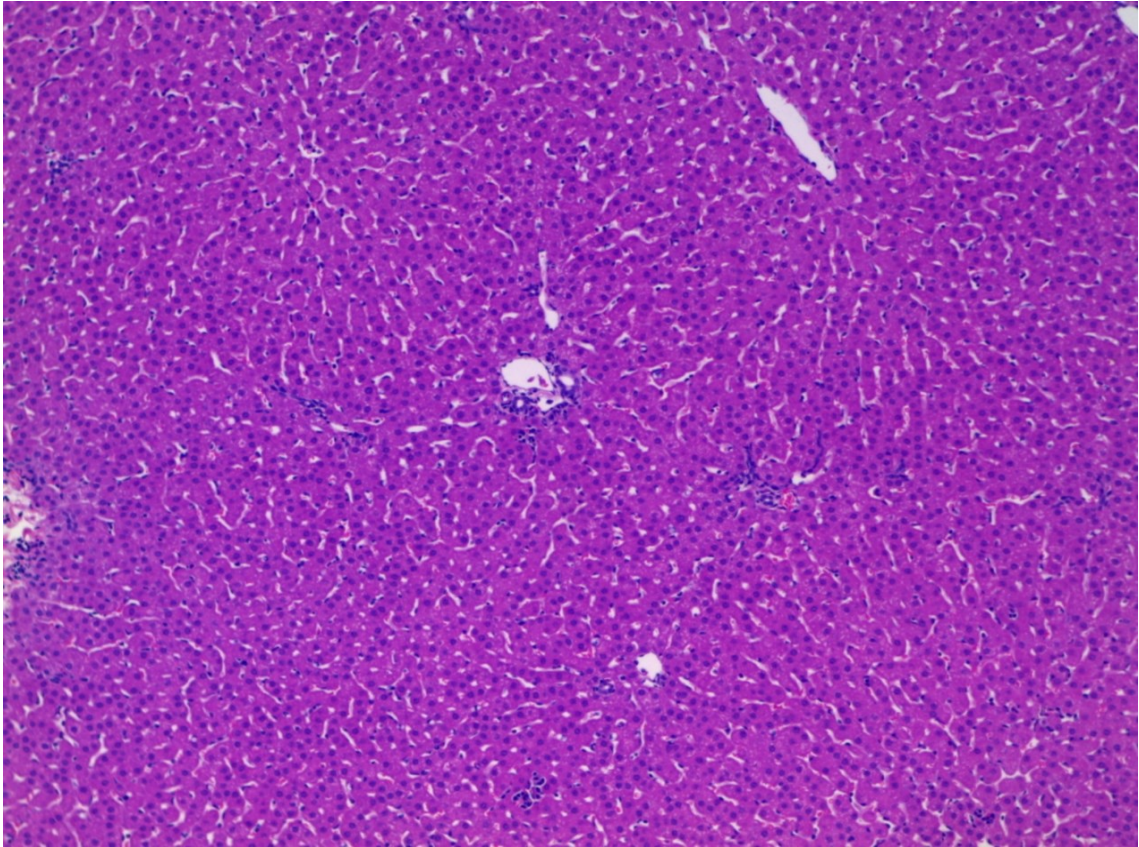
Figure 4. 10: Liver morphology in OA-CTRL+WPH mink. Liver morphology returns to normal. Plates of hepatocytes are orderly and radiating outward from the central vein to the lobule periphery. Magnification 100×
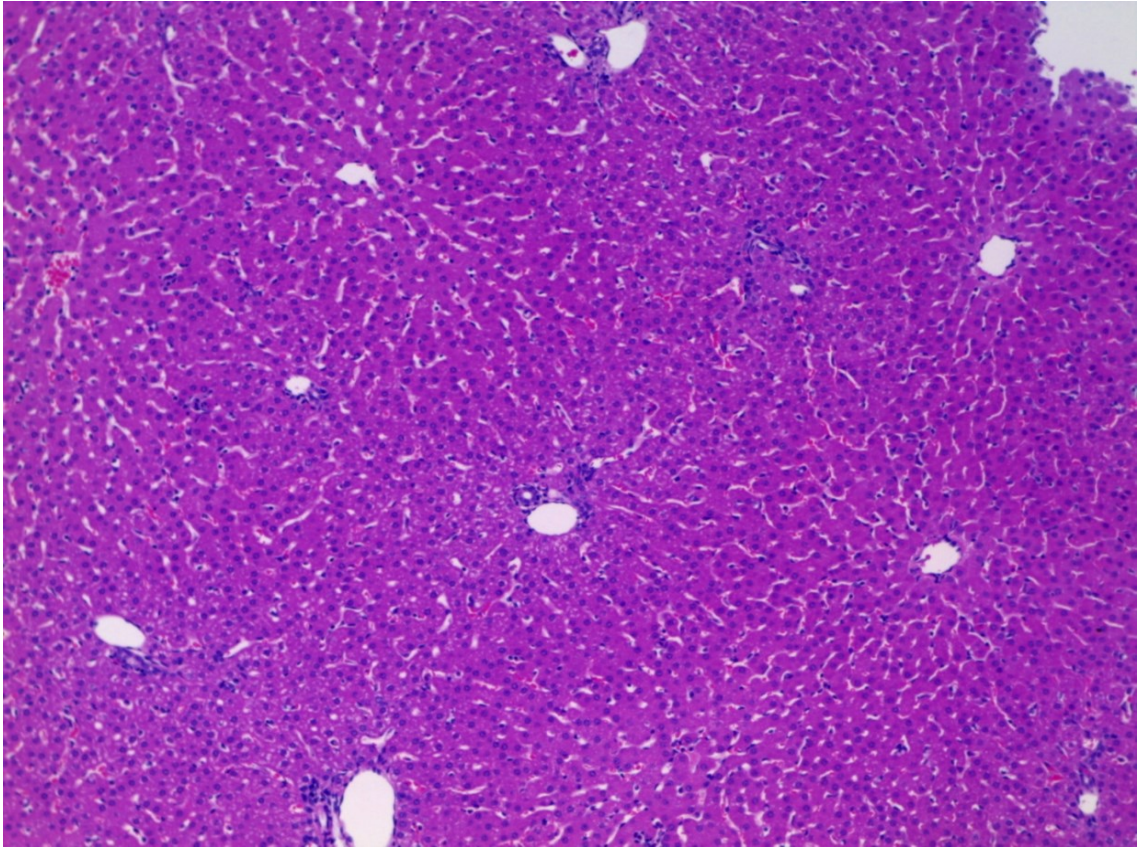
Figure 4. 11: Liver morphology in OA-CTRL+Arg+WPH mink. Liver morphology returns to normal. Plates of hepatocytes are orderly and radiating outward from the central vein to the lobule periphery. Magnification 100×

Table 4. 6: Total lipid content in the mink liver in response to the different dietary treatments.

