THE EFFECT OF GRAPE SEED POLYPHENOLS AND DELTA TOCOTRIENOL AS SUPPLEMENTS IN IMPROVEMENT OF NASH HISTOPATHOLOGY IN C57BL/J6 MICE FED HIGH FAT DIET

A DISSERTATION

SUBMITTED IN PARTIAL FULFILLMENT OF REQUIREMENTS

FOR THE DEGREE DOCTOR OF PHILOSOPHY

IN THE GRADUATE SCHOOL OF THE

TEXAS WOMAN'S UNIVERSITY

DEPARTMENT OF NUTRITION AND FOOD SCIENCES

COLLEGE OF HEALTH SCIENCES

BY

SHRADHA SODHANI, B.SC.

DENTON, TEXAS

MAY 2012

TEXAS WOMAN'S UNVERSITY
DENTON, TEXAS

December 8, 2011

To the Dean of the Graduate School:

I am submitting herewith a dissertation written by Shradha Sodhani entitled "The effect of grape seed polyphenols and delta tocotrienol as supplements in improvement of NASH histopathology in C57BL/J6 mice fed high fat diet". I have examined this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy with a major in Nutrition.

Dr. Victorine Imrhan, Ph.D, RD, Major Professor

We have read this dissertation and recommend its acceptance:

Nathaniel Mills, Ph.D (Co-Chair)

Parakat Vijayagopal, Ph. D

Shanil Juma, Ph. D

Chandan Prasad, Ph.D
Department Chair

Accepted:

Dean of the Graduate School

ACKNOWLEDGMENTS

I take this opportunity to thank all those who have made this possible. I start with my advisor and mentor, Dr. Victorine Imrhan, for accepting me into her research group and for her guidance, encouragement, advice and support through the years. I would also like to extend my heartfelt appreciation to Dr. Nathniel Mills who has inspired me and given me advice and support for patiently teaching me the research techniques for enriching my future career.

I thank Dr. Andie Hseuh, Dr. Parakat Vijayagopal, Dr. Shanil Juma, Dr. Owen Kelly and Dr. Shazia Ahmed for offering their expertise, advice and time.

I am especially grateful to my colleagues and friends, Filsy Samuel, Shamali Ayyar and Kathleen Davis for being there every day for seven years. I could not have asked for better people to work with. I also thank Dr. Mills lab team for being such an excellent group and with all their help.

I am thankful to the Texas Woman's University Graduate Scholarship, The Reitch Scholarship for International students for supporting my education and research.
I thank my parents who taught me there is nothing more important in life than good education and have always encouraged me. Thank my Grandparents for their blessings. I thank my sister and brother-in law, Neha and Rupesh for giving me their constant love and support. Finally, I deeply share my love and trust in God.

ABSTRACT

SHRADHA SODHANI

THE EFFECT OF GRAPE SEED POLYPHENOLS AND DELTA TOCOTRIENOL AS SUPPLEMENTS IN IMPROVEMENT OF NASH HISTOPATHOLOGY IN C57BL/J6 MICE FED HIGH FAT DIET

MAY 2012

Nonalcoholic Fatty Liver Disease (NAFLD) and Nonalcoholic Steatohepatitis (NASH), which are histologically similar to alcoholic hepatitis are the most common liver conditions in USA. Diets high in fat have been associated with the development of obesity and NASH. Currently there are no therapeutic drugs approved by FDA available as treatment for these diseases; therefore attention has been directed towards the role of bioactive compounds like polyphenols and fat-soluble vitamin E in the treatment of NAFLD/NASH.

The objective of this study was to investigate the effects of dietary grape seed polyphenols (GSP, 1% and 2%) and δ- tocotrienol (δ3T, 0.025% and 0.05%) supplementation alone and in combination, incorporated into a high fat diet (HF) on NASH-like histopathologic features and liver fibrosis in C57BL/6J mice.

Mice were placed on HF diets which were supplemented with two doses of GSP, δ-tocotrienol or their combination. Histopathological changes of the liver were assessed using Hematoxylin and Eosin stain, periodic acid Schiff stain (P.A.S) and Masson's

trichrome stain and hepatic fibrosis by immunostaining. The morphometric analysis showed that the NASH -like histologic features increased in HF diet fed group, and reduced both by (i) GSP supplementation alone and (ii) in combination with δ3T. Steatosis was reduced by GSP with reduction of the lipid droplet area ($p \leq .01$) (cells/mm2). Overall, the HF diet was found to induce steatosis in the mice. The photomicrographs obtained from the three different histology stains showed the beneficial effects of GSP supplementation with reduction in mice mean liver weight, steatosis and absence of collagen accumulation and reduction in the activation of hepatic stellate cells (HSC) and liver fibrosis. Thus dietary GSP supplementation alone and in combination with δ3T in HF diet may ameliorate the NASH-like histopathological features in C57BL/6J mice.

TABLE OF CONTENTS

Page

LIST OF TABLES

Table Page

LIST OF FIGURES

Figure Page

CHAPTER I

INTRODUCTION

Chronic liver disease is the third most common cause of deaths in persons with nonalcoholic fatty liver disease (NAFLD) (Ong J.P et al, 2007). In the United States, NAFLD affects an estimated 30 million Americans (Clark J.M et al, 2003; Ruhl C.E et al 2003 and Browning J et al, 2004). The detection of NAFLD, liver fat content, possible histological changes related to NAFLD and disease activity is still an area with limited information, as the only available means of diagnosis is liver tissue evaluation from biopsy. A histological subtype of NAFLD is Non-alcoholic Steatohepatitis (NASH), with necroinflammation resulting from hepatocyte injury due to accumulation of excess lipids in liver. NASH is known to be a silent syndrome with unknown etiology but is found in 70-80% of persons with type 2 diabetes mellitus (Targher et al, 2007). People diagnosed with NASH are 70% obese and tend to be 40% heavier than their desirable body weight.

Approximately 20 to 80% patients with NASH have hyperlipidemia, either high plasma triglyceride levels and or high plasma total cholesterol levels. Obese persons who are diagnosed with hyperlipidemia often have insulin resistance; these two conditions are the features of metabolic syndrome (Met S) commonly observed in NASH (Chávez-Tapia NN et al, 2009 and Fabbrini E et al, 2010). NASH otherwise known as a benign

condition is on the rise in conjunction with obesity and type 2 diabetes mellitus (Williams R et al 2006;Ong J.P et al, 2007; Angulo P et al, 2007 and Fan J.G et al, 2009). NASH is one of the leading causes of liver fibrosis (Paschos P et al, 2009). Liver biopsy studies in obese individuals have demonstrated that 30-40% of patients develop simple steatosis with presence of inflammation and or necrosis (McCullogh A.J et al, 2006). It is estimated that about 30% of Americans are affected to some degree by hepatic fibrosis and cirrhosis (Ogden et al, 2006). In patients with chronic liver disease, 74% have fibrosis and approximately 30% have cirrhosis at the time of their initial liver biopsy (Clark J. M et al, 2006). NASH currently comprises of steatohepatitis of nonalcoholic origin that may progress to end-stage liver disease, that is, liver fibrosis, cirrhosis and or hepatocellular carcinoma (Powell E. E et al, 1990 and Fassio E et al, 2004).

Obesity related hepatic fat accumulation leads to increased secretion of inflammatory cytokines (Diehl et al, 2004 and Li Z et al, 2005). These cytokines are involved in activation of hepatic stellate cells (HSCs) which then secrete increased amount of collagenous substances. This causes the hepatic cellular morphology to be less contractile and more fibrocontractive with increased accumulation of alpha smooth muscle actin (α-SMA) (Friedman 2008). These changes can be used as a biomarker to detect the onset of liver fibrosis by immunohistochemistry (Cohen P.A et al, 1997; Feldstein A.E et al, 2005 and Lau D.T et al, 2005). Thus NASH may be defined as progressive condition with hepatic lipid accumulation, hepatocyte damage, lobular

inflammation, fibrosis and finally cirrhosis or even hepatocellular carcinoma (HCC) (Brunt E.M, 2004).

Both epidemiological and clinical studies on subjects with NAFLD provide evidence that diet restriction leads to overall body weight loss and reversal of fatty liver conditions in humans (Ueno T et al, 1997; Sahai A et al, 2004; Kim B.J et al, 2006 and Sato F et al, 2007). Nutrition is an important environmental factor and a cornerstone in therapy, while over-nutrition has known implications in the progression of NASH (Musso G et al, 2003; Solga S et al, 2004; Kechagias S et al, 2008 and Marchesini G et al, 2008). Many bioactive compounds found naturally in food have been reported to have various beneficial health effects. Reports have suggested that they play a role as antioxidants, anticarcinogens, antihypertensive and anti-artherogenic agents (Ko S.H et al, 2005; Reddy J.K et al, 2006; Day C.P et al, 2006; Qureshi K et al, 2007 and Shoelson S.E et al, 2007). Pilot trials using antioxidants; polyphenols and vitamin E have shown to act as lipid-lowering and hepato- protective agents with preliminary results showing reduction in appearance of characteristic features typical of NASH via histology (Neuschwander-Tetri B.A et al, 2003 and Vuppalanchi R et al, 2009).

Recent evidence from scientific studies report a moderate to strong relationship between chronic liver steatosis and diet low in antioxidants particularly vitamin E (Cave M et al, 2007 and Allard JP et al, 2008). Clinical long term intervention studies have shown vitamin E (as alpha tocopherol) to be the most promising agent for amelioration of NASH. (Sanyal AJ et al, 2010). Tocotrienols share their main property as natural

antioxidants with tocopherols, where the delta (δ) isoform of tocotrienol (δT3) with the unsaturated isoprene side chain has been reported to be very effective hypocholestrolemic agent (Song B.L et al, 2006). In a long term multicenter, randomized, double-masked, placebo-controlled trial in children with NASH (TONIC study) 400 IU of vitamin E, twice a day for two years, was beneficial in reversing NASH by reduction in hepatic cell size and also apoptosis (Lavine J.E et al, 2000).

Various phenolic compounds found in food are reported to have bioactive functions, the most potent being antioxidant properties. Some phenolic compounds may be included in the possible treatment of metabolic conditions like obesity, cardiovascular disease and different types of cancers (Aron M et al, 2008). Phenolic compounds promote lipid excretion or aid in reduced absorbance of fat from food items high in fat via the cytocrome P450 system during 'xenobiotic' digestion of phenolic compounds in liver (Osada K et al, 2006). Grapes and seed extract from grapes are rich in polyphenols. Grape seed proanthocyanidin extract (containing monomers and oligomers of flavan-3-ols) have been found to prevent oxidative injury (Puiggros F et al, 2005). Grape seed proanthocyanidins mimic the action of insulin, which aids in reduction of insulin resistance and improve insulin sensitivity in adipocytes (Pinent M et al, 2006) and therefore is beneficial in modulation of glucose homeostasis (Montagut et al, 2010). In an *in vivo* study with normolipidemic rats, grape seed extract decreased plasma triglycerides levels (TG) and apolipoprotein B (apoB) (Del Bas et al, 2005). In an *in-vitro* study GSP behaved similarly to insulin and improved insulin sensitivity in

adipocytes treated with different concentrations of grape extract (Terra X et al, 2007& 2009). Thus inclusion of food bioactive compounds into the diet can be convenient alternative to use of drugs/medications for the treatment of a disease condition.

Hypothesis

The hypothesis of this research study is that dietary grape seed polyphenols (GSP, 1% and 2%) and δ- tocotrienol (δ3T, 0.025% and 0.05%) supplementation alone and in combination incorporated into a high fat diet (HF) may improve the NASH-like histopathologic features and liver fibrosis in C57BL/6J mice. The high fat diet was used to induce NASH-like characteristic features and later the dietary supplements were added to high fat diet alone and in combination at two different concentrations.

CHAPTER II

REVIEW OF LITERATURE

Nonalcoholic fatty liver disease (NAFLD) and its advanced stage Nonalcoholic Steatohepatitis (NASH) both are defined as pathological liver diseases. They occur in the absence of alcohol, drugs or a specific cause (Chitturi S et al, 2002 and Angulo P et al, 2007). NASH is broadly defined as lipid accumulation resulting in cellular damage and inflammation, with or without scarring and fibrosis (Brunt E.M, 2004). NASH progresses from steatosis to fibrosis, cryptogenic cirrhosis, end stage liver disease, and or hepatocellular carcinoma (HCC) (Ludwig et al, 1980). The frequency of NAFLD in the US population is highest amongst Hispanic (45%) population followed by Caucasian whites (33%) and African Americans (24%) (Browning J.D et al, 2004).

Estimates obtained from clinical studies suggest 20% to 30% of individuals in Western countries have liver steatosis as the most common liver disorder (Hilden M et al, 1977 and Wanless I.R et al, 1990). NASH is prevalent in approximately 2-3% of the general population and 37% among morbidly obese individuals (Neuschwander-Tetri B.A et al, 2003; McCullogh A.J et al, 2006 and Machado M et al, 2006). Currently patients with hepatic steatosis are at an increased risk for developing NASH and related mortality (Portincasa et al, 2005). Approximately 25% of patients with NASH progress to cirrhosis and its associated complications such as liver failure and HCC (Adams L.A.et

al, 2005, Bugianessi E et al, 2002 & 2007, Ekstedt M et al, 2006 and Ogden et al, 2006). Liver fibrosis may or may not be present in individuals with steatosis but NASH may progress to fibrosis (Sheth S.G et al, 1997 and Contos M.J et al, 2004). Tissue architectural remodeling develops in 10-15% of NASH and 15-25% of cirrhosis patients (Matteoni CA, et al, 1999; McCullough A.J et al, 2004; Powell E.E, et al, 2005 and Farrell G.C et al, 2006).

Pathophysiology

The presence of hepatic steatosis, generally known as fatty liver (macrovesicular fat), is the most common histological finding in human liver biopsies. Sustained lipid peroxidation due to oxidation of free fatty acid (FFA) initiates inflammation in NASH. Steatosis progresses to steatohepatitis due to inflammation characterized by necro-inflammatory changes to fibrosis followed by cirrhosis and or HCC (Kashireddy P.V et al, 2004). Histologically, the liver of NASH patients exhibits micro- and macrovesicular steatosis and hepatocyte injury (mostly degeneration of cells) usually in the form of ballooning (Matteoni C.A et al, 1999). Inflammation may be lobular (Brunt E.M et al, 2005 and Hubscher S.G et al, 2006) or periportal typically localized in the acinar zone 3. There is occasional presence of mononuclear cells which replace the regular stellate cells or leukocyte infiltration at the initial stages of fibrosis (Brunt E.M et al, 2007; Yeh M.M et al, 2007 and Tiniakos D.G et al, 2009)

When fibrosis in the liver occurs there is the activation of hepatic stellate cells (HSC's) with accumulation of sinusoidal collagen which is part of the extracellular matrix (ECM) (Washington K et al, 2000 and Cortez-Pinto H et al, 2001). With the activation of HSCs there is loss in their contractile function and are transformed to myofibroblastic cells which secrete alpha smooth muscle actin (α-SMA). The α-SMA is used as a biomarker for early hepatic fibrosis detection via immunohistochemistry (Cohen P.A et al, 1997; Feldstein A.E et al, 2005 and Lau D.T et al, 2005). The change in the hepatic tissue promotes proliferation, migration and synthesis of various ECM products such as fibrillar collagens, fibronectin and cytokines. These are known to be responsible for the maintenance of balance between degree of inflammation and inflammatory cell recruitment (Reeves H.L et al, 2002 and Miele L et al, 2007). Other histological lesions in NASH are the occasional presence of (i) metamitochondria (enlarged mitochondria) or needle shaped, specifically seen in microvesicular steatosis, (ii) glycogenated nuclei, and (iii) iron deposition (Le Tri. H et al, 2004 and Zatloukal K et al, 2007).