Treatment	CTRL0	CTRL	OA+CTRL	OA+Arg	OA+WPH	OA+Arg+ WPH	OA-CTRL+Arg	OA-CTRL+WPH	OA-CTRL+Arg+WPH
Total Lipid Content (%)	5.19±1.13	5.24±1.01	7.77±0.77	6.32±1.07	5.36±0.88	5.21±1.09	5.46±0.96	5.97±1.10	6.29±0.93

Control, CTRL; Orotic acid 1%, OA; L-Arginine 2%, Arg; Whey Protein Hydrolysates, WPH.
Values are means ± SE.

Table 4. 7: Hematological variables in the mink in response to the different dietary treatments.

Group	WBC	RBC	PLT	LYM	MON	MCV	MCH	MCHC
CTRL0	6.7 ± 1.2^{ab}	7.0 ± 1.2	579.3 ± 2.5^{d}	18.6 ± 1.5^{ab}	5.5 ± 1.1^{ab}	69.0 ± 1.1	21.5 ± 0.8	30.4 ± 0.8
CTRL	7.9 ± 1.2^{ab}	6.8 ± 1.2	640.7 ± 2.4^{cd}	18.9 ± 1.1^{ab}	4.9 ± 0.9^{ab}	67.2 ± 1.4	20.5 ± 1.1	30.4 ± 0.8
OA+CTRL	4.0 ± 1.2^{b}	6.6 ± 1.3	662.7 ± 4.4^{bcd}	16.4 ± 1.4^{ab}	5.2 ± 1.1^{ab}	68.3 ± 1.3	20.8 ± 0.8	30.5 ± 0.7
OA+Arg	5.8 ± 1.2^{ab}	8.3 ± 0.9	674.5 ± 2.1^{bcd}	21.0 ± 1.2^{ab}	6.0 ± 1.0^{a}	70.6 ± 1.1	21.7 ± 0.9	30.7 ± 0.8
OA+WPH	6.2 ± 1.2^{ab}	8.2 ± 0.8	699.8 ± 2.2^{abcd}	19.4 ± 1.5^{ab}	4.9 ± 1.0^{ab}	69.3 ± 1.0	20.6 ± 0.9	29.6 ± 0.9
OA+Arg+WPH	8.5 ± 1.2^{a}	8.2 ± 1.0	706.0 ± 2.1^{abc}	18.6 ± 1.4^{ab}	4.8 ± 1.0^{ab}	70.8 ± 1.1	21.9 ± 0.8	31.0 ± 0.8
OA-CTRL+Arg	7.1 ± 1.0^{ab}	8.6 ± 0.8	786.3 ± 2.4^{ab}	25.9 ± 1.5^{a}	6.4 ± 0.9^{a}	68.4 ± 1.1	20.4 ± 0.8	29.7 ± 0.7
OA-CTRL+WPH	7.9 ± 1.0^{ab}	7.6 ± 1.0	821.5 ± 2.2^{a}	14.1 ± 1.5^{b}	3.6 ± 1.0^{b}	68.6 ± 1.2	20.5 ± 0.9	29.8 ± 0.8
OA-CTRL+Arg+WPH	6.9 ± 1.1^{ab}	7.1 ± 1.1	721.8 ± 2.5^{abc}	18.0 ± 1.3^{ab}	5.1 ± 1.1^{ab}	69.6 ± 1.1	20.8 ± 0.9	29.9 ± 0.7

Control, CTRL; Orotic acid 1%, OA; L-Arginine 2%, Arg; Whey Protein Hydrolysates 5%, WPH.

Values are means ± SE. Labeled means with no common letters differ within the column (P ≤ 0.05).

WBC: White blood cells; RBC: Red blood cells; PLT: Platelets; LYM: Lymphocytes; MON: Monocytes; MCV: Mean Corpuscular Volume; MCH: Mean Corpuscular Hemoglobin; MCHC: Mean Corpuscular Hemoglobin Concentration.

Table 4. 8: Hepatosomatic index of the mink in response to the different dietary treatments.

Treatments	Body Weight (g)	Hepatosomatic Index	Body Mass Index (BMI)/ (g/cm^2)
CTRL0	1087.8±2.7	2.8±0.9	0.76±0.6
CTRL	1203.0±2.8	2.8±0.7	0.73±0.5
OA+CTRL	1043.3±3.7	2.9±0.6	0.63±0.3
OA+Arg	1217.8±2.6	2.6±0.6	0.75±0.6
OA+WPH	1033.2±2.8	2.9±0.7	0.63±0.6
OA+Arg+WPH	1297.2±2.9	2.6±0.9	0.71±0.6
OA-CTRL+Arg	1284.0±2.4	2.4±0.6	0.72±0.5
OA-CTRL+WPH	1122.4±2.7	3.2±1.1	0.66±0.5
OA-CTRL+Arg+WPH	1237.8±2.5	2.5±0.0.7	0.71±0.6

Control, CTRL; Orotic acid 1%, OA; L-Arginine 2%, Arg; Whey Protein Hydrolysates, WPH. Values are means ± SE. Labeled means values of the groups are not statistically significant (P>0.05).

Table 4. 9: Variables of clinical chemistry in the mink plasma in response to the different dietary treatments.

Treatment	Urea (mmol L^{-1})	Glucose (mmol L^{-1})	Cholest (mmol L^{-1})	Alk. Phos (U L^{-1})	ALT (U L^{-1})	Total Protien (g L^{-1})	Albumin (g L^{-1})	Globulin (g L^{-1})
CTRL0	4.10±0.9^b	9.24±0.8^{ab}	6.45±0.9^{bcd}	49.6±1.6^{ab}	77.0±1.7	59.2±1.2^{ab}	31.6±1.1	26.5±1.1^{ab}
CTRL	4.80±1.0^b	7.66±1.0^b	7.41±0.9^{abc}	46.6±1.4^{ab}	93.7±1.7	59.0±1.1^{ab}	32.0±0.9	26.0±1.0^{ab}
OA+CTRL	5.07±1.1^b	8.24±0.9^{ab}	8.29±0.6^a	57.5±1.8^a	92.8±1.6	60.3±1.2^a	31.4±1.3	27.0±1.2^a
OA+Arg	4.40±0.8^b	9.24±1.0^{ab}	5.67±0.8^d	43.2±1.5^{ab}	70.7±1.3	56.1±1.0^{bc}	30.4±0.9	25.6±1.0^{ab}
OA+WPH	7.66±0.8^a	8.93±1.0^{ab}	7.47±0.9^{abc}	57.5±1.6^a	97.3±1.4	59.0±1.2^{ab}	31.3±0.9	26.3±0.9^{ab}
OA+Arg+WPH	4.14±0.8^b	9.92±1.0^a	5.95±1.(	38.5±1.3^b	79.0±1.4	53.6±1.0^c	31.5±1.1	23.8±1.1^b
OA-CTRL+Arg	4.54±0.9^b	9.12±1.0^{ab}	7.60±0.9^{ab}	41.7±1.4^{ab}	84.7±1.7	55.8±1.1^{bc}	30.4±1.1	25.4±0.9^{ab}
OA-CTRL+WPH	5.17±0.8^b	8.84±1.0^{ab}	6.57±0.7^{bcd}	49.0±1.5^{ab}	81.8±1.5	57.5±1.0^{ab}	31.8±0.9	25.5±0.9^{ab}
OA-CTRL+Arg+WPH	4.42±0.9^b	9.40±0.8^{ab}	6.32±0.9^{bcd}	40.0±1.6^{ab}	76.5±1.8	57.0±1.1^{abc}	30.8±1.0	26.2±1.0^{ab}

Control, CTRL; Orotic acid 1%, OA; L-Arginine 2%, Arg; Whey Protein Hydrolysates, WPH; Values are means ± SE. Labeled means with no common letters differ within the column (P ≤ 0.05). Alk. Phos: Alkaline phosphatase; ALT: Alanine Aminotransferase; Choles: Cholesterol.

Total lipid content (Table 4.6) was highest in the positive control, whereas lowest liver lipid found in the control groups. White blood cell concentration (Table 4.7) was lowest in the positive control group and fatty liver treatment groups showed elevated red blood cells concentration compared with rest of the treatment groups. Plate concentration in blood was elevated in the preventive groups and highest was observed in OA-CTRL+WPH mink. The mink of OA-CTRL+WPH group had highest HSI (Table 4.8) and the lowest in the mink fed arginine in treatment group. In the positive control group, plasma cholesterol level (Table 4.9) was highest, and the levels were lower in the OA+Arg and OA+Arg+WPH combination groups. Blood glucose levels were higher at the start of the feeding trial and elevated in diet contains arginine. Plasma ALT and Alk. Phosphate levels did not differ among the controls and treatment groups. Highest ALT and Alk. Phosphate levels was observed in the group of OA+WPH mink and lowest concentrations observed in OA-CTRL+Arg+WPH group.
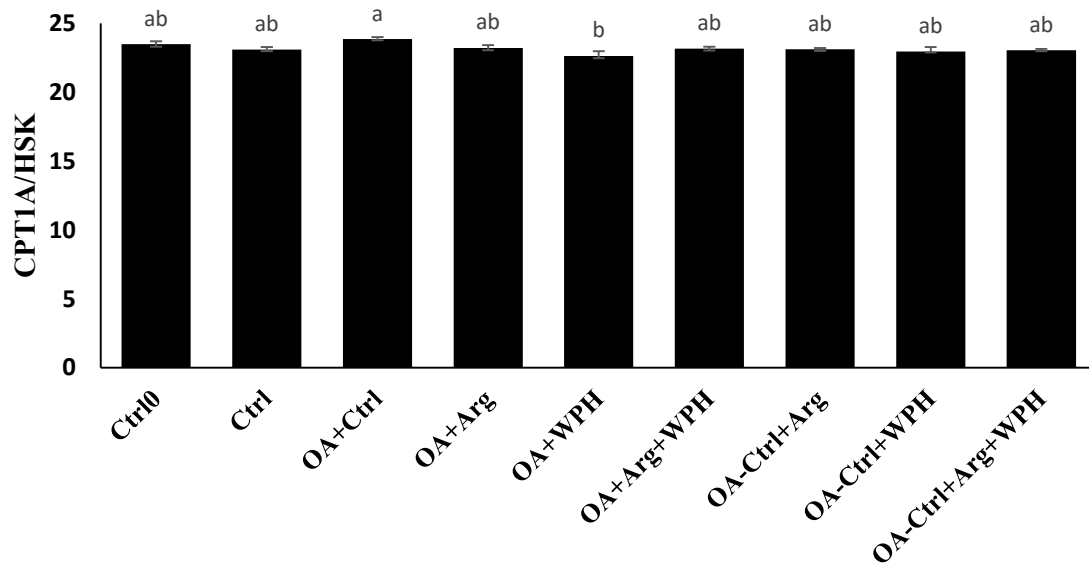
Figure 4. 12: Effect of Arginine and Whey protein hydrolysates (WPH) on expression of carnitine palmitoyltransferase 1A (CPT1A) relative to the geometric mean of the expression of housekeeping genes GAPDH, RP2 and HPRT1 in mink liver. Different letters indicate significant differences between groups (P<0.05). HSK: Housekeeping genes.

4.2.1 Carnitine palmitoyltransferase 1A mRNA levels

The CPT1a qRT-PCR assay had an efficiency of 1.829 with $R^2=0.94$ and all samples were peaking within the standard curve. Mink in positive control resulted in highest liver mRNA expression of CPT1a, whereas lowest in mink fed OA+WPH.
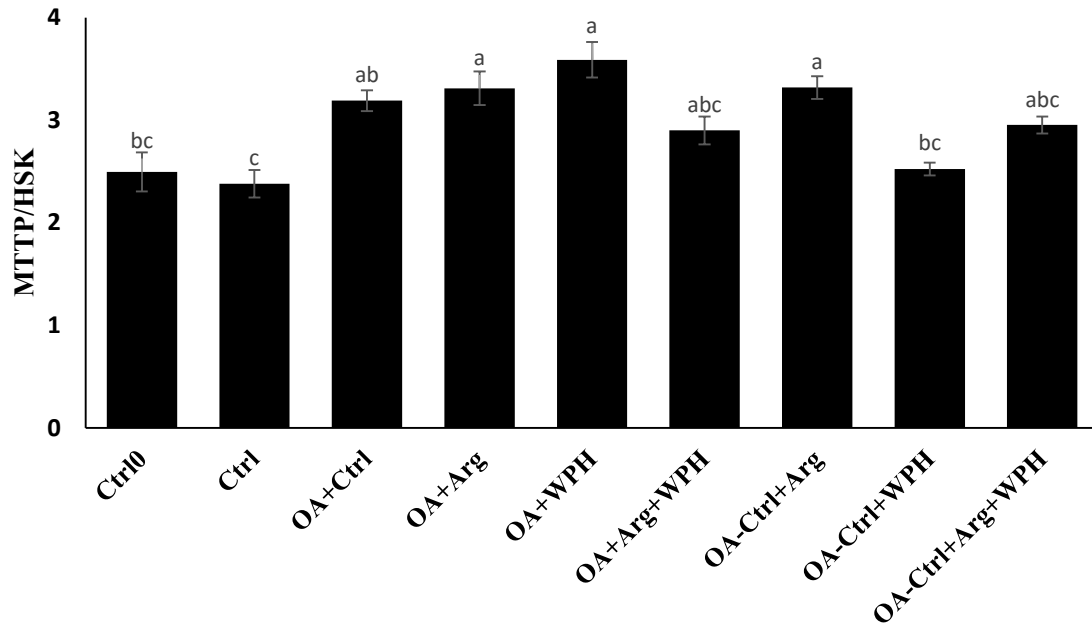
Figure 4. 13: Effect of Arginine and Whey protein hydrolysate (WPH) on expression of Microsomal triglyceride transfer protein (MTTP) relative to the geometric mean of the expression of housekeeping genes GAPDH, RP2 and HPRT1 in mink liver. Different letters indicate significant differences between groups (P<0.05). HSK: Housekeeping genes.