Detection and Diagnosis of NASH

Histology

The extent of hepatocellular steatosis (percent of liver weight or percent of affected hepatocytes), the size of lipid droplets (macro-versus microvesicular) and zonal distribution (periportal, pericentral or diffused) can be distinguished only by histology

(Straub B.K et al, 2010). Thus the differentiation between steatosis and steatohepatitis can be confirmed by a histological approach (Oh M.K et al, 2008).

Grading and staging in NASH and fibrosis. The progression from steatosis to steatohepatitis and steatohepatitis with fibrosis and/ or liver cirrhosis is gradual. Therefore, histopathological staging systems have been developed to evaluate and monitor the progression of fibrosis by using the peculiar pattern of NASH and associated fibrosis (Kleiner D.E et al, 2005 and Longerich T et al, 2008). The histopathological classification of NASH has evolved in the last two decades from a two-step to a four step model (Wanless I.R et al, 2004). The classification of liver steatosis uses a four stage method with the first being characterized by fatty infiltration of the liver, second fatty infiltration with inflammation, third fatty infiltration with ballooning degeneration, and the last stage characterized by fatty infiltration with lesions similar to alcoholic hepatitis and sinusoidal fibrosis with or without Mallory's hyaline (Matteoni et al, 1999 and Angulo P et al, 2002). Stages three and four together were classified as NASH which cannot be distinctly observed. Brunt et al (1999 &2001) have proposed a histopathological grading and staging of NASH. It evaluates the degree of steatosis, the presence of hepatocellular ballooning and the lobular and portal inflammation in order to determine the grade and stages of the disease according to the extent of the fibrosis. The National Institutes of Diabetes and Digestive and Kidney Diseases (NIDDK) –sponsored NASH Clinical Research Network (CRN) has developed the histological scoring system which is uniformly used for all clinical trials involving NASH (Kleiner D.E et al, 2005).

Three distinct histologic lesions are necessary to make the diagnosis of NASH. These are zone 3 macrosteatosis, hepatocyte ballooning and mixed lobular inflammation. The CRN group proposed a NAFLD activity score (NAS) which is derived by addition of individual scores for steatosis, lobular inflammation, and hepatocellular ballooning and ranges from 0-8. Steatosis is scored (0-3), lobular inflammation 0-3 and ballooning 0-2. A 'NAS' score $\geq$5 correlates with a diagnosis of NASH whereas a score of<3 is not NASH.

Earlier grading system for fibrosis was staged from 0 to 4, where F1 is foci or extensive zone 3 perisinusoidal or pericellular fibrosis. This grading system for fibrosis in liver hepatitis includes *Grade 0*, no staining or very rare staining; *Grade 1*, staining in <30% of stellate cells in sinusoidal liver cells; *Grade 2*, staining between 31% and 60% cells; *Grade 3*, staining between 61% and 90% cells; and *Grade 4*, diffuse staining more than 90% of sinusoidal liver cells (Batts K.P et al, and Ludwig J.et al, 1995). While the fibrosis scoring with the NASH CRN system is based on the prototype staging method proposed by Brunt et al (1999), with a single difference that stage 1 is further sub-divided into three sub-stages to differentiate between delicate perisinusoidal zone 3 fibrosis (stage 1a), dense perisinusoidal zone 3 fibrosis (stage 1b), and portal fibrosis only (stage 1c) (Kliener D.E et al, 2005).

Treatment Options

Despite recent gains in understanding of NASH, several issues related to its progression and effective treatment remain unresolved (Sang Hoon Park et al, 2008). The US Food and Drug Administration (FDA) have not approved any medications for use specifically in NASH, and all medications are considered experimental. Based on the present knowledge of the pathogenesis of the disease, several human studies have highlighted the importance of multiple treatments. These include lifestyle modifications, nutritional supplements and prescription medications separately, or in conjunction with diet therapy.

Weight Loss

There is evidence that a 7% reduction in weight results in histological improvements in patients with NASH (Promrat K et al, 2010) and improves insulin resistance and intra-hepatic lipid content (Sato F et al, 2007). Weight loss can be achieved by combination of caloric restriction, exercise, and / or prescription drugs as well as surgical methods like bariatric surgery, in patients with morbid obesity (Dixon J.B et al, 2004; Suzuki. A et al, 2005; Klein S et al, 2006 and Chavez-Tapia NC et al, 2010).

Exercise

Studies have shown improvements in subjects with or without weight reduction suggesting beneficial effects of exercise with improvements in insulin sensitivity and

glucose homeostasis (O'Leary V.B et al, 2006). Data from exercise training shows prevention of diet-induced hepatic steatosis in rats (Gauthier M.S et al, 2003) via AMP-activated protein kinase (AMPK).

Pharmacological Treatment Options

Most of the anti-diabetic drugs which are available today are known to improve insulin sensitivity with possible use in liver steatosis and NASH. Recent meta-analyses demonstrate increased cardiovascular risk with pioglitazone, a class of thiazolidinedione which helps to improve insulin sensitivity (Chiquette E et al, 2004 and Mannucci E et al, 2008). There is a risk of edema, heart failure, and bladder cancer associated with long-term thiazolidinedione therapy (Dormandy J.A et al, 2005; Boden G et al, 2006, Gerstein H.C et al, 2006 and Rubenstrunk A et al, 2007). Other studies show that lactic acidosis associated with metformin drug therapy especially in patients with liver disease have a higher risk of bone fracture (Lalau J.D et al, 1995; Kruse J.A et al, 2001; Chang C.T et al, 2002 and Fitzgerald E et al, 2009).

Antioxidants as Treatment

The various side effects from the use of pharmacologic therapy (Dormandy J.A et al, 2005; Gerstein H.C et al, 2006) emphasize the need for alternatives like the intake of antioxidants in patients with NASH. Certain nutrients are being recognized to enhance insulin sensitivity via diet alone. These include vitamin E and its isomers and polyphenols namely resveratrol from grapes (Ko S H et al, 2005; Reddy J.K et al, 2006;

Day C.P et al, 2006; Qureshi K et al, 2007 and Shoelson S.E et al, 2007). Since these nutrients are known to be antioxidants and are found naturally in plants in abundance, they may have a high benefit to risk ratio which can be beneficial in treating liver steatosis and NASH with reduced side effects. *In vivo* studies in NASH show improvement in major histological features of disease activity, grade of steatohepatitis and occasionally of fibrosis following dietary therapy (Neuschwander-Tetri B.A et al, 2003 and Vuppalanchi R et al, 2009). Antioxidants, mainly vitamin E (Lavine J.E et al, 2000, Hasegawa et la 2001; Sanyal AJ et al, 2004; Bugianesi et al 2005;Yakaryilmaz et al 2007 and Sanyal AJ et al, 2010), betaine (Abdelmahek M.F et al, 2001), and ursodeoxycholic acid (Lindor K.D et al, 2004) have been proposed as effective supplements in patients with NASH.

Animal Models In Experimental Use

There is a lack of appropriate study models to understand the development of NASH and therefore provide data for efficient prevention and therapeutic options for treatment of NASH (Wanless I.R et al, 2004; Farrell G.C et al, 2006 and London R.M et al, 2007). Animal models to study NASH can be used to reproduce the *in vivo* results and may provide vital mechanisms in the pathophysiology of steatohepatitis (Fan J. G et al, 2009). Rodent models are known to effectively test the therapeutic function of certain natural food components like polyphenols and vitamin E isomers in our diet (Sanyal AJ et al, 2004 and Aron et al, 2008). The use of animal models with dietary modifications

similar in humans (high fat high caloric diets) may overcome one of the major limitations to reproduce human NASH-like characteristic features. These rodent models may study both the combined features of steatohepatitis and the metabolic abnormalities associated with NASH.

One of the best approaches to induce NASH via *in vivo* models is nutritional or dietary approach which involves over-nutrition, overfeeding of saturated and unsaturated fat, and/ or methionine and choline deficient (MCD) diet (Musso G et al, 2003; Sundaram S.S et al, 2005; Carmiel- Haggai M et al, 2005; Deng Q.G et al, 2005; Svegliati-Baroni G et al, 2006; Zou Y et al, 2006; Romestaing C et al, 2007; Baumgardener J.N et al, 2008 and Rinella M.E et al, 2008). Studies have reported that C57BL/J6 mice develop obesity, insulin resistance, diabetes mellitus, hypertriglyceridemia (Fraulob J. C et al, 2010) and advanced fatty liver disease when fed a high fat diet (Gallaou-Kabani et al, 2007 and Cong et al 2008). Rinella and Green (2004) reported that the methionine and choline deficient (MCD) diet-induced mice model reproduced most of the histological features of human steatohepatitis; however their mice model did not develop insulin resistance and obesity. Apart from the MCD diet, diets containing high fat have been widely used to induce steatosis and NASH in animals with characteristic features including oxidative stress and fibrosis. Thus a high fat diet (HF) mouse model may promote a better experimental system to investigate NASH, to examine treatment methods that prevent progression of steatosis and to correlate the degree of liver steatohepatitis with obesity and metabolic syndrome. Compared with other animal models, such as Zucker obese

rats, ob/ob or db/db mice or other gene deletion animal models, C57BLJ6 mice on high fat diet reproduces human obesity, serving as a research model based on diet with high caloric intake from increased fat intake (Gallaou-Kabani et al, 2007). The C57BLJ6 mouse model was used to induce NASH with the use of modified high fat in the rodent diet (Cong et al 2008). This rodent strain appropriately mimics the spectrum of pathologic and pathophysiologic features associated with NASH (Fraulob J.C et al, 2010). Study of C57BL/J6 mice has provided information about the pathogenesis of diet induced nonalcoholic steatohepatitis features (Sundaresan S et al, 2010). These studies with high fat diet were able to show the characteristic pathophysiologic features of NASH including an enlarged pool of FFAs, insulin resistance, subclinical inflammation and oxidative stress (Fraulob J.C et al, 2010, Sundaresan.S et al, 2010).

Vitamin E-Tocopherols and Tocotrienols

Vitamin E ($C_{29}H_{50}O_2$) is a general term used to refer to a group of naturally occurring compounds including tocopherols and tocotrienols (Kamal- Eldin & Appelqvist, 1996). Vitamin E has a chroman ring structure with an isoprene side chain and is divided into two groups, i.e., tocopherol with a saturated isoprene side chain and tocotrienol with three double bonds in the farnesyl isoprenoid side chain. This may account for differences in their efficacy and potency *in vitro* and *in vivo* (Serbinova E et al, 1991; Suzuki Y.J et al, 1993; Sen C.K et al, 2006; Roy S et al, 2002 and Yoshida Y et al, 2003). Each group has four isomers (α, β, γ and δ), making a total of 8 forms of vitamin E. The isomeric forms of tocotrienol are

distinguished by the number and location of the methyl groups on the chromanol rings: δ tocotrienol is 8- monomethyl. Tocotrienols are natural vitamin E isomer found in select vegetable oils, cereal grains like rye, barley, wheat germ, oats, saw palmetto, palm, annatto seeds, rice bran and certain nuts. It is a potent antioxidant that is used as a nutritional supplement. Patients with various types of liver disease frequently have low serum vitamin E concentrations. The antioxidant property depends primarily on the phenolic group in the chromanol ring, rather than the side-chain (Niki E et al, 1985 and Steiner M et al, 1995). Some evidence suggests that enzymes of the gut bacteria in chickens can convert tocotrienols to tocopherols (Qureshi A.A et al, 2001 and 2002).

Tocotrienols, like tocopherols, are capable of scavenging and quenching reactive oxygen species, also known as free radicals. Their antioxidative activity, however, resides mainly with the ability to neutralize peroxyl and alkoxyl radicals generated during lipid peroxidation (Burton G.W et al, 1990 and Kamal-Eldin A et al, 1996). Serbinova et al (1991) observed higher antioxidant activity with tocotrienols against lipid perioxidation in rat liver microsomes than with α-tocopherol (Sebrinova E et al, 1991). Suarna et al (1993) reported that when rats or humans were treated with tocotrienols and tocopherols, tocotrienols provided higher oxidative protection compared to tocopherols (Suarna C et al, 1993). Tocotrienols in the tocotrienol rich fraction (TRF) of palm oil were found to reduce total cholesterol and LDL- cholesterol levels through down regulation of hepatic HMG-CoA reductase activity (Iqbal J et al, 2003). Chou et al

(2009) observed that rice bran oil rich in tocotrienols improved lipid abnormalities, reduced the artherogenic index and suppressed the hyperinsulinemic response in rats with streptozotocin/nicotinamide-induced type-2 diabetes mellitus (Chou T.W et al, 2009). In a similar study, TRF intake from palm oil in rats not only reduced serum glucose and glycated hemoglobin concentrations, it also reduced plasma total cholesterol, LDL-cholesterol and triglyceride levels, and increased levels of high- density lipoproteins (HDL-cholesterol), compared to the untreated group (Budin S.B et al, 2009). A combination of insulin and tocotrienol treatment prevented biochemical and molecular changes in the diabetic condition and reversed neuropathic pain through modulation of oxidative and reactive nitrogen stress and release of inflammatory cytokines in streptozotocin (STZ) induced diabetes in rats (Kuhad A et al, 2009 and Kuhad A, Bishnoi M et al, 2009). In a large study, a number of mechanisms were shown to contribute to its higher antioxidant activity compared to α- tocopherol. First, because of structural differences, tocotrienols may be more uniformly distributed in the lipid bilayer. Second, the chromanol ring of tocotrienols may interact more efficiently with the lipid bilayer than that of tocopherols. Third, tocotrienols may have a higher recycling efficiency (Sebrinova E et al, 1991). Fourth, cellular uptake of tocotrienols is 70 times higher than that of tocopherols (Saito Y et al, 2004). These factors may contribute to tocotrienol's greater efficacy. These results indicate that tocotrienols have anti-lipidemic and anti-diabetic potential, which are the major factors in the etiology of NASH.