4.2.2 Microsomal triglyceride transfer protein mRNA levels

The Microsomal triglyceride transfer protein qRT-PCR assay had an efficiency of 1.949 with $R^2=0.882$. Highest MTTP mRNA expression was observed in OA+WPH mink and while lowest levels noted in control mink. Mink fed OA-CTRL+WPH resulted in a lower expression of MTTP compared with the other treatment groups and not significantly different from the control mink.
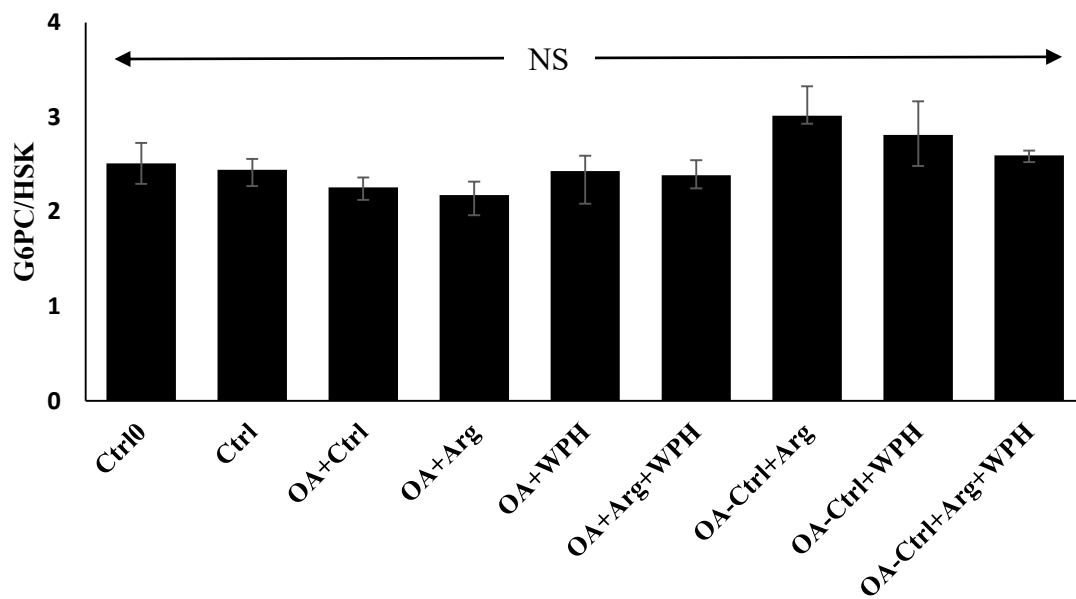
Figure 4. 14: Effect of Arginine and Whey protein hydrolysate (WPH) on expression of Glucose-6-phosphatase (G6PC) relative to the geometric mean of the expression of housekeeping genes GAPDH, RP2 and HPRT1 in mink liver. NS, mean values of the groups are not statistically significant (P>0.05). HSK: Housekeeping genes.
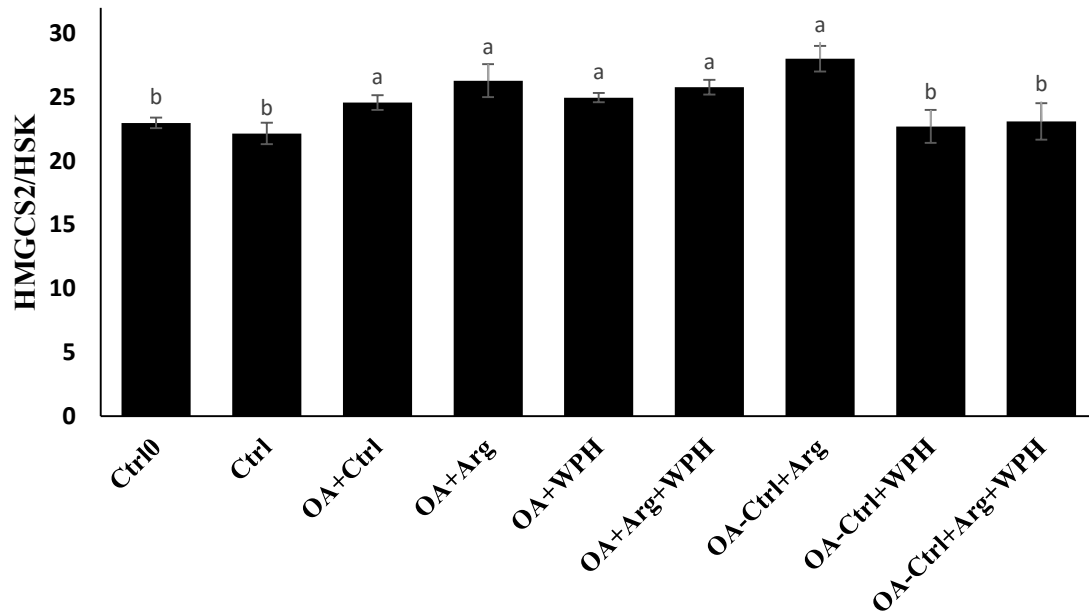
Figure 4. 15: Effect of Arginine and Whey protein hydrolysate (WPH) on expression of 3-hydroxy-3-methylglutaryl-CoA synthase 2 (HMGCS2) relative to the geometric mean of the expression of housekeeping genes GAPDH, RP2 and HPRT1 in mink liver. Different letters indicate significant differences between groups (P<0.05). HSK: Housekeeping genes.

4.2.3 3-hydroxy-3-methylglutaryl-CoA synthase 2 mRNA levels

The 3-hydroxy-3-methylglutaryl-CoA synthase 2 qRT-PCR assay had an efficiency of 1.927 with $R^2=0.876$ and all samples peaking within the standard curve. Mink fed in treatment groups resulted in the higher HMGCS2 mRNA expression and significantly different from the control mink group. Highest HMGCS2 mRNA expression was observed in OA-CTRL+Arg mink, while lowest in control mink.
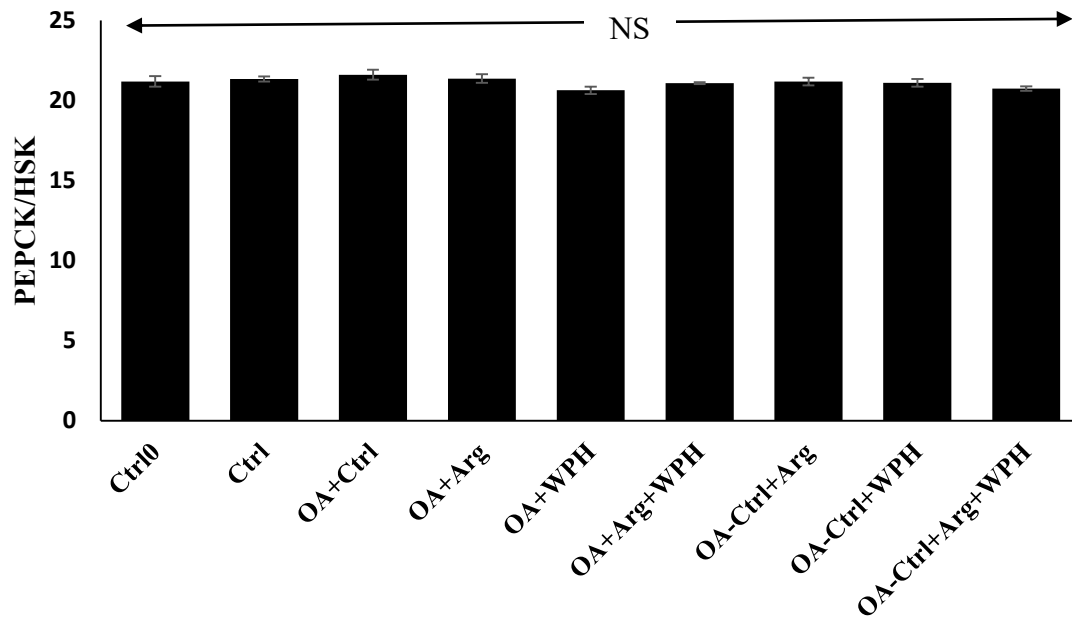
Figure 4. 16: Effect of Arginine and Whey protein hydrolysate (WPH) on expression of Phosphoenolpyruvate carboxykinase (PEPCK) relative to the geometric mean of the expression of housekeeping genes GAPDH, RP2 and HPRT1 in mink liver. NS, mean values of the groups are not statistically significant (P>0.05). HSK: Housekeeping genes.
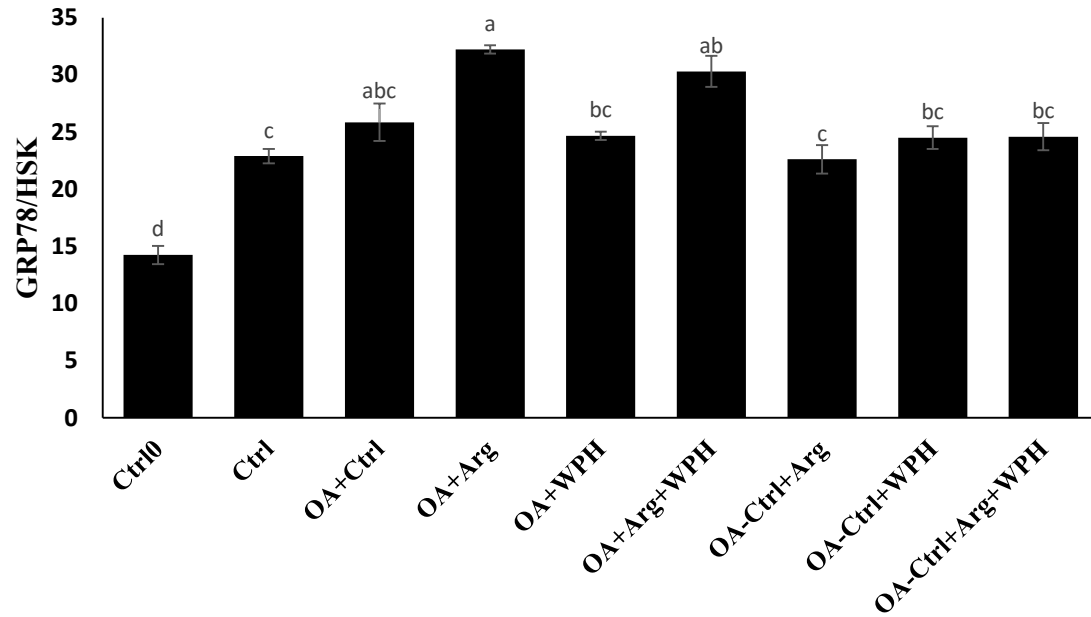
Figure 4. 17: Effect of Arginine and Whey protein hydrolysate (WPH) on expression of Glucose-regulated protein 78 kDa (GRP78) relative to the geometric mean of the expression of housekeeping genes GAPDH, RP2 and HPRT1 in mink liver. Different letters indicate significant differences between groups (P<0.05). HSK: Housekeeping genes.

4.2.4 Glucose-regulated protein 78 kDa mRNA levels

The Glucose-regulated protein 78 kDa qRT-PCR assay had an efficiency of 1.937 and $R^2=0.911$. GRP78 mRNA expression was lower in the preventive group mink and no significantly different compared with control mink. Highest expression of GRP78 was in the OA+Arg mink, while lowest in control mink.

CHAPTER 5: DISCUSSION

5.1 Progression of Fatty Liver in Mink

5.1.1 Pilot Research

In the pilot project, means for the different treatments of the clinical chemistry responses are shown in Table 4.1. OA intake through the diet resulted in significant increase in mink plasma glucose levels at both OA doses, and plasma cholesterol levels at the high OA dose, resulting in typical simple hepatic steatosis. Similar research findings reported by Tetri et al. (2008) indicated that trans-fat fed mice developed hepatic steatosis and glucose intolerance within 2 to 4 weeks of receiving the diet. This glucose intolerance and disruption of glucose homeostasis directly correlate with the development of insulin resistance (Shoelson et al. 2007).

Mallory-Denk bodies are identified by the characteristic hyaline deposits in the hepatocyte cytoplasm. Neither Mallory-Denk bodies nor fibrosis were observed in the hematoxylin and eosin stain prepared liver sections of mink in the three treatment groups (Figures 4.1, 4.2, & 4.3). Moreover, as shown in Table 4.2, the total lipid content of the mink liver was not different in the treatment groups, which suggest the absence of clear fatty liver as a result of OA treatment. Furthermore, analysis of liver NAI showed that both OA5 and OA10 groups were not significantly different (Table 4.3). However, OA5 and OA10 showed higher NAI than that of the control mink, and values higher than 1, indicating the accumulation of lipids in the liver and development of mild fatty liver.

As shown in Table 4.4, there was no statistically significant effect of OA treatment on the hematological variables in the mink. Putting all the results together, it is possible that feeding the mink 1% OA for 10 days resulted in an increase in liver inflammation, which

can explain the elevated plasma glucose and plasma cholesterol levels in the liver. An increase in liver inflammation is associated with excess mobilization of glucose, resulting in a pro-inflammatory state and an increase of liver lipids (Browning and Horton 2004). Based on the evidence of mild fatty liver, OA was administered to the mink for 10 days and the model was used to test the effects of bioactive whey peptides for prevention and treatment.

5.1.2 Experiment 2: Influence of Bioactive Peptides on OA Induced Fatty Liver Disease

Several studies on different animal species have shown that OA promotes liver hepatocellular carcinoma when initiated by a variety of chemical reactions (Griffin et al. 2004; Jung et al. 2011b). These studies reveal that even though OA is a normal cellular constituent, when ingested in excess, it can promote the development of liver disease and accumulation of liver fat.