Tocotrienol isoforms. The isoforms of tocotrienols, which differ in their number of methyl groups on the prenyl side chain, also differ in their biological activities. *In vitro* studies suggest that there may be as much as a 30-fold difference in the ability of α, γ, and δ isomers of tocotrienol to inhibit cholesterol biosynthesis (Pearce B.C et al, 1992). Alpha-tocotrienol has higher antioxidant capacity than γ-tocotrienol followed by δ-tocotrienol (Kamal- Eldin A et al, 1996). This was reported in a study when γ tocotrineol was administered orally in mice; γ tocotrienols appeared faster in the plasma but at lower levels than tocopherols due to increased intestinal epithelial cell absorption (Tsuzuki W et al, 2007). The anti-proliferative potencies of the three isomers are; δ-tocotrienol higher than γ-tocotrienol followed by α-tocotrienol (McIntyre B.S et al, 2000 and Inkouchi H et al, 2003). Tocotrienol rich fractions (TRFs) from palm oil and desmethyl tocorienols (γ and δ) from annatto extract inhibit the enzyme HMG-CoA reductase (HMGR) in the *de novo* synthesis of cholesterol (Song B.L et al, 2006). Palm oil TRF improved whole body glucose utilization and insulin sensitivity in C57BLKS/J-Lepr db/db mice by regulating PPAR target genes (Fang et al, 2010). In an *in vivo* study, treatment of low-density lipoprotein receptor (LDLr)-deficient mice with 1mg/day (50mg/kg/day) of γδ – tocotrienols for one month showed a 28% and 19% reduction in cholesterol and triglyceride levels, respectively, whereas high-density lipoprotein (HDL) level was unaltered (Zaiden N et al, 2010). These results were confirmed in an *in vitro* model of HepG2 liver cell lines. The use of TRF from palm oil as treatment suppressed the upstream regulators of lipid homeostasis in STZ treated diabetic rats leading to the

suppression of triglycerides, cholesterol and VLDL biosynthesis as the primary benefit caused by ingestion of TRF (Budin S.B et al, 2009). The delta (δ) isomer of tocotrienol (δ3T) has been reported to be an effective hypocholestrolemic agent (Pearce B.C et al, 1992; Qureshi et al, 2001 and Zaiden N et al, 2010).

Grape Seed Polyphenols

Polyphenols from grape seed especially oligomeric proathocyanidins, have been known to have high antioxidant activity (Koga T et al, 1999 and Shi J et al, 2003). Foods rich in proanthocyanidins have high oxygen radical absorbance capacity which has been linked to numerous health benefits such as weight management, cell health, and cardiovascular health. Studies have shown that phenolic compounds from plants may aid in the maintenance of lipid homeostasis by increasing anti-lipogenic activities such as increased lipid excretion or reduced absorption (Osada et al, 2006). Grape seed extract consists of compounds known as flavanoids, and flavan-3-ols or the proanthocyanidins. Polyphenols including falvonoids, flavan-3-ols (Appendix A, Figure 1) and their oligomeric forms (proanthocyanidins) have been reported to be successful in exhibiting several health beneficial effects including antioxidant, antimicrobial, cardiopreventive, anti-viral and neuro-protective (Aron et al, 2008). Antioxidant substances such as polyphenols have been widely recognized to hinder degenerative processes that underlie chronic diseases.

Polyphenols found in grapes alter activities of important cell signaling enzymes such as tyrosine kinase, phosphodiesterases and phosphoinositide kinases (Harmon A.W

et al, 2001; Pinent.M et al, 2004 and Gruzman, A et al, 2009). Recent data show that the polyphenols from grapes decrease plasma triglycerides and cholesterol accumulation in the aorta of ovariectomized guinea pigs (Zern T.L et al, 2005). They produce results similar to resveratrol (Rayalam S et al, 2008). Both *in vitro* and *in vivo* studies show positive effects on reducing lipid synthesis, inflammation and therefore overall attenuation of steatohepatitis (Pinent. M et al, 2004). In another study atherogenic diet was used to induce steatohepatitis in C57BL/J6 mice, and resveratrol was used as treatment, resulted in amelioration of dyslipidemia and steatohepatitis induced by atherogenic diet (Ahn. J et al, 2008). The dimethylnitrosamine induced liver fibrosis in rats was reversed by resveratrol which was confirmed by histology showing improvement in hepatic steatosis, necro-inflammation, and fibrosis with reduction in alpha smooth muscle actin (α-SMA) (Lee E.S et al,2010 and Hong S.W, et al 2010). In a single-blinded crossover trial reported, that consumption of lyophilized grape powder per day (equivalent of 1.5 cups/d of grapes) lowered triacylglycerides by 15% and 6%, ($P < 0.002$) in pre- and postmenopausal women (Zern T.L et al, 2005). In an *in vitro* study grape seed extract prevented the differentiation of adipocytes (Pinent M et al, 2005 and Baiges I et al, 2010). This was repeated in an *in vivo* study in female Wistar rats fed high-fat diet and treated with grape seed polyphenol extract (25 mg/kg-1 of body weight), which improved dyslipidemia by repressing lipogenesis and very low density lipoprotein (VLDL) assembly in the liver (Quesada. H et al, 2009). Another study used Sprague Dawley rats and fed them a high-fat diet supplemented with procyanidin-rich

polyphenols (1%) from tea (TP), hops (HP) or apples (AP). The metabolites of procyanidins from apples, which are oligomers of (−)-epicatechin, as well as catechin monomer, modulated hepatic cholesterol 7a-hydroxylase (CYP7) enzyme activity and consequently increased the excretion of acidic steroids into feces (Osada et al, 2006). The hypo-cholesterolemic activity of GSP was most likely mediated by enhancing bile acid excretion (Feng Q et al, 2002, and Tsang C et al, 2005). These strongly suggest that polyphenols from grape seeds may act as functional agent for preventing and treating lipid altered metabolic states. Polyphenols from grape seeds may be beneficial for treatment and prevention of liver steatosis and inflammation and / or liver fibrosis (Sun G.Y et al, 1999; Khoshbaten M et al, 2010 and Aoun M et al, 2010).

Increasing research has been done on the different forms of vitamin E, alone (Lavine et al, 2000) or in combination, with insulin sensitizers (Sanyal A.J et al, 2010), to reveal the most effective vitamin E isomer in nonalcoholic steatosis treatment. The general possible mechanisms that explain how grape seed polyphenols (GSP) (Puiggros et al, 2005; Del Bas et al, 2005; Osada et al, 2006; Cave M et al, 2007 and Allard JP et al, 2008) exert protective effects on nonalcoholic steatohepatitis (NASH) have been studied and identified. However, knowledge of the mechanism of action of grape seed polyphenols and delta tocotrienol in steatohepatitis and fibrosis prevention is limited. Keeping in view the reviewed literature, the present study aims to investigate the effect of dietary supplementation of grape seed polyphenols (GSP) and delta tocotrienols (δ3T) in improvement of NASH histopathology in C57BL/J6 mice fed high fat diet

CHAPTER III

MATERIALS AND METHODS

The present study is part of a larger study.

Animals

Briefly Sixty-four 5-week old C57/BL/J6 male mice, were obtained from Jackson Laboratories (Maine, US) and housed 4/cage. Mice were acclimated to the TWU vivarium with water and food *ad libitum* for 4 weeks at 25° C with a 12-hour light/dark cycle. All animals were cared for in accordance with the Texas Woman's University Institutional Animal Care & Use Committee (IUCAC) guidelines (Denton, TX).

Diets

A diet modeled on the high fat diet (HF) with 42% calories from fat (Teklad Diet 88137) was utilized. The control diet followed American Institute of Nutrition (AIN), growth purified (all contents defined) diet (AIN-93 G, Normal fat – NF-C) recommendations with 7% calories from fat (Teklad Diet 09752). All diets were purchased from Harlan Teklad (Table 1, Appendix B). Food intake was measured and animal weights were recorded twice per week using a digital balance (Sartorius, Germany). Diets (experimental and control) were freshly prepared weekly by addition of supplements, either, δ tocotrienols (δ 3T, DeltaGold® 70, American River Nutrition, Inc, MA) or grape seed extract (GSP,

Leucoselect,® Indena S.p.A, Spain) to TD 88137 (Table 1, Appendix B). Diets were stored at 4° C and feeding cups were replenished every 3rd day. Concentrations of δ 3T and GSP were used in accordance to Qureshi et al, 2001; Yu.G et al, 2006; Pinent et al, 2004 and Osada et al, 2006 for all groups.

Experimental Design (Figure 1)

All animals were acclimated for 4 weeks and were fed mouse chow; at this time point seven of the eight groups were started on high fat diet, with 42% calories from fat (HF) and were continued on the diet for 16 weeks. At the end of ten weeks, six of the seven groups on HF were continued on HF that was supplemented with δ 3T (0.025% & 0.05%) and or GSP (1% &2%) respectively.

Post acclimatization, all animals remained on their respective diets until the termination of the study. At the end of twenty weeks, all mice were fasted overnight, and sacrificed the next morning. Following decapitation, trunk blood of mice was collected. Two-hundred milligrams of tissue from each liver was immediately harvested for histopathological analyses and the remaining liver tissue was stored at -80°C for later analyses.

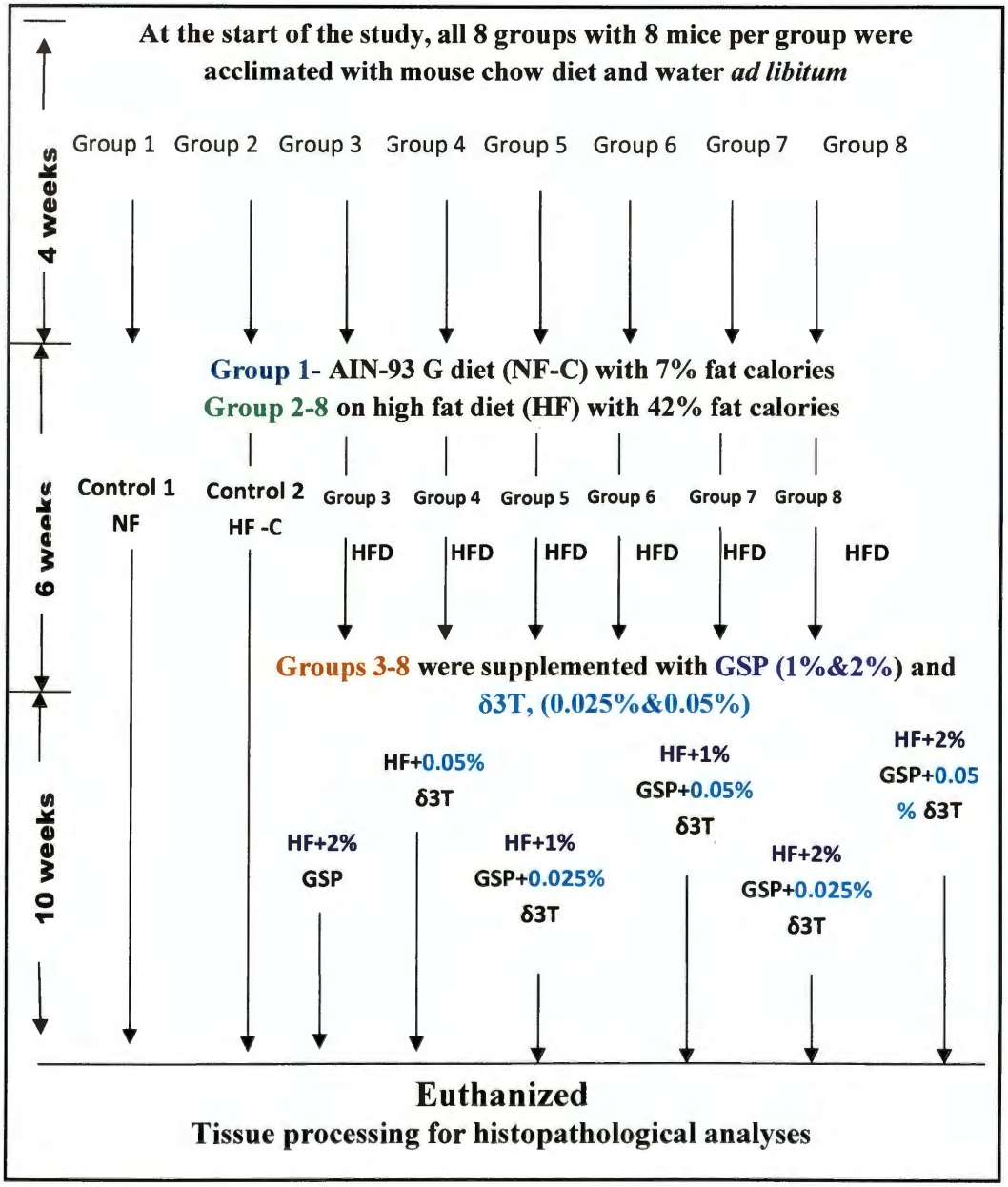


Figure 1: Flow chart of study design and time line

HF – High-fat diet

Group 1- AIN-93 G diet- Control 1, 7% calories from fat **(NF-C)**

Group 2- high-fat diet –Control 2, 42% calories from fat **(HF-C)**

Group 3- high-fat diet + grape seed poly phenols (2%) **(HF+G2)**

Group 4- high-fat diet + δ-tocotrienol (0.05%) **(HF+ T0.05)**

Group 5- high-fat diet + grape seed poly phenols (1%) + δ-tocotrienol (0.025%) **(HF+ G1+T0.025)**

Group 6- high-fat diet + grape seed poly phenols (1%) + δ-tocotrienol (0.05%) **(HF+ G1+T0.05)**

Group 7- high-fat diet + grape seed poly phenols (2%) + δ-tocotrienol (0.025%) **(HF+ G2+T0.025)**

Group 8- high-fat diet + grape seed poly phenols (2%) + δ-tocotrienol (0.05%) **(HF+ G2+T0.05)**

Histopathological Analyses

Fresh liver samples were washed with phosphate buffered saline (IX PBS, pH 7.4), cut into 3-4 mm cubes, and then immersed in modified Davidson's fluid and fixed at 4° C for 24 hours with a change of fixative at 2-3 hours following the initial immersion (Latendresse et al, 2002). This was followed by serial dehydration of tissues in 70% ethanol for 3 hours twice, 90% ethanol for 2 hours, and then 100% ethanol twice for 2 hours each, all at 4°C. The solution changes were carried out sequentially to facilitate the transition of tissue samples from more polar to less polar solutions in the process of chemical dehydration in preparation for paraffin wax embedding. Then the tissue was placed in, a 50-50 ethanol –xylene solution for two hours at room temperature, followed

by 2 changes of 100% xylene for 2 hours each and then followed with a 50-50 xylene-wax solution at 60 °C for two to three hours. Then after two changes of 100% paraffin wax, the processed liver samples were placed in heated molds and then embedded in paraffin wax placed in molds with labels. After removal from the molds, the wax blocks with the embedded tissue were stored at 10° to 15°C until sectioned. Paraffin embedded tissue was than sectioned at six micron using a rotary microtome (Reichert Inc.). Ribbons were then floated onto a 40° C water bath. After spreading, sections were then collected on a collagen coated subbed glass slides. Each glass slide had three serial liver sections. Liver from each of six mice for all eight groups were sectioned and analyzed. Ten to fifteen slides were prepared from each liver sample and the slides with sections were stored at 15°C until used (Appendix C).

Analysis of histological changes was conducted by using slides from all the eight groups. Slides were stained in triplicate from each of six animals for each of the three stains used (1) Hematoxylin and Eosin; (2) Periodic Acid Schiff; and (3) Masson's trichrome. To conduct each staining procedure the slides were deparaffinized in three changes of xylene and sequentially rehydrated in graded ethanol (100, 90% and 70%) for 3 to 5 minutes each. After the slides were stained they were serially dehydrated in graded ethanol (70, 90% and 100%) xylene and were than mounted with a cover-slip using permount as the mounting medium.