The histology images (Figure 4.5) clearly indicate the development of fat in the liver under different treatment conditions. There was no substantial difference in the liver samples from mink in different treatment groups (Figures 4.6–4.11), except for the mink that received OA. The latter showed clear evidence of fat accumulation in the liver (Figure 4.5). Except for the positive control treatment group, all of the groups had mean NAI values between 0 and 1 (Table 4.5), which put them in the category of no fatty liver, or mild fatty liver. However, mink fed with OA had NAI of 4-5, which indicates the development of moderate fatty liver in the mink. This finding is also supported by the substantially increased liver lipid content in the OA mink group compared to mink that received no OA or different treatments of Arg and WPH (Table 4.6). The control mink had liver NAI of

0.82±0.41 (Table 4.5) and total lipid of 5.19±1.88% (Table 4.6) at the onset of this experiment, representing the healthy state of the mink. Consequently, the subsequent NAI and lipid content data suggest that OA causes progression of fatty liver in mink from a normal to a mild to moderate stage.

Nursing sickness and fatty liver can lead to increase in liver weight, but this was not clearly shown in the results of this experiment. A previous study suggested that the total lipid level for fatty liver in mink would be between 9 to 12% (Rouvinen-Watt et al. 2010). Based on that, the control and treatment group appeared to have healthy hepatic lipid levels. Similar results were observed (Table 4.8) in the hepatosomatic index (HSI) and Body Mass Index (BMI). Since hepatic lipid content were not increased in mink, liver weight was not significantly increased and this led to the lack of difference in HSI.

In Table 4.9, plasma glucose levels in the combination group was significantly elevated compared with rest of groups. Insulin resistance is directly associated with fatty liver in human and feline models (Bugianesi et al. 2010; Hoenig 2002). However, it is difficult to interpret the results as handling stress and ketamine can also increase blood glucose levels (Hsu and Hembrough 1982). In feline hepatic lipidosis and mink in nursing sickness, do not shows severe impairment of glucose levels and appear to have normal glycemic conditions. Plasma ALT and Alk. phosphate levels are major indicators of liver dysfunctions (Kerner et al. 2005). However, plasma ALT and Alk. phosphate levels were not significantly different in treatment groups, indicating that 1% OA diet for 10 days was not enough for the development of fatty liver in mink. Plasma cholesterol levels in 2% arginine group and the combination group were significantly decreased compared to the control group. Female mink body weight changes are greater, compared to males, because

female are often pregnant from March onwards and the body weights of females tends to decrease the amount of body fat in autumn months (Tauson and Aldén 1984; Tauson 1993). This is an adaptive response to possible food scarcity in the winter months. In this study, NAI suggest that liver fat percentage progressed with 1% OA in the diet for 10 days, and a return to baseline level was possible with a mink diet composition of 2% Arg or 5% WPH or a combination of the two. The effects of the Arg and/or WPH were similar when administered before or after OA intake, indicating their roles in the prevention and treatment of OA-induced fat accumulation in the liver.

The major potential mechanism for the decrease in inflammation is bioactive compounds which are small proteins that are stimulated with digestion, suppressing the oxidative stress and inflammatory response. The antioxidant properties of whey have been linked to their high content of sulphur-containing amino acids such as methionine, cysteine, homocysteine, and taurine. However, there is some evidence that, whey protein hydrolysates can improve inflammation conditions. Calcitriol is a hormone that can be suppressed by high dietary calcium, favors fatty acid synthesis and inhibits lipolysis (Zemel and Sun 2008). Whey protein hydrolysates suppress calcitriol by calcium, which reduces reactive oxygen species and lack of availability of nutrients.

As a free amino acid, arginine functions as an intermediate in the urea cycle. Urea production takes place mainly in hepatocytes in mitochondria and continues in the cytosol. Ornithine accept a carbamoyl group from carbamoyl phosphate forming citrulline, which passes from the matrix to the cytosol further accepting a second amino group from aspartate, which yields argininosuccinate. The proceeding of the urea cycle involves two steps of cleavage; argininosuccinate is cleaved into fumarate and arginine, which is further

cleaved into urea and ornithine. Different type of metabolic and physiological conditions (triggered by illness or stress) may result partial deficiencies of enzyme production in urea cycle. These disturbances can be reducing the activity of the enzymes, which disrupts the urea cycle and prevents the body from processing nitrogen effectively. In particular, deficiency of argininosuccinate synthase can trigger the development of hypoglycemia, dislipidemia and fatty liver (Komatsu et al. 2008). Impairments of urea cycle and chronic liver failure are linked to systemic hyperammonemia (Machado and Pinheiro da Silva 2014), loss of β-oxidation and progression of hepatic fibrosis (Yaplito-Lee et al. 2013).

Jabecka et al. (2012) study confirms that increased concentration of arginine stimulates NO biosynthesis which leads to reduction of oxidative stress. Morris (2002) reviewed that, elevated arginase activity may reduce intracellular arginine concentration and regulate arginine availability for NO synthesis (Mori and Gotoh 2000). Moreover, high arginase activity in macrophages can induce reduction in NO production (Rutschman et al. 2001). Study conducted by Shi et al. (2001), elevated plasma arginine in arginase knockout mice model indicates that arginase contributes to maintaining arginine hemostasis. Pesce et al. (2009) study shows that, arginase as an essential suppressive mediator of alternatively activated macrophages and critical mediators of immune down-modulation in chronic schistosomiasis, which cause long term liver damage. According to the Morris (2002), in this study, elevated arginase activity triggered by induced inflammation condition in liver, resulted to regulate dietary arginine to induce NO synthesis.

5.2 Gene Expression in Mink Liver

5.2.1 Carnitine Palmitoyltransferase1A (CPT1A) Expression in Liver

Carnitine Palmitoyltransferase1A regulates the transfer of long chain acyl-CoA from the cytosol into the mitochondria for oxidation. From our study, the expression of the CPT1A genes tended to be increased with fatty liver development; Figure 4.12 shows that administering WPH before OA treatment resulted in significantly decreased CPT1A mRNA expression compared to the OA group. CPT1A activity is inhibited by malonyl-coA (McGarry et al. 1978), the product of the first step of FA synthesis, preventing simultaneous FA synthesis and degradation. Decreased FA oxidation in the liver, together with the mobilization and transport of free FAs from visceral adipocytes, are the main factors in the development of liver steatosis (Gentile et al. 2015). Several studies have documented the involvement of CPT1A in the development of liver inflammation in various fatty liver models (Koteish and Diehl 2001; Woo et al. 2014; Yang et al. 2014). Chronic starvation also increases the expression of β-oxidation genes via transcriptional mechanisms. Oxidative stress can reduce β-oxidation of fatty acids and prolonged fasting has been associated with higher level of β-oxidation (Kneeman et al. 2012). Peroxisome proliferator-activated receptor (PPAR)-alpha and its co-activator PPAR gamma co-activator 1 (PGC-1)-alpha are critical in enhancing the expression of CPT1A (Reddy and Hashimoto 2001). The qRT-PCR results of increased mRNA expression, which were observed in the liver, led to the hypothesis that early stages of fatty liver can increase hepatic TG accumulation, resulting in an increase in β-oxidation in the hepatocytes.

In contrast, subjects with NAFLD have hepatic mitochondrial structural and functional abnormalities including loss of mitochondrial cristae and paracrystalline inclusions (Sanyal

et al. 2001), a decrease in mitochondrial respiratory chain activity (Perez-Carreras et al. 2003), impaired ability to resynthesize ATP after a fructose challenge (Cortez-Pinto et al. 1999), and increased hepatic uncoupling protein 2. These abnormalities could represent an adaptive uncoupling of fatty acid oxidation and ATP production, which allows the liver to oxidize excessive FA substrates without generating unneeded ATP.

5.2.2 Microsomal triglyceride transfer protein (MTTP) Expression in Liver

The effects of OA and Arg/WPH treatments on fat content and NAI in mink was further investigated by measuring the expression of genes (mRNA) related to lipid metabolism in the liver. Microsomal triglyceride transfer protein belongs to a family of lipid transfer proteins that also includes apoB, lipophorin and vitellogenin. MTTP is a large 97 kDa protein that has three structural domains; the N-terminal β-barrel that binds to apoB, the middle α-helical region able to associate with both apoB and protein disulfide isomerase, and the C-terminal β-sheet that mediates the lipid binding and lipid transfer activity of MTTP (Hussain et al. 2003). The tissue expression of MTTP is highest in the liver and in the epithelial cells of the small intestine, the main organs producing apoB-containing lipoproteins, but MTTP is also expressed by other tissues including kidney, heart, retina, neurons, yolk sac, and immunological cells. The expression of MTTP is related to MTTP's role in lipid antigen presentation by cluster of differentiation 1 (CD1) proteins (Brozovic et al. 2004). Studies have shown that during the lipidation process, the affinity of MTTP for apoB decreases, allowing at a certain point the dissociation of MTTP from apoB, and the formation of a secretion-competent primordial VLDL (Hussain et al. 1997).

Figure 4.13 results show that feeding OA to the mink resulted in a significant treatment effect, with the OA mink having higher liver mRNA expression of MTTP compared to the control

group. Arg, WPH or a combination of both treatments did not have any preventative effect on the OA-induced elevated MTTP mRNA level. In fact, administering WPH before OA treatment resulted in the highest level of MTTP expression, and this may be responsible for the lower total liver lipid content in the mink group. However, MTTP expression levels was found to decline in the mink that received OA before WPH treatment, to a value similar to those of the control mink before and after the 10-day trial. The finding indicates that the whey peptide treatment played a role in modulating the lipid transport gene expression. The role of MTTP in the progression of NAFLD has been previously documented by several studies. In human models, Higuchi et al. (2011) reported that MTTP protein expression increases in cells proximal to the central vein and lessens toward the periphery of the lobule opposing the portal triad (Swift et al. 2003). The major function of MTTP in hepatocytes, as well as the previously discussed enterocytes, is to mobilize dietary and endogenous fat to other tissues through their incorporation into apoB-lipoprotein particles. Minehira et al. (2008) demonstrated that inhibition of VLDL secretion, through deletion of hepatic MTTP, resulted in hepatic steatosis in the absence of changes in peripheral lipid stores, along with an insulin sensitivity in mice. In our study, the increased MTTP expression in OA mink group could be due to the need for increased lipid transport due to liver fat accumulation in the mink, and vice versa for the OA-Ctrl+WPH group.

It has been reported that insulin resistance is associated with an increase in liver fat (Rector et al. 2010). Expression of MTTP, which is necessary for proper lipoprotein secretion, is regulated by the transcription factor Forkhead box O1 (FoxO1), which is in turn, also negatively regulated by insulin. Consequently, with normal insulin signaling, the inhibition of FoxO1 leads to decreased expression of MTTP, resulting in an inhibitory effect on VLDL secretion (Perilhou et al. 2008). An insulin-resistant state, on the other hand, would result in

increased expression of FoxO1, enhanced MTTP expression and finally, greater production of VLDL, as is seen in disease states. Hepatic FoxO1 abundance and MTTP expression are increased in mice with abnormal TG metabolism, suggesting that increased levels of MTTP play a role in the overproduction of VLDL. The effect observed for WPH indicate the possible beneficial role of the peptide treatment on lipid transport and metabolism, even after OA-induced hepatic lipid accumulation.

5.2.3 Glucose 6-Phosphatase (G6PC) Expression in Liver

Glucose 6-phosphatase is an important enzyme in glucose production. It catalyzes the conversion of glucose-6-phosphate to glucose in the last step of glucose production by glycogenolysis and gluconeogenesis in the liver. G6PC is located inside the lumen of the endoplasmic reticulum with the catalytic site facing the lumen. The function of G6PC is coupled to a transporter protein encoded by SLC37A4, which transports glucose-6-phosphate from the cytoplasm to the lumen. There are three isoforms of the G6PC gene family called G6PC 1, 2 and 3. The liver isoform known as G6PC or G6PC1 is expressed in hepatocytes, nephrocytes, enterocytes, and in β-cells. Although the major function of G6PC is the production of glucose, another function in the liver is to maintain intracellular homeostasis of glucose-6-phosphate by its preventing excessive elevation.

No significant different was observed for hepatic G6PC mRNA expression in the different treatment groups (Figure 4.14). G6PC is one of the key enzymes in gluconeogenesis and catalyzes the terminal step in both the gluconeogenic and glycogenolytic pathways. Long-chain fatty acids are known to increase G6PC mRNA and protein levels (Massillon et al. 1997). The Phosphatidylinositide 3-Kinases/ Protein kinase B (PI3K/AKT)-pathway also controls hepatic glucose metabolism in hepatocytes by the inhibition of the FOXO1, which

normally exists in an active form in the cell nucleus where it binds to the G6PC promoter and stimulates G6PC transcription (Nakae et al. 2001; Taniguchi et al. 2006). Additionally, G6PC activity can be inhibited by some amino acids (Mithieux 1997) and by fructose, which is probably mediated by fructose-1-phosphate (Robbins et al. 1991). Unsaturated free fatty acids reduce G6PC activity (Daniele et al. 1997), which is also seen at low concentrations of acyl-CoAs (Fulceri et al. 1995; Mithieux and Zitoun 1996).