The hematoxylin and eosin (H&E) stain was used to assess liver morphology. Hematoxylin stain (American Master*Tech Scientific Inc. USA) allows evaluation of nuclear localization in the tissue; Eosin is used as a counterstain for protein and allows general assessment of hepatocyte cytoplasmic morphology. These stained sections were also used for morphometric analysis of lipid droplets in hepatocytes.

Masson's Trichome stain (One Step Trichrome kit # KTTRGPT, American Master*Tech Scientific Inc. USA) was used to distinguish hepatocytes from the surrounding connective tissue. The collagen fibers secreted by connective tissue stains bluish, while nuclei are stained blue or black (Modified Mayer's Hematoxylin, American Master*Tech Scientific Inc. USA) and the background is stained red. In the trichrome stains, that employ more than one acid dye, use is made of ***dye competition*** to distinguish different cellular distributions.

The periodic acid Schiff stain (PAS) (PAS stain kit # KTPAS, American Master*Tech Scientific Inc. USA) plus hematoxylin is used to detect glycogen stores and glycoproteins. In hepatic tissues sugars with adjacent hydroxyl groups (1, 2 glycols) or amino and hydroxyl groups are oxidized to aldehyde groups with periodic acid (0.5%). Schiff's reagent reacts with the aldehyde groups to produce a reddish or magenta appearing precipitate. This technique identifies a number of polysaccharides or carbohydrate-containing proteins with hydroxyls on adjacent carbons. The PAS sections were counter stained with hematoxylin for nuclear identification and light green stain for

cytoplasm. With PAS stain, the glycogen and glycoproteins were stained magenta or reddish purple and the nuclei are stained dark blue (Appendix C).

All of the above staining techniques were carried out in triplicates for each liver sample of 8 mice per group. These observations under the light microscope were captured by the camera to get photomicrographs from each mouse per group which is represented as a single photomicrograph from each group as a panel from 4x to 40x magnification for comparisons and histopathological patterns and changes from them as qualitative data to analyze levels of steatosis and fibrosis using the grading scale (Appendix D). H&E stained slides with transparent area as lipid vacuoles within the hepatocytes were quantified using software (NIS-Element) for the light microscope (Eclipse 90i ® Nikon Inc., USA). The measurements were performed using area radii and circularity as the two main factors of selection on the H & E stained sections for counting the hepatic cells which had lipid vacuoles. Cells with lipid droplets were counted in ten randomly chosen areas, at 100X magnification for 0.56 mm^2 of each area of a section selected. Each value is multiplied by 1.786 to correct to 1.0 mm^2 (1.0 mm^2/0.56 mm^2). The total number of lipid droplet was divided by 10 to determine the mean number of lipid filled cells per mm^2.

Staining with the above dyes therefore resulted in a known color and pattern based in part on the acid base characteristics of the tissue. Color varies not only with specific stains used, but also with the conditions that exist during preparation of the slide.

These include stages from the initial fixing in solution to the ionic strength of the staining solution and the differentiating solvents utilized after staining.

Immunohistochemical Analysis

To evaluate fibroblast infiltration that is indicative of fibrosis liver sections from three mice each of the eight, one control and remaining treated groups (six μm-thick sections of formalin-fixed, paraffin-embedded) were used for immunofluorescence staining, using rabbit anti-mouse alpha smooth muscle actin (α-SMA) antibody. Positive and negative controls on one of the three adjacent sections for each slide for each group were used for statistical analyses. Slides were deparaffinized in five changes of xylene and serially rehydrated in graded ethanol (100, 90% and 70%). Slides were treated with 0.1 mM EDTA for 15 minutes, and then rinsed in 1X Phosphate buffered saline (PBS buffer) for 10 minutes. Slides were incubated with 10% donkey serum for one hour at 37°C for blocking; primary rabbit polyclonal anti-α-SMA was added to sections only (1:200 dilution, sc-130619, SantaCruz Biotechnology Inc.CA. USA) and then incubated overnight at 4° C, followed by the secondary antibody donkey anti-rabbit DyLight 594 (1:200, 711-495-152, Jackson Immuno Research Inc, PA, USA) for at least one hour at 37° C. The slides were rinsed with 1X PBS buffer solution for 5 minutes thrice, nuclear staining was done using 4, 6-diamidino-2-phenylindole (DAPI) diluted into the mounting media (1.2μg/ml, VECTASHIELD, Vector Labs, CA USA). Negative controls were

performed by omitting the primary antibody. The presence of fibrosis as HSC-reactive - α-SMA was scored (Cohen P.A et al, 1997 and Lau D.T et al, 2005) (Appendix E).

Statistical Analysis

All samples were analyzed using a one-way analysis of variance (ANOVA) to compare diet and intervention mediated effects on whole body and tissue parameters. $P < 0.05$ was considered as significant followed by the post hoc test known as least significance difference (LSD). Simple correlation and linear regression tests were performed to identify potential relationships among experimental parameters using SPSS, version 15.

CHAPTER IV

RESULTS

The present study utilized a high fat diet (HF) to induce hepatic steatosis and NASH in C57BL/J6 mice to investigate the effect of supplementing the HF diet with grape seed polyphenols (GSP) and delta tocotrienol (δ 3T) for the last ten of the sixteen week HF diet treatment. The effects of HF diet with supplementation were evaluated by analysis of tissue weights and histopathological changes to features of NASH. Across this study of twenty weeks, sixty one C57BLJ/6 mice out of sixty four survived. This resulted in three groups with seven mice and five groups with eight mice each. The AIN-93G, normal fat control (NF-C) group, high fat diet control (HF-C) group and one of the combination HF supplemented treatment group (HF+1% G1+T 0.025) had a total of seven mice while the remaining five supplemented groups had eight mice in each respective groups HF+ G2, HF+ T0.05, HF+G1+T0.05, HF+G2+T0.025 and HF+G2+T0.05.

Liver Weight and Combined Adipose Tissue Weight

The mean liver weights for each group were calculated at termination of the treatment at 16 weeks. Mean liver weight was significantly higher ($p<0.05$) in HF-C (3.87±0.53 g) group compared to NF-C group (2.12±0.36 g). The liver weight of the

HF+T0.05 group was comparable to HF-C group. However, the liver weights of all HF groups supplemented with grape seed polyphenols (GSP) were significantly smaller than HF-C and HF+T0.05 (Table.1 and Figure.2). The liver weights of HF+G2 group was 1.62±0.14 g followed by the four combination groups; (HF+G1+T0.025, 2.04±0.33 g; HF+G1+T0.05, 1.99±0.14 g; HF+G2+T0.025, 1.61±0.17 g and HF+G2+T0.05group, 1.64±0.10 g) compared to HF-C as 3.87±0.53 g indicating that GSP supplementation at either concentration (1% &2%) produced smaller liver weights. Overall this suggests that no benefit was obtained from HF fed mice with δ3T supplementation, and may provide little amelioration in the gross liver weight increase observed with NASH.

The adipose tissue from each mouse was excised from epidydimal fat pad and peri-renal depots and weighed as combined adipose tissue weight from each group. The mean combined adipose tissue weights of each group were calculated for all groups (Table. 1 and Figure.2). There was increase in epididymal fat pad and peri-renal fat weight, which was clearly observed in HF-C mice (2.62±0.50 g). The adipose tissue weight of HF+ G2 group was 1.96±0.10 g and the four combination groups; HF+G1+T0.025 was 2.43±0.48 g; HF+G1+T0.05 was 2.43±0.17 g and; HF+G2+T0.025 was 1.52±0.31 g; HF+G1+T0.025 was 2.02±0.03 g which was decreased compared to HF-C fed groups ($p<0.05$) (Table.1 and Figure.2).

The increase in liver weight and combined adipose weight was significant in HF-C fed mice compared to NF-C fed mice ($p<0.05$). There was significant reduction in liver

weight and combined adipose weight in HF+G2 fed group compared to HF-C fed ($p<0.05$) mice followed by all four combination supplemented groups. The data for mean liver weight and combined mean adipose weight are summarized in Table 1.

Table 1

Liver Weight and Combined Adipose Tissue Weight in C57BL/J6 mice

Parameters	**Mean Liver weight (g)**	**Combined Adipose weight (g)**
Group (n)		
NF-C	2.12±0.36	2.20±0.44
HF-C	3.87±0.53#	2.62±0.50#
HF+G2	1.62±0.14*	1.96±0.10*
HF+T0.05	3.75±0.38	2.77±0.23
HF+G1+T0.025	2.04±0.33*	2.43±0.48
HF+G1+T0.05	1.99±0.14*	2.43±0.17
HF+G2+T0.025	1.61±0.17*	1.52±0.31*
HF+G2+T0.05	1.64±0.10*	2.02±0.03*

*P<0.05 compared to HF-C group, # P<0.05 compared to NF-C group, (n=8)

Data points obtained by two-way paired ANOVA, SPSS. (Mean ±SE)

NF-C, low fat control diet (n=7) ; **HF-C**, high fat control diet (n=7) ; **HF+G2**, HF+ 2% GSP; **HF+T0.05**, HF-C+ 0.05% delta tocotrienol; **HF+G1+T0.025**, HF+1%GSP+0.025% δ3T (n=7); **HF+G1+T0.05**, HF+1%GSP+0.05% δ3T; **HF+G2+T0.025**, HF+2%GSP+0.025% δ3T; **HF+G2+T0.05**, HF+1%GSP+0.025% δ3T.

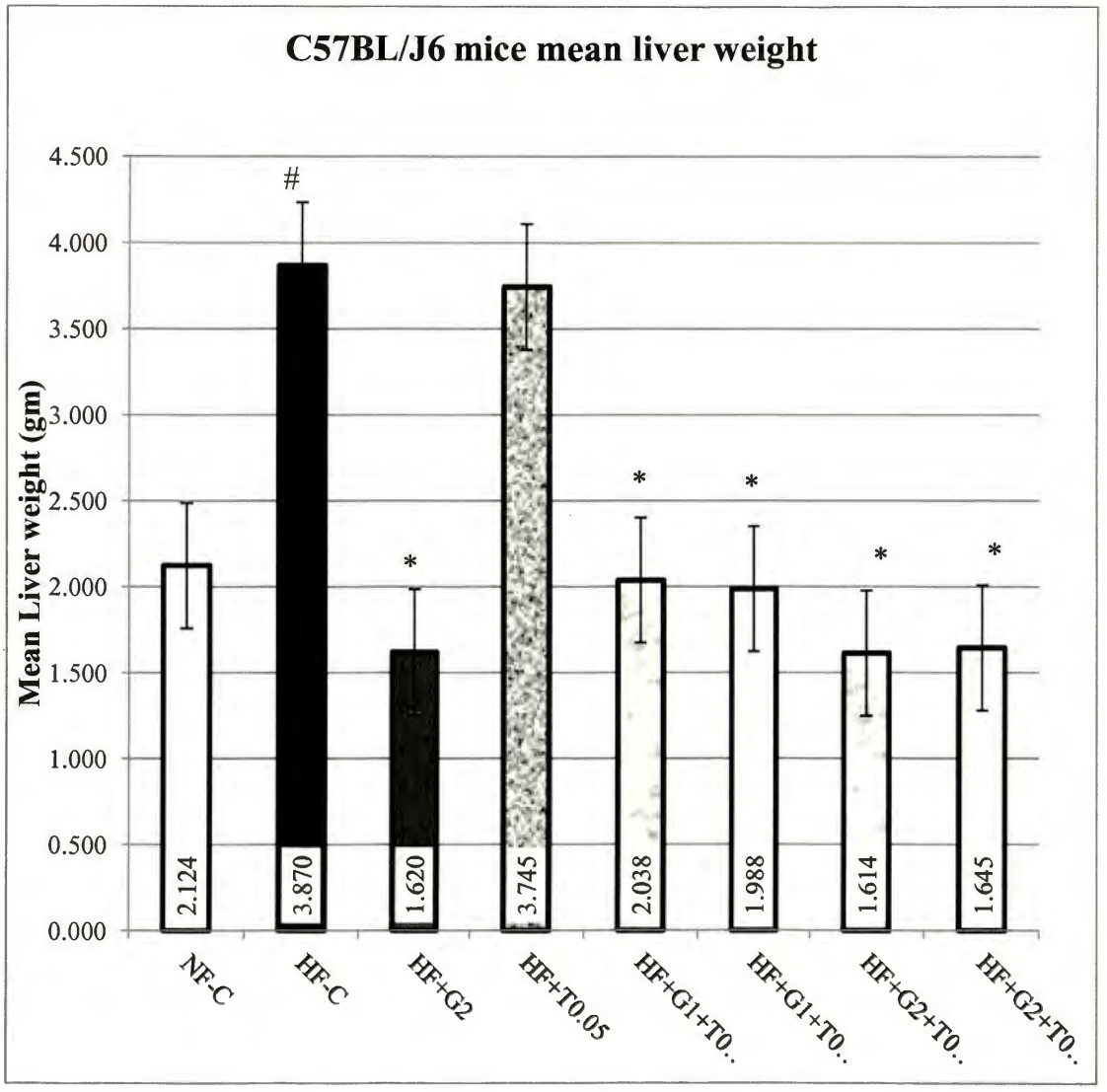


Figure 2: Mean liver weight in C57BL/J6 mice with fatty livers in 8 groups

*P≤0.05, compared to HF-C, # P<0.05, compared to NF-C, Mean ± S.D, (n=8).

NF-C control group (n=7); **HF-C** group (n=7); **HF+G2** ,HF+ 2% GSP ; **HF+T0.05**, HF+ 0.05% δ 3T; **HF+G1+T0.025**, HF+1% GSP+0.025% δ 3T (n=7); **HF+G1+T0.05** HF+1% GSP+0.05% δ 3T ; **HF+G2+T0.025** HF+2% GSP+0.025% δ 3T; **HF+G2+T0.05** HF+2% GSP+0.05% δ 3T .