5.2.4 3-Hydroxy-3-Methylglutaryl-CoA Synthase 2 (HMGCS2) Expression in Liver

Figure 4.15 shows that the expression of HMGCS2 in the liver of OA-fed mink was significantly different from the control groups. Administering Arg, WPH or Arg+WPH prior to OA did not result in any beneficial effect on the liver gene expression. Conversely, mink groups that received WPH or Arg+WPH after OA treatment had significantly reduced expression of HMGCS2, to levels similar to the control groups. Robust ketogenesis is limited to the hepatocytes due to the relatively restricted expression of the ketogenic enzyme, HMGCS2, under normal circumstances (Hegardt 1999). HMGCS2 gene and encoded protein are regulatory targets during conditions such as transition to starvation, diabetes, during adherence to low carbohydrate/high fat diets, ketogenic factors, and aging (Balasse and Fery 1989; Cahill 2006; Girard et al. 1992; Sengupta et al. 2010). Insulin suppresses HMGCS transcription, possibly via phosphorylation-induced sequestration of FOXA2 from the nucleus, while glucagon induces it via activation of the AMP regulatory element binding protein (Ayte et al. 1993; Hegardt 1999; Wolfrum et al. 2004). In addition, free fatty acids induce HMGCS2 in a peroxisome proliferator activated receptor (PPAR)-α dependent manner (Rodriguez et al. 1994; Wentz et al. 2010). The HMGCS2 mRNA

expression results suggests that OA-induced ketogenesis may be occurring in the mink hepatocytes, and that WPH may be primarily responsible for the modulation since the gene expression level was still high in the OA-Cntrl+Arg group. The findings raise the possibility that OA-induced ketogenesis could latently result in fatty liver disease and attest to the critical fine-tuning role that hepatic ketogenesis plays in the regulation of lipid metabolism.

5.2.5 Phosphoenolpyruvate carboxykinase (PEPCK) Expression in Liver

Gluconeogenesis, required to generate energy, occurs in periportal hepatocytes where the rate limiting enzyme of the pathway and PEPCK are zonally expressed (Giffin et al. 1993). Results of Figure 4.16 show that PEPCK mRNA expression was not significantly different in the mink groups. PEPCK expression has been reported to change in response to nutritional signals (Jungermann 1986). In contrast, glucose-6-phosphatase is found in pericentral hepatocytes where it converts glucose to glycogen for storage, when energy is not needed (Jungermann et al. 1982). While the focus of this study has been mainly concerned with pericentral genes, not much is known about the zonal regulation of periportal genes. PEPCK is regulated by many nutritional and hormonal signals (Roesler 2001), but the factor to silence it within the pericentral hepatocytes remains unknown. Linking the promoter to a heterologous gene drives the expression to a zonal pattern, much like that of the endogenous gene.

5.2.6 Glucose Regulated Protein 78 kDa (GRP78) Expression in Liver

As shown in Figure 4.17, GRP78 mRNA levels have been shown to be elevated in control mink after the 10-day study, and also in mink that received OA. Administration of Arg or Agrg+WPH before OA treatment resulted in further increase in GRP78 mRNA expression. Studies by Hanada et al. (2007) suggested that oxidative stress and the endoplasmic reticulum

(ER) resident chaperone glucose regulated protein 78 (GRP78) are associated with ER stress in human liver disease (Gonzalez-Rodriguez et al. 2014; Kammoun et al. 2009). Activation of the unfolded protein response (UPR) in the liver, as a result of ER stress, is a key feature in the development of steatohepatitis (Videla et al. 2004), insulin resistance, and type-2 diabetes (Ozcan et al. 2004). These elevated GRP78 mRNA levels in our study suggest that due to the initiation of the unfolded protein, mink may be developing endoplasmic reticulum stress, which can lead to oxidative stress. Hence, this may be indicative of entrance into the earliest stages of steatohepatitis development. Interestingly, this effect was observed in the mink regardless of OA treatment, and was further exacerbated in mink the received Arg alone or in combination with WPH. This suggests that Arg may be responsible in inducing this effect. Moreover, GRP78 mRNA expression in different treatment groups did not differ from that of the control group at the end of the trial (Figure 4.17). The increased GRP78 expression observed in this study can be due to any of the following physiological and environmental conditions: glucose deprivation, accumulation of unfolded proteins, intracellular calcium efflux, and any other pathological conditions that result in the disruption of normal ER function and homeostasis (Lee 2005).

Interestingly, decreased expression of genes of the two key enzymes of gluconeogenesis G6PC and PEPCK, which might be due to higher activity of Sirt1, leads to an increased phosphorylation (Wang et al. 2011a) and decreased acetylation of FoxO1 (Motta et al. 2004). The GRP78, HMGCS2 and MTTP mRNA expression results, taken together with the increased liver NAI, and the higher glucose and cholesterol concentrations (suggest the existence of fatty liver in the OA-treated mink). However, a lack of the presence of fibrosis and Mallory-Denk bodies suggest that the mink were possibly at the early stage of developing steatohepatitis. If the mink have been fed with a higher level of OA, it is likely

that they would have developed fatty liver disease. However, human development of NAFLD (Dam-Larsen et al. 2004) occurs over a long period of time and this may also be the reason for its occurrence in the mink.

The accumulation of lipids, in particular, lipids characterized by increased saturated fatty acids, can be toxic to cells, a condition termed lipotoxicity (Borradaile et al. 2006). Lipotoxicity is not a result of triglyceride accumulation, but rather, lipid-derived metabolites can activate inflammatory and ROS generating pathways. Saturated fatty acids, such as palmitate, are poorly incorporated into triglyceride and readily cause apoptosis in human and rat hepatocytes (Weigert et al. 2004). In the event that triglyceride accumulation is impaired, free fatty acids may no longer be safely incorporated into triglyceride pools and instead, act to increase susceptibility to liver injury and triggers, leading to NASH development (Borradaile et al. 2006). Simple fatty liver condition has been shown to be reversible in humans (Tiikkainen et al. 2003) and mice (Gonzalez-Periz et al. 2009). Based on results from this study, it is evident that 1% OA-induced mild steatohepatitis can be reversible within 10 days with diets containing 2% Arg or 5% WPH, or a combination of both.

CHAPTER 6: CONCLUSION

In this study, feeding of OA to female mink resulted in an increase in liver inflammation condition, which were accompanied by the elevated mRNA expression in HMGCS2 and GRP78 in the liver. Moreover, an increase in liver inflammation can be explained by the excess mobilization of hepatic glucose, which can result in a pro-inflammatory state, and an increase of liver lipids, which has been related to mRNA expression of different genes involved in lipid metabolism. However, comparing NAI, liver lipid and mRNA expression levels in the different treatments and prevention groups, Arg and WPH have demonstrated the ability to modulate the OA-induced conditions in the liver. In general, this research has offered insight on the prospects of using dietary L-arginine and whey peptide supplement in improving the production performance and health of female mink. Future studies would focus on evaluating if prolonged OA treatment, beyond 10 days, or higher OA levels would result in the development of severe fatty liver condition in mink, and whether there would be negative effects on the regulation of different biological interactions in the hepatocytes. Furthermore, the beneficial roles of L-arginine and the whey peptides need to be further studied towards the development of functional ingredients for health promotion.

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