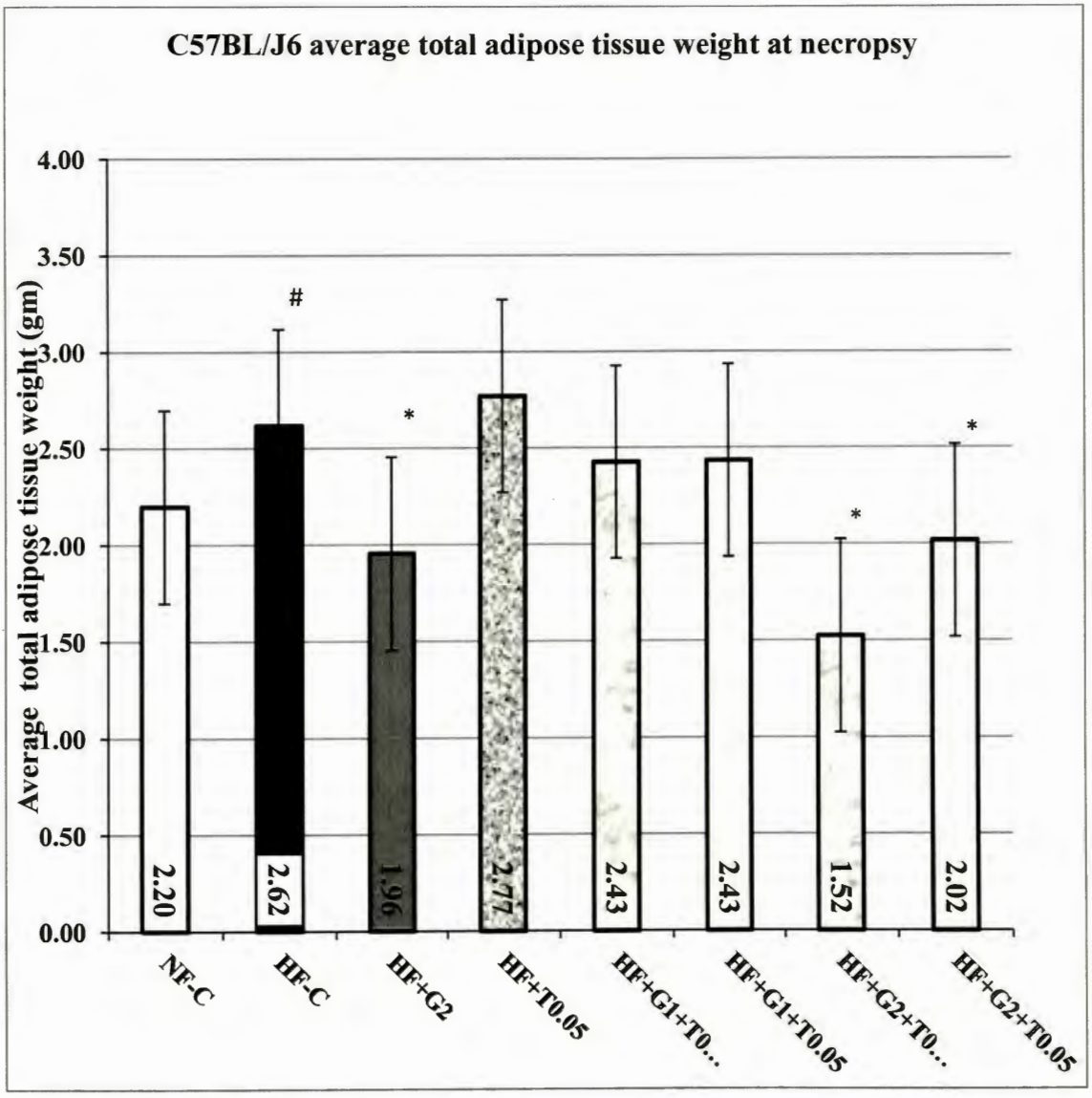


Figure 3: Mean combined adipose tissue weight in C57BL/J6 mice in 8 groups

*P≤0.05, compared to HF-C, # P≤0.05, compared to NF-C, Mean ± S.D, (n=8)

NF-C, control diet n=7; **HF-C**, high fat control diet n=7; **HF+G2**, HF+2% GSP ; **HF+T0.05**, HF+0.05% δ 3; **HF+G1+T0.025**, HF+1% GSE+0.025% δ 3T n=7; **HF+G1+T0.025** HF+1% GSP +0.05% δ 3T; **HF+G2+T0.025** HF+2% GSE+0.025% δ 3T ; **HF+G2+T0.05** HF+2% GSE+0.05% δ 3T.

Histopathological Analyses with Stains

Liver histology of all the groups was evaluated to investigate the occurrence of NASH and effect of supplementation of HF-C in amelioration of steatosis and or liver fibrosis by immunohistochemistry.

Hematoxylin and Eosin (H&E) Stained Photomicrographs

Hepatic steatosis analysis. H&E stained liver sections (Figures 4.1 to 4.7) demonstrated the morphological changes of the liver tissue where hematoxylin stained the nuclei as bluish-black and eosin as the counter stain with pink to light red for the cytoplasm of the hepatocytes. Figure 4.1 shows representative images of liver H&E stained sections from NF-C (A-D) and HF-C (E-H) groups and figure 4.2-4.7 show sections of liver from HF supplemented groups. Sections from HF-control showed fat accumulation in the liver cells (Figure.4.1, E-H). The photomicrographs of the livers from HF-C fed mice had enlarged hepatocytes with ballooning degeneration, micro and macrovesicular steatosis. After 20 weeks of continued high fat feeding, lipid droplets became visible. The number of the droplets increased with time and on calculation as fraction area in mm^2 was 31.02±0.87 for HF-C group (Table 2). Steatosis advanced to a more severe degree with continued HF feeding. Thus the mice in the HF-C fed group developed grade 2 (30 to 60%) steatosis. Overall, no abnormalities were observed in the liver tissues of the NF-C fed mice (Figure 4.1, A-D). The lipid droplet fractional area was lower 18.70 ± 0.94 (Table 2, p# 44)

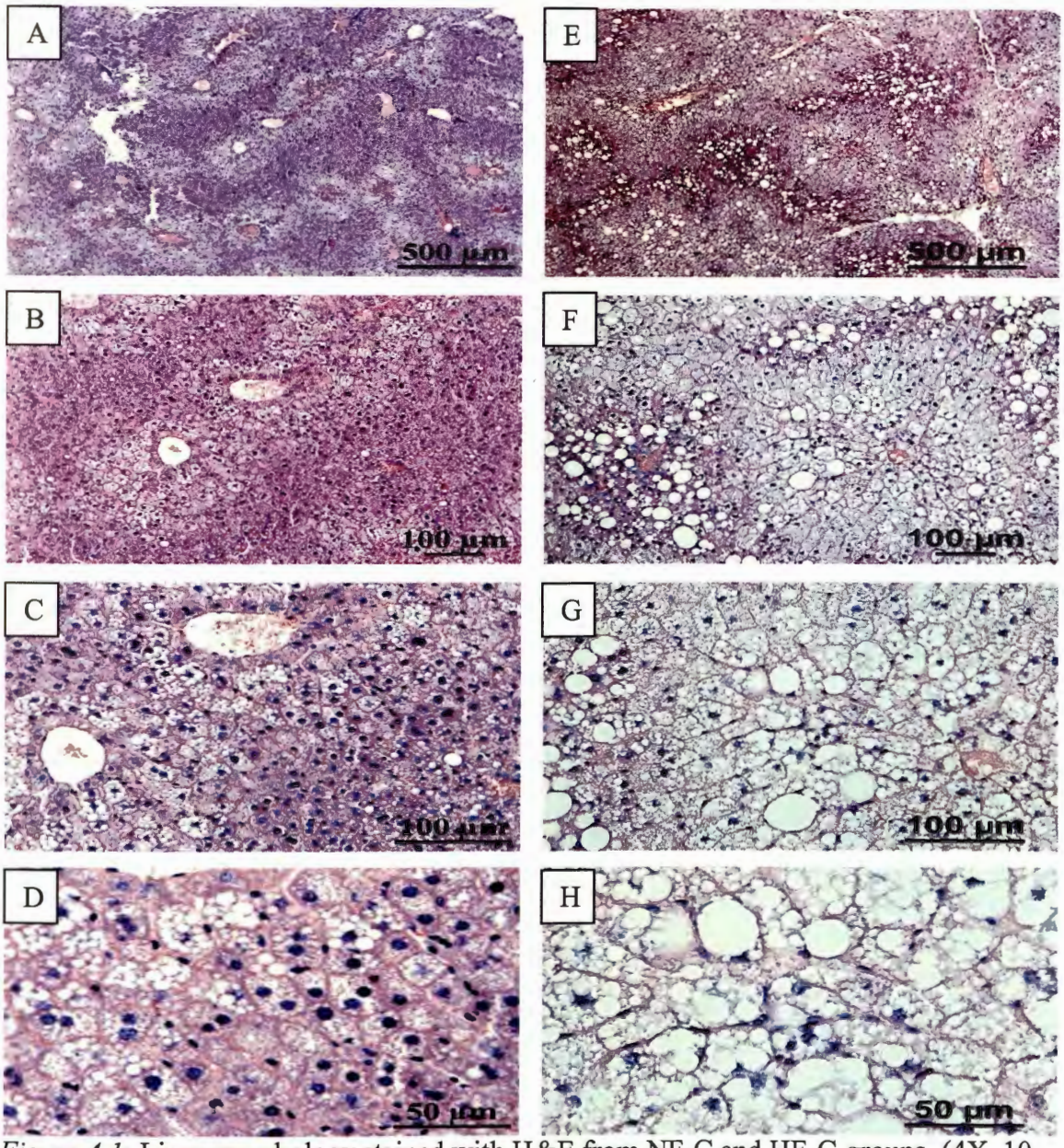


Figure 4.1: Liver morphology stained with H&E from NF-C and HF-C groups. (4X, 10 X, 20X &40X). (A-D) NF control group and (E-H) HF control group. Normal liver parenchyma with hepatocytes (pink), sinusoids and portal tracts were observed in control AIN-G group (A-D).Macrovesicular and microvesicular steatosis and ballooning degeneration.

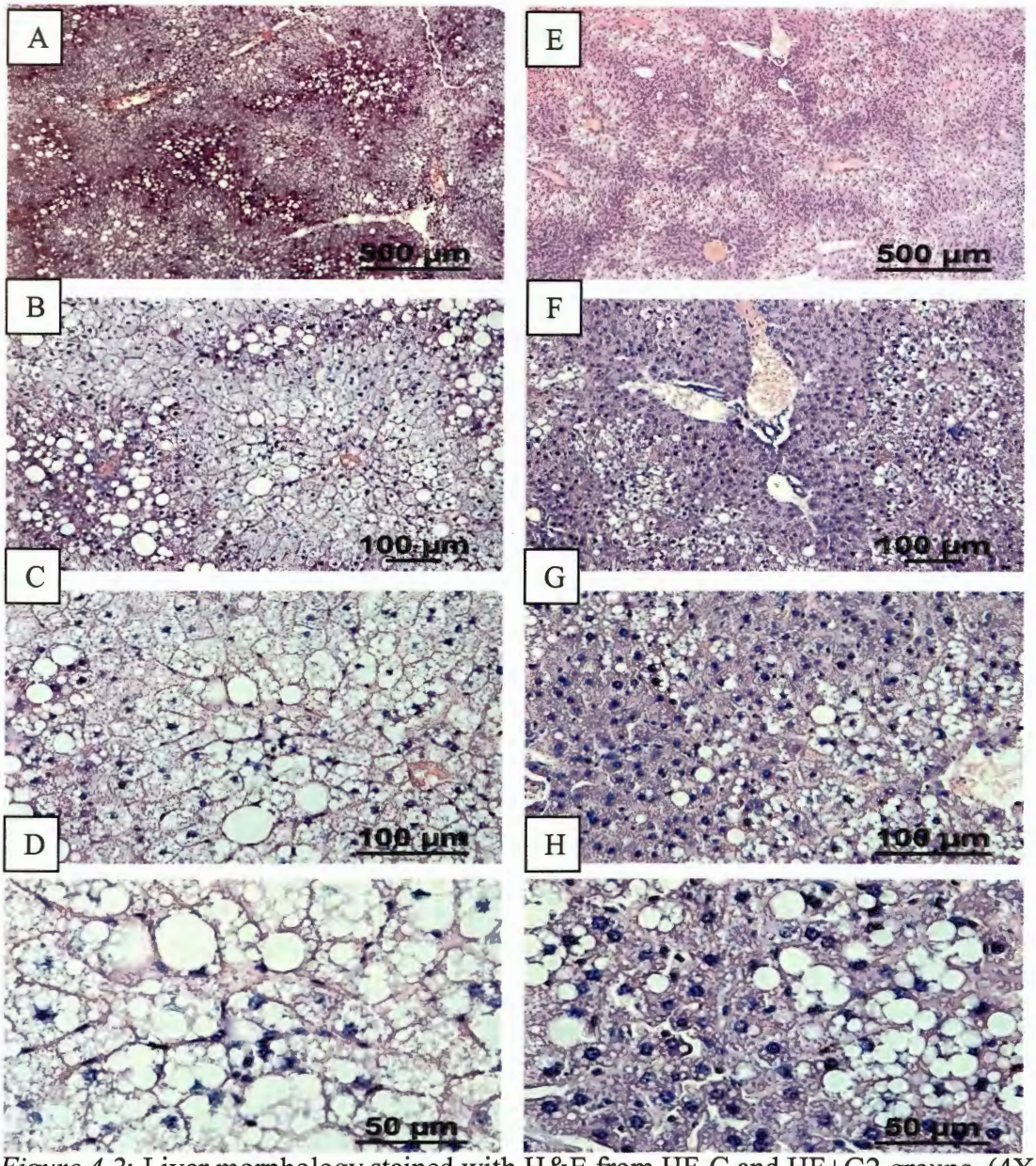


Figure 4.2: Liver morphology stained with H&E from HF-C and HF+G2 groups. (4X, 10 X, 20X &40X). (A-D) HF control group versus (E-H) HF+G2 group; Macrovesicular and microvesicular steatosis and ballooning degeneration central vein in the liver (A-D). Marked reduced macrovesicular and microvesicular steatosis and ballooning degeneration in hepatocytes (E-H).

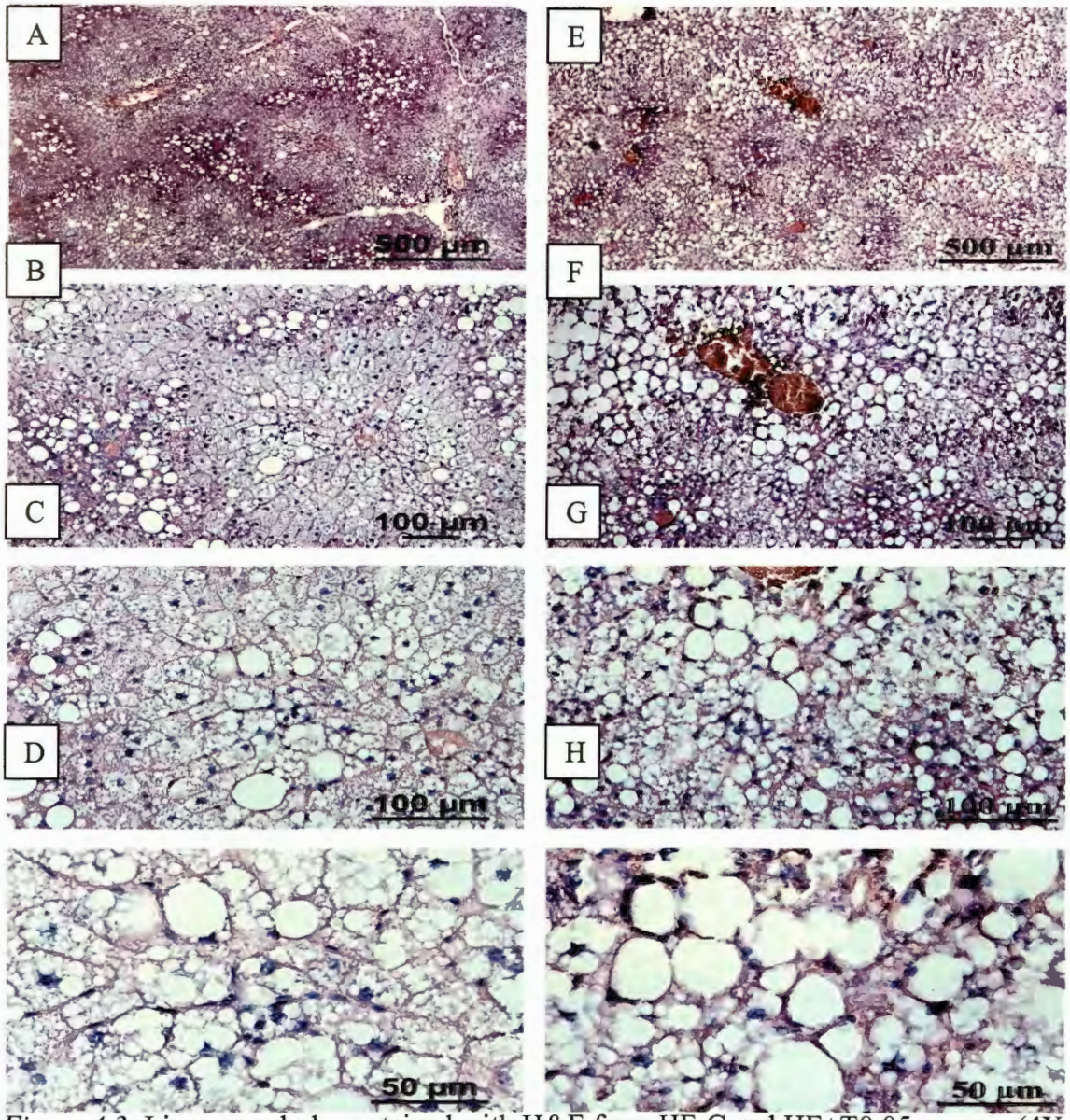


Figure 4.3: Liver morphology stained with H&E from HF-C and HF+T0.05 groups. (4X, 10 X, 20X &40X). (A-D) HF control group versus (E-H) HF+T0.05 group. Disorganization of hepatic chords, macrovesicular and microvesicular steatosis and ballooning degeneration central vein in the liver (A-D). No reduction in macrovesicular and microvesicular steatosis and ballooning degeneration in hepatocytes (E-H).

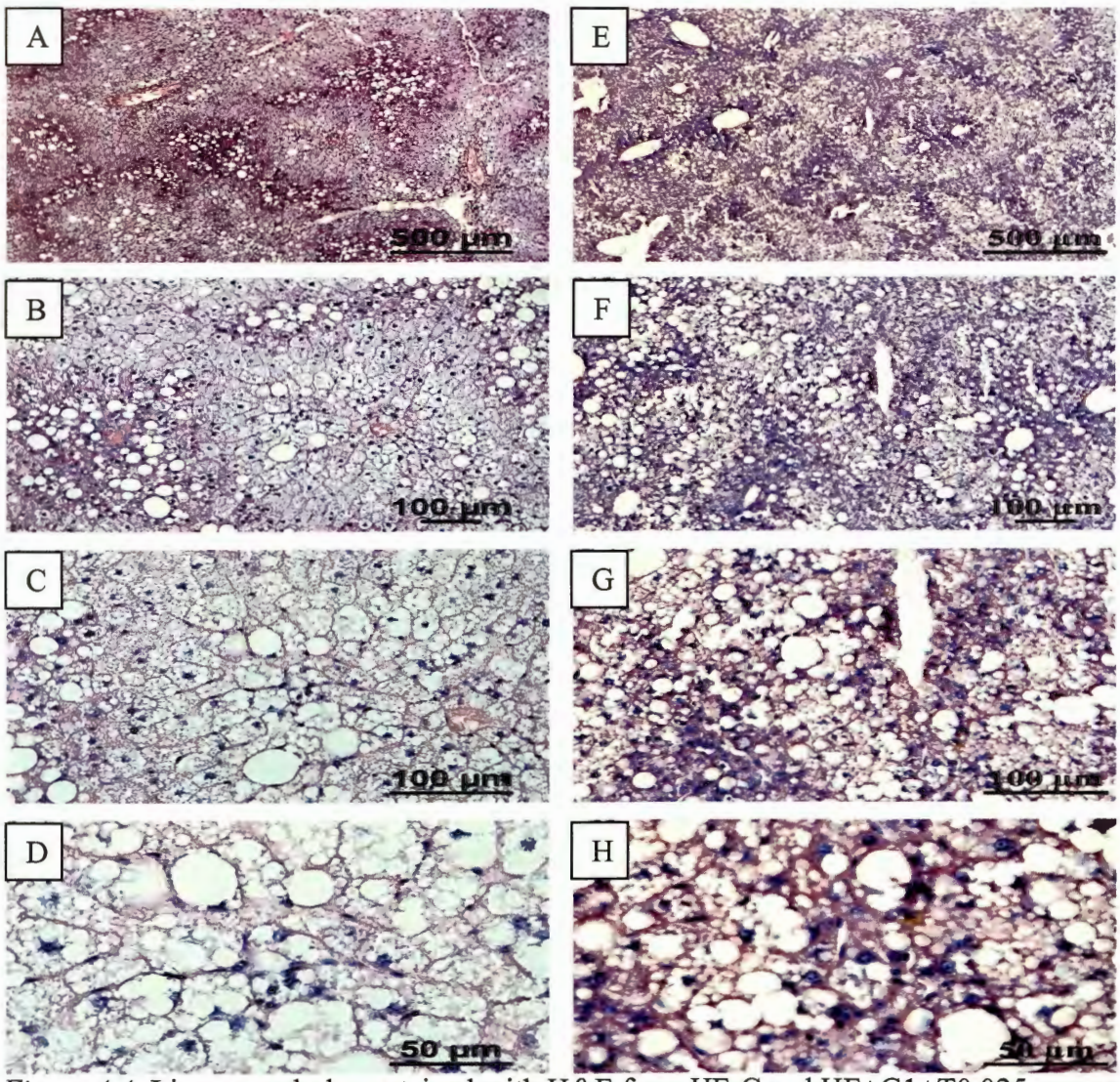


Figure 4.4: Liver morphology stained with H&E from HF-C and HF+G1+T0.025 groups. (4X, 10 X, 20X &40X). (A-D) HF control group versus (E-H) HF+G1+T0.025 group; Macrovesicular and microvesicular steatosis and ballooning degeneration central vein in the liver (A-D). Reduced macrovesicular and microvesicular steatosis and ballooning degeneration in hepatocytes (E-H).

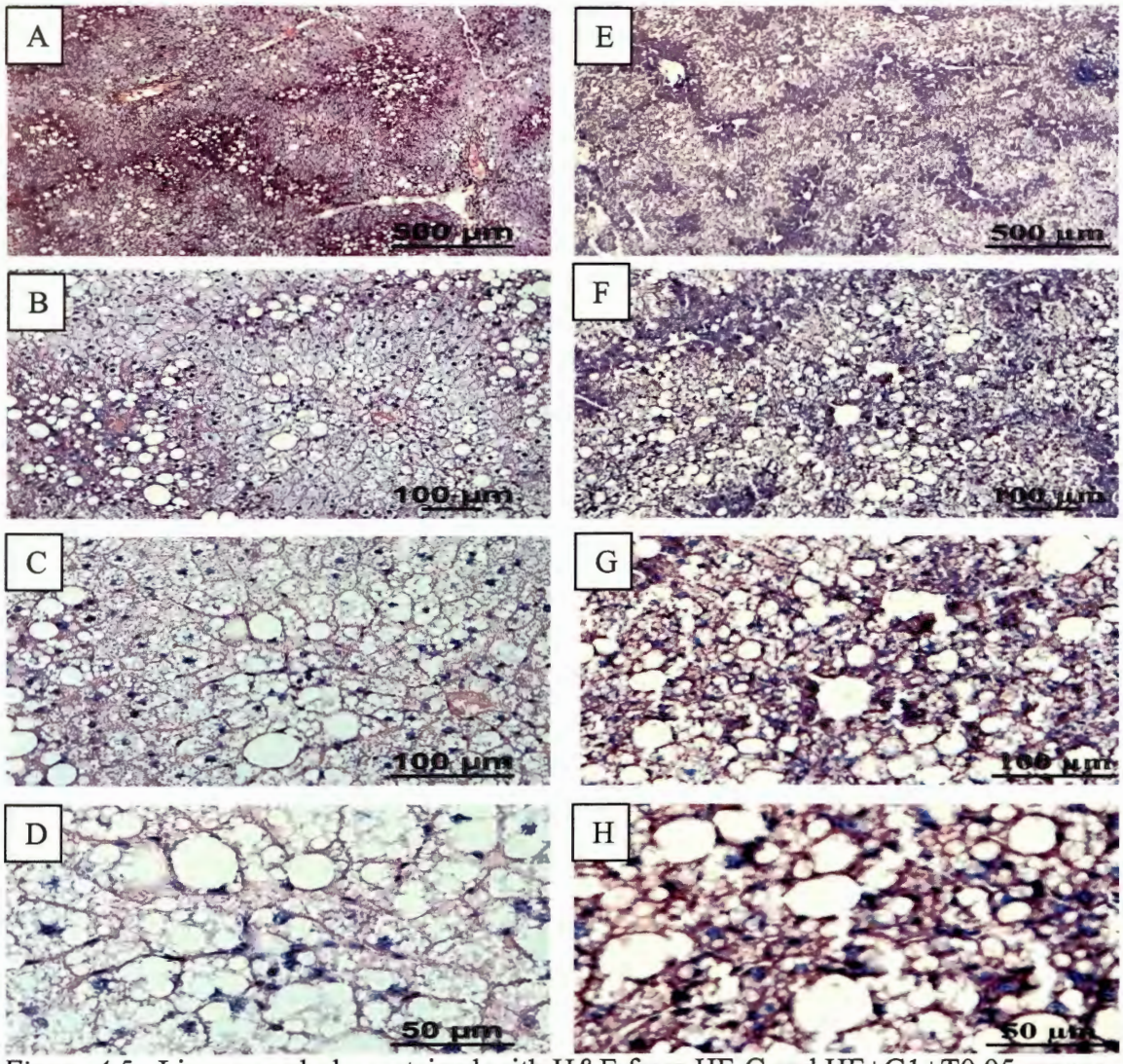


Figure 4.5: Liver morphology stained with H&E from HF-C and HF+G1+T0.05 groups. (4X, 10 X, 20X &40X). (A-D) HF control group versus (E-H) HF+G1+T0.05 group. Macrovesicular and microvesicular steatosis and ballooning degeneration central vein in the liver (A-D). Mild macrovesicular and microvesicular steatosis and ballooning degeneration in hepatocytes (E-H).

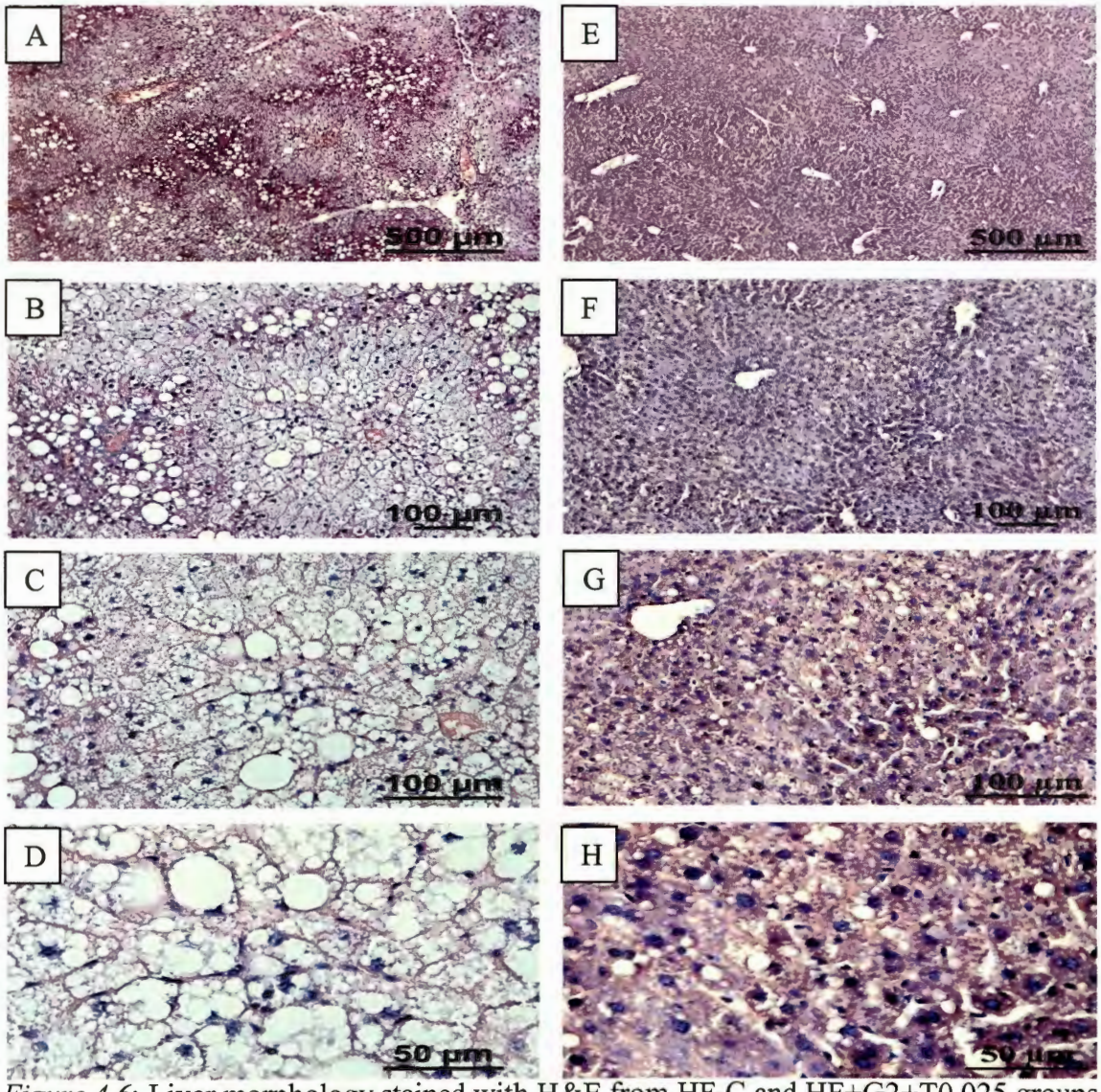


Figure 4.6: Liver morphology stained with H&E from HF-C and HF+G2+T0.025 groups. (4X, 10 X, 20X &40X). (A-D) HF control group versus (E-H) HF+G2+T0.025 δ3T group. Macrovesicular and microvesicular steatosis and ballooning degeneration central vein in the liver (A-D). Mild macrovesicular and microvesicular steatosis and ballooning degeneration in hepatocytes (E-H).

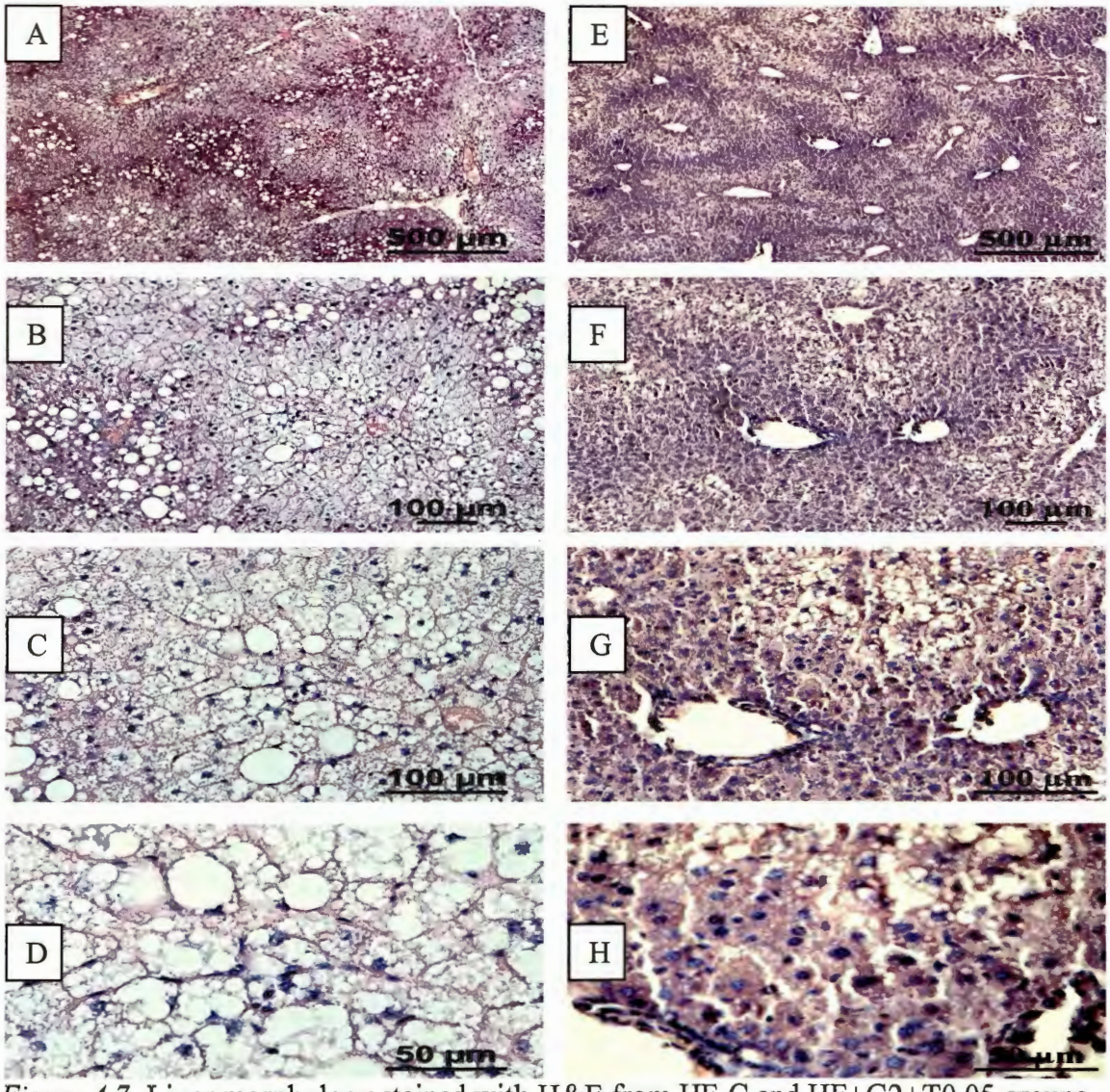


Figure 4.7: Liver morphology stained with H&E from HF-C and HF+G2+T0.05 groups. (4X, 10 X, 20X &40X). (A-D) HF-C control group versus (E-H) HF+G2+T0.05 group. Macrovesicular and microvesicular steatosis and ballooning degeneration central vein in the liver (A-D). Mild macrovesicular and microvesicular steatosis and ballooning degeneration in hepatocytes (E-H).

On microscopic observation liver specimens from mice fed HF with dietary supplements were different in the morphology and showed reduced steatosis between the control and treatment groups (Figure 4.1-4.7). Liver sections from HF with GSP supplemented groups showed significant changes as compared to HF-C group. There was reduced fat accumulation. After 10 weeks of HF supplementation with 2% GSP (Figure 4.2, E-H) there was a visible reduction of steatosis and ballooning of hepatocytes and lower tissue degeneration. In the HF+G2 fed sections; hepatocytes had no microvesicular and or macrovesicular steatosis. There was presence of mild to moderate hepatic lobular degeneration. The lipid droplets fractional area in mm^2 reduced substantially to 14.04 ±0.98 (Table 2) compared to HF-C with 31.02±0.87. Since the steatosis was initially due to HF feeding, GSP supplementation reduced it from a severe to a mild grade (grade 1, >10% steatosis). It showed mild to zero lobular degeneration and focal necrosis. The H+T0.05 group (Figure 4.3, E-H) had moderate fat accumulation in the hepatocytes with ballooning, microvesicular and macrovesicular steatosis. The lipid droplet fractional area was 29.60±1.015 with moderate steatosis (30%) (Table 2), which was close to HF-C fed group with the fractional area of 31.02±0.87.

All the combination supplement groups (Figure 4.4 to 4.7) showed histopathological changes similar to HF+G2 group; they were significantly different compared to HF-C group. They had observable reduction in steatosis with mild microvesicular and or macrovesicular, low ballooning degeneration with random distribution of mild to moderate hepatic lobular degeneration. The lipid droplet fractional

area was similar to HF+G2 fed diet and lower than HF-C group, with a mild steatosis at grade 1 (>10% steatosis) (Table 2). The histological images therefore are comparable and follow the trend similar with data obtained as mean liver weights and adipose tissue weights, where the HF-C group had significantly higher tissue weights compared to NF-C fed group while HF supplemented with GSP demonstrated reduction in tissue weights.

Table 2

Results of Histopathological Findings for Liver Steatosis

Groups	**Liver Steatosis**
	fraction area / total area
	(cells/mm2)
NF-C, n=7	18.70±0.94
HF-C, n=7	31.02±0.87#
HF+G2, n=8	14.04±098*
HF+T0.05, n=8	29.60±1.01
HF+G1+T0.025, n=7	16.56±1.75*
HF+G1+T0.25, n=8	8.69±0.64*
HF+G2+T0.025, n=8	8.85±093*
HF+G2+T0.25, n=8	8.62±0.64*

*P <0.01 is the significant difference from HF-C group, n=8.

(**NF-C**, low fat control, **HF-C**, **HF+G2**-HF +2%GSP, **HF+T0.05**- HF +0.05%delta tocotrienol, **HF+G1+T0.025**- HF-C+1% GSE+0.025% delta tocotrienol, -**HF+G1+T0.05**-HF-C+1% GSE+0.05% delta tocotrienol, **HF+G2+T0.025**-HF-C+2% GSE+0.025% delta tocotrienol, **HF+G2+T0.05**-HF-C+2% GSE.05% delta tocotrienol)

The values represent the means ±SEM. Data points obtained by using two way paired ANOVA, SPSS. The fractional area (mm^2) from total fat droplet area in each liver section for eight groups stained with H&E was calculated to evaluate steatosis.

Morphometric Analysis of H&E Stained Photomicrographs

Morphometric analysis of the lipid droplets using the software (NIS- Element, Nikon, USA) with H & E stained tissue sections was done. The calculated numerical values for all groups are shown in Table 2 and Figure 5.1 and 5.2. The percent lipid droplet as fractional area for HF-C group (n=7) was the highest with 31.02±0.87 and NF-C modified group (n=7) as 18.70 ± 0.94 ($p<0.05$). This shows that the HF+T0.05 group was comparable to HF-C group. The GSP supplemented group and all the four combination groups had the lowest values compared to HF-C where the HF-C+ G2 group (n=8) as 14.04 ±0.98 was lower than HF–C. The remaining 3 out of 4 combination groups also had lower numerical values with (HF+G1+T0.025) had a mean area of 16.56 ±1.75 lowest among the combination groups (Figure 5.1 and 5.2). This suggests that GSP may reduce steatosis in liver tissue of the mice in the HF + GSP supplemented groups.

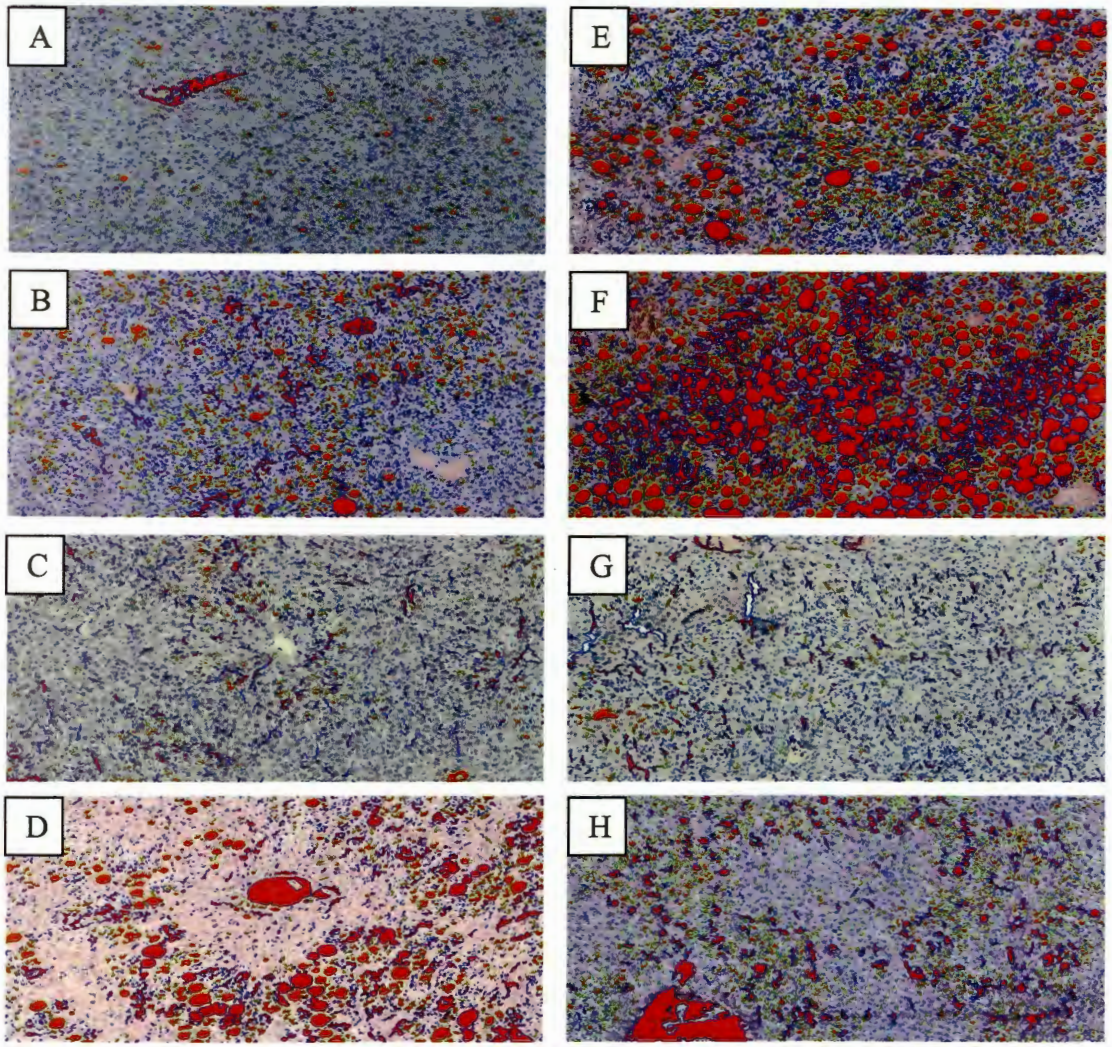


Figure 5.1: Photomicrogaph with percent lipid droplet area analysis stained with H&E (10 X) from all groups. H&E stained mice liver sections (10X) histomicrograph with percent lipid droplet area analysis by using software NIS-elements, Nikon, USA to calculate percent lipid droplet area (cells/mm^2). (A) **NF-C** control group (E) **HF-C** control group, (B) **HF-C+ G2** group (F) **HF-C+T0.05** group,(C) **HF+G1+T0.025**,(G) **HF+G1+T0.05** group, (D) **HF+G2+T0.025** group and (H) **HF+G2+T0.05** group.

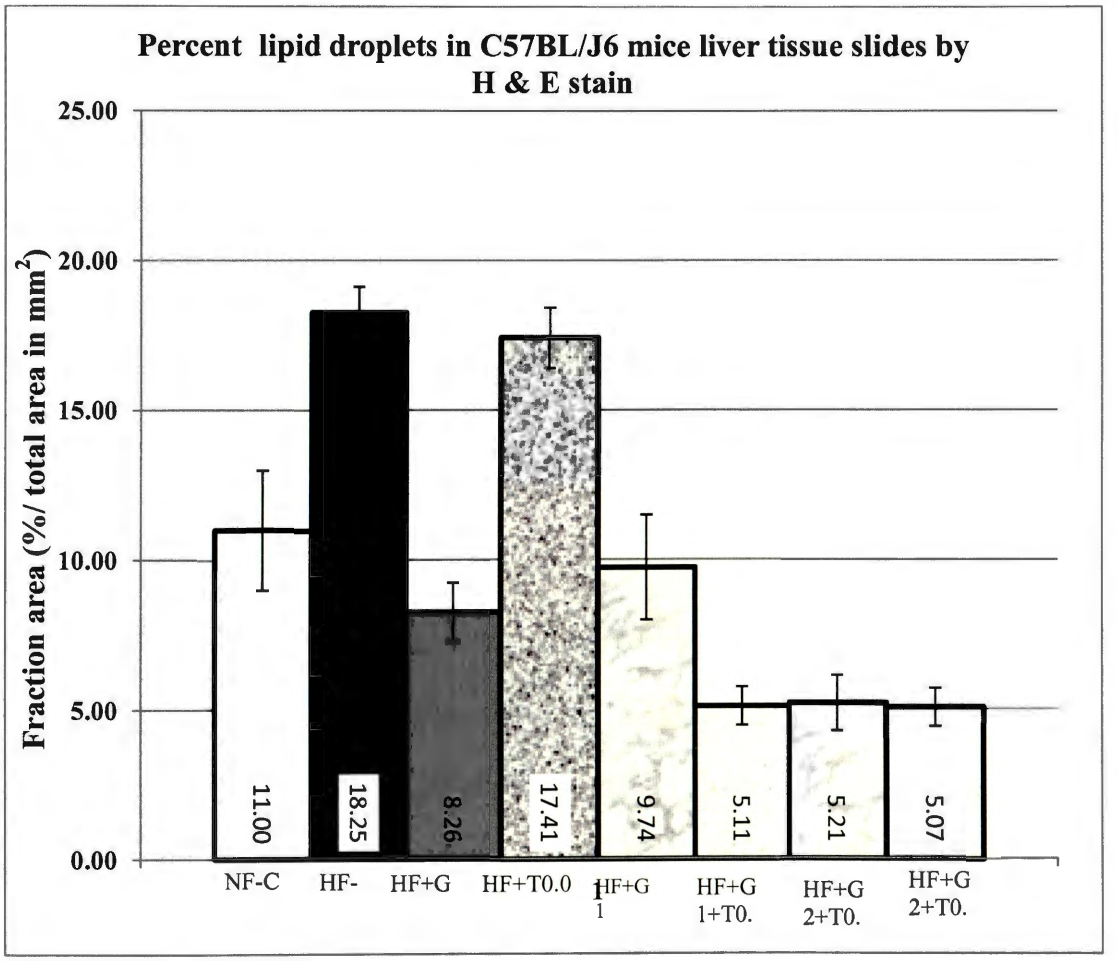


Figure 5.2: Area plot (cells/ mm^2) of C57BL/J6 mice liver tissue sections after H & E stain.*P≤0.05, compared to HF-C control, # P≤ 0.05, compared to AIN-G control) ± S.D, (n=8). Data points obtained by using two-way paired ANOVA, SPSS.**NF-C** control (white bar), **HF-C** control (black bar), **HF+G2**-HF-C+ 2% GSP (gray bar), **HF+T0.05**-HF-C+ 0.05% δ 3T (gray textured bar), **HF+G1+T0.025**(white textured bar), **HF+G1+T0.05** (white gradient bar), **HF+G2+T0.025**(white textured bar), **HF+G2+T0.05** (white gradient bar).

Periodic Acid Stain (P.A.S) Stained Photomicrographs

Hepatic glycolipid storage analysis. P.A.S staining was performed to investigate the effect of high fat diet on liver glycogen and or glycolipids stores (Figures 6.1 – 67, (A-H) 4x, 10x, 20x & 40x). The HF-C (Figure 6.1, E-H) group showed moderate staining for glycogen stores as sporadic aggregation of pink color areas (Figure 6.1, E-H 4x, 10x, 20x & 40x) compared to NF-C (Figure 6.1, A-D) control group. There were no other morphological changes in the liver sections with P.A.S staining. The hepatocytes were stained light green with heamotoxylin stained nuclei throughout the sections. The sections showed uniform staining for cytoplasm and the nuclei for HF-C group and NF-C group. The liver sections from the HF-C supplemented with GSP (HF+G2) (Figure 6.2, E-H) had green cytoplasm and bluish-black nuclei and no pink color aggregates compared to HF-C group. While the HF+T0.05 (Figure 6.3, E-H) group showed moderate sporadic pink aggregates which show the presence of glycogen and or glycoprotein stores. There were no observable changes in the histology of the liver sections from combination groups (Figure 6.4 to 6.7).

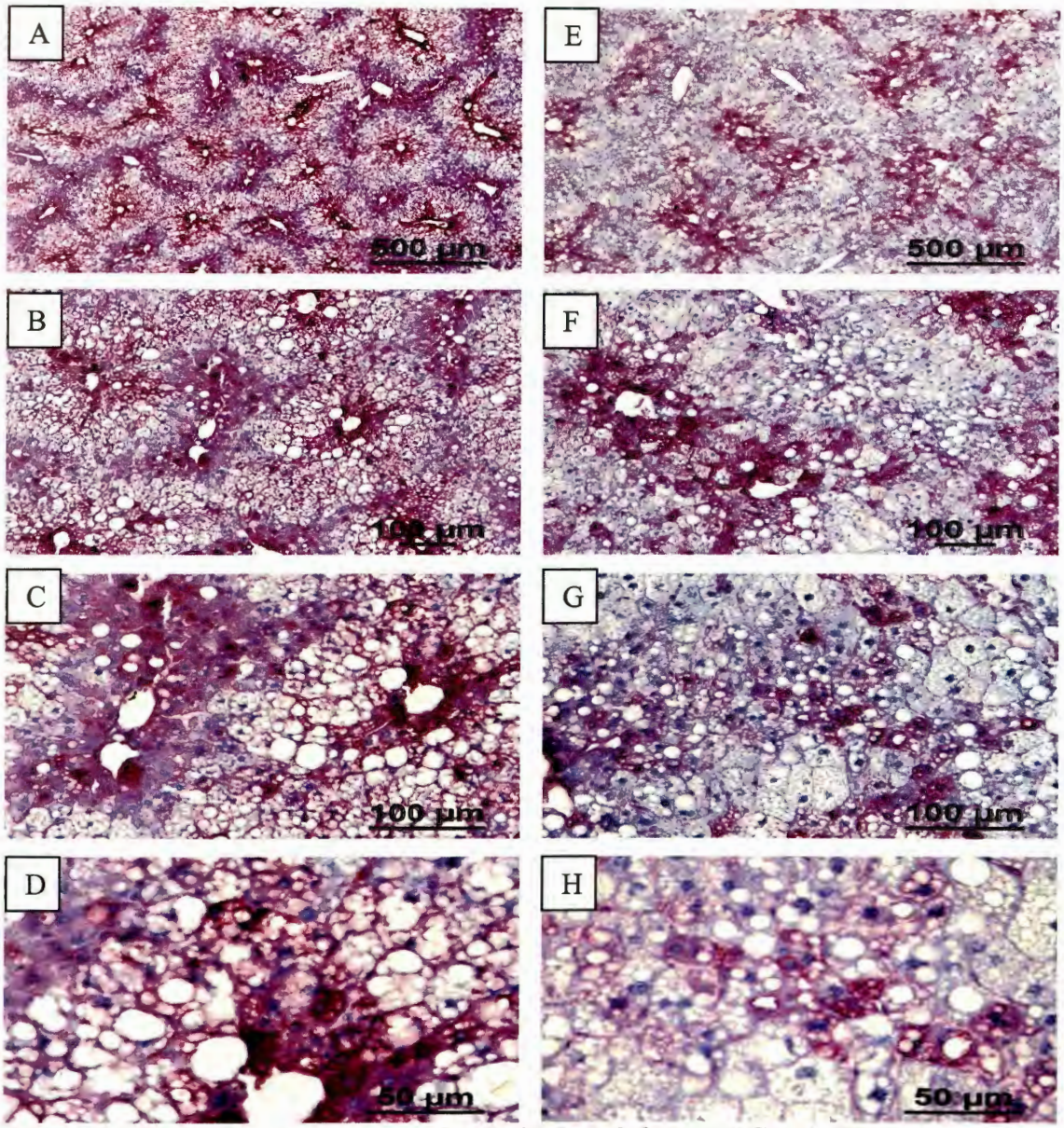


Figure 6.1: Liver glycogen stores stained with P.A.S from NF-C and HF-C group. (4X, 10 X, 20X &40X). (A-D) AIN-G control group and (E-H) HF-C control group, HF-C group; Normal liver parenchyma with hepatocytes (light green), sinusoids observed in control AIN-G group (A-D).Aggregates of storage form of carbohydrates mostly glycogen (pink) in HF-C group.

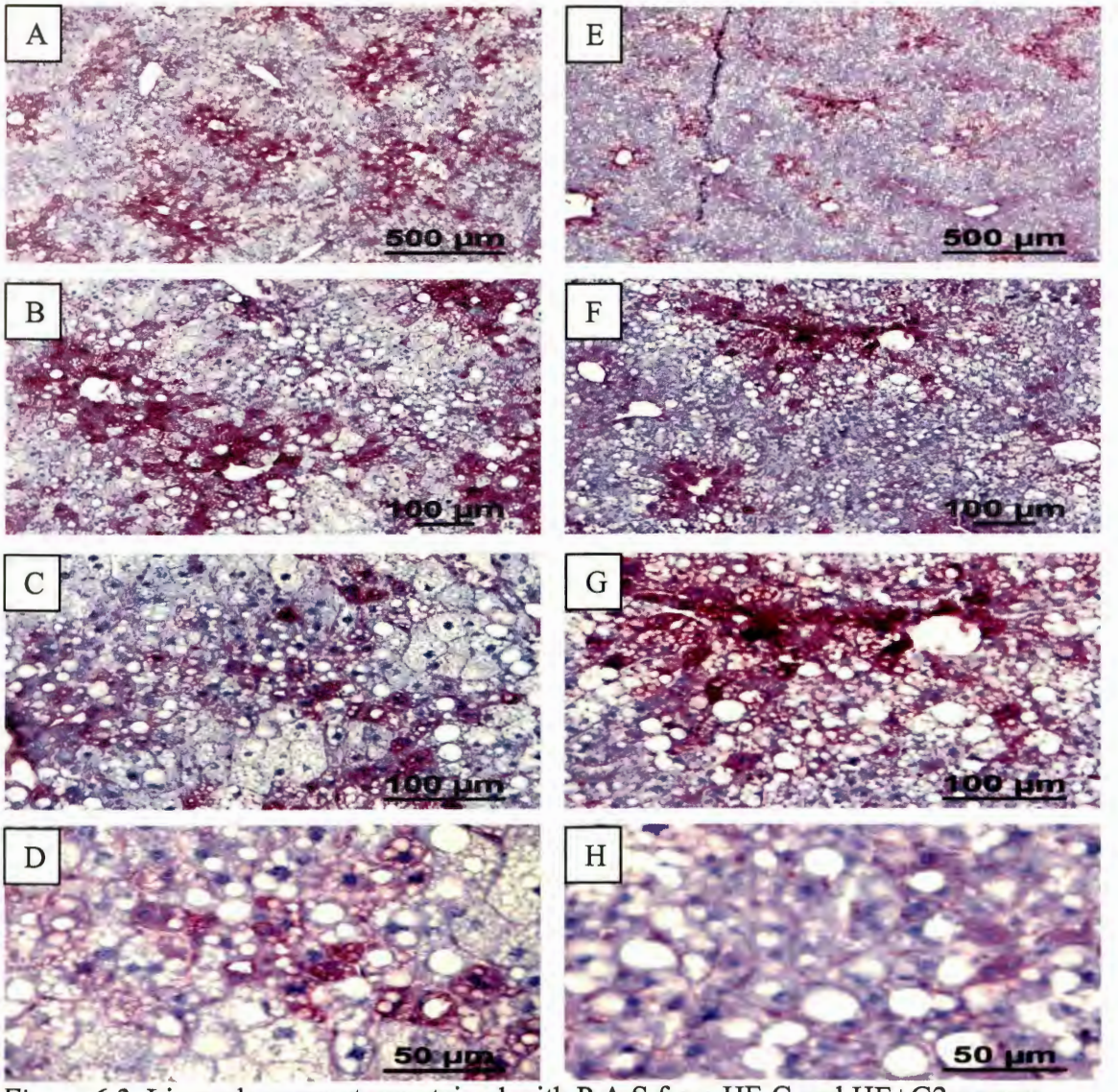


Figure 6.2: Liver glycogen stores stained with P.A.S from HF-C and HF+G2 groups. (4X, 10 X, 20X &40X). (A-D) HF-C control group and (E-H) HF-C + 2% GSE group; aggregates of storage form of carbohydrates mostly glycogen (pink) in HF-C group (A-D).Diffused storage form of carbohydrates mostly glycogen (pink) in HF+G2 group.

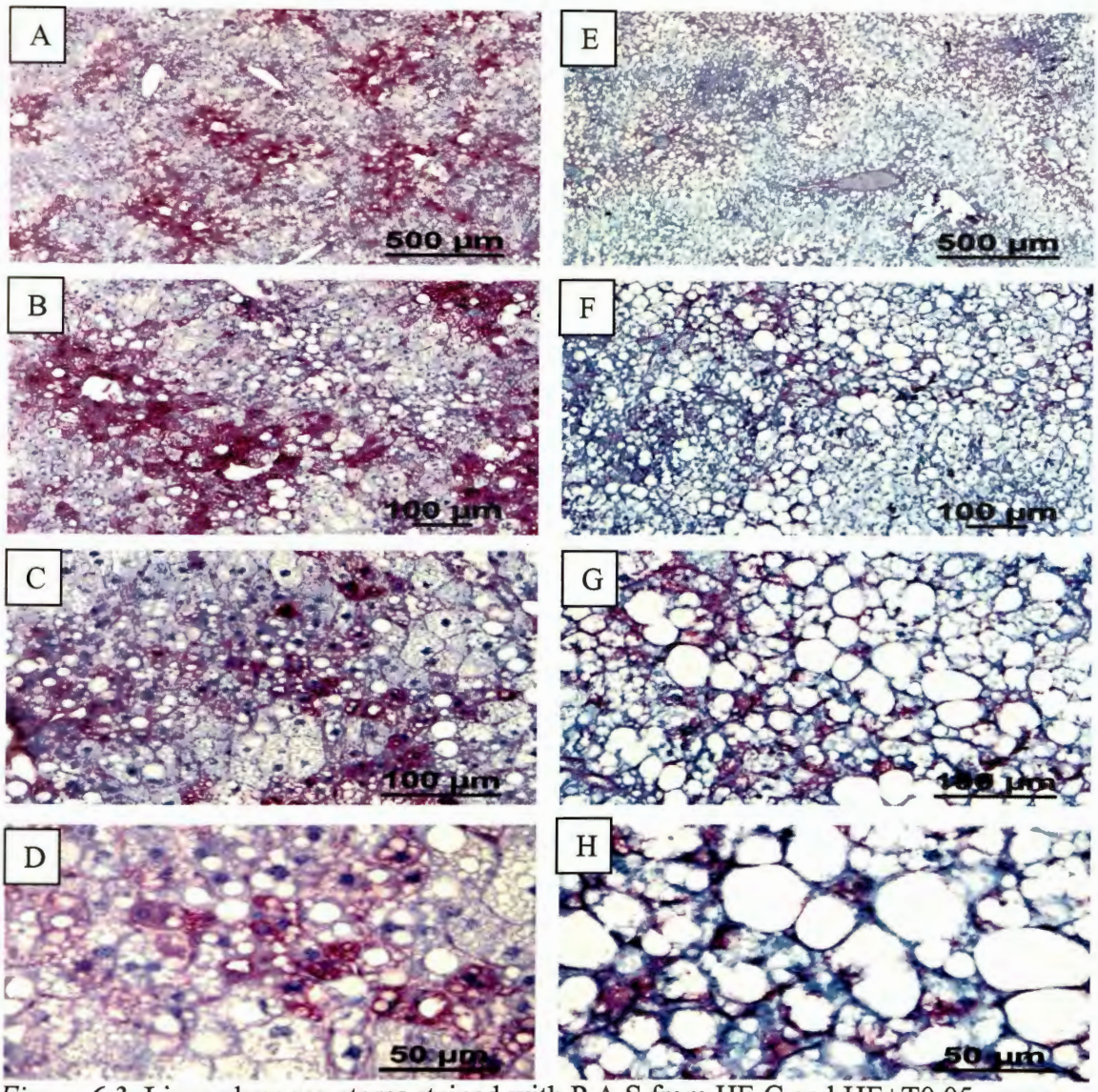


Figure 6.3: Liver glycogen stores stained with P.A.S from HF-C and HF+T0.05 groups. (4X, 10 X, 20X &40X). (A-D) HF-C control group and (E-H) HF-C + 0.05% δ3T group; aggregates of storage form of carbohydrates mostly glycogen (pink) in HF-C group (A-D).Mild aggregates of storage form of carbohydrates mostly glycogen (pink) in HF+T0.05 group.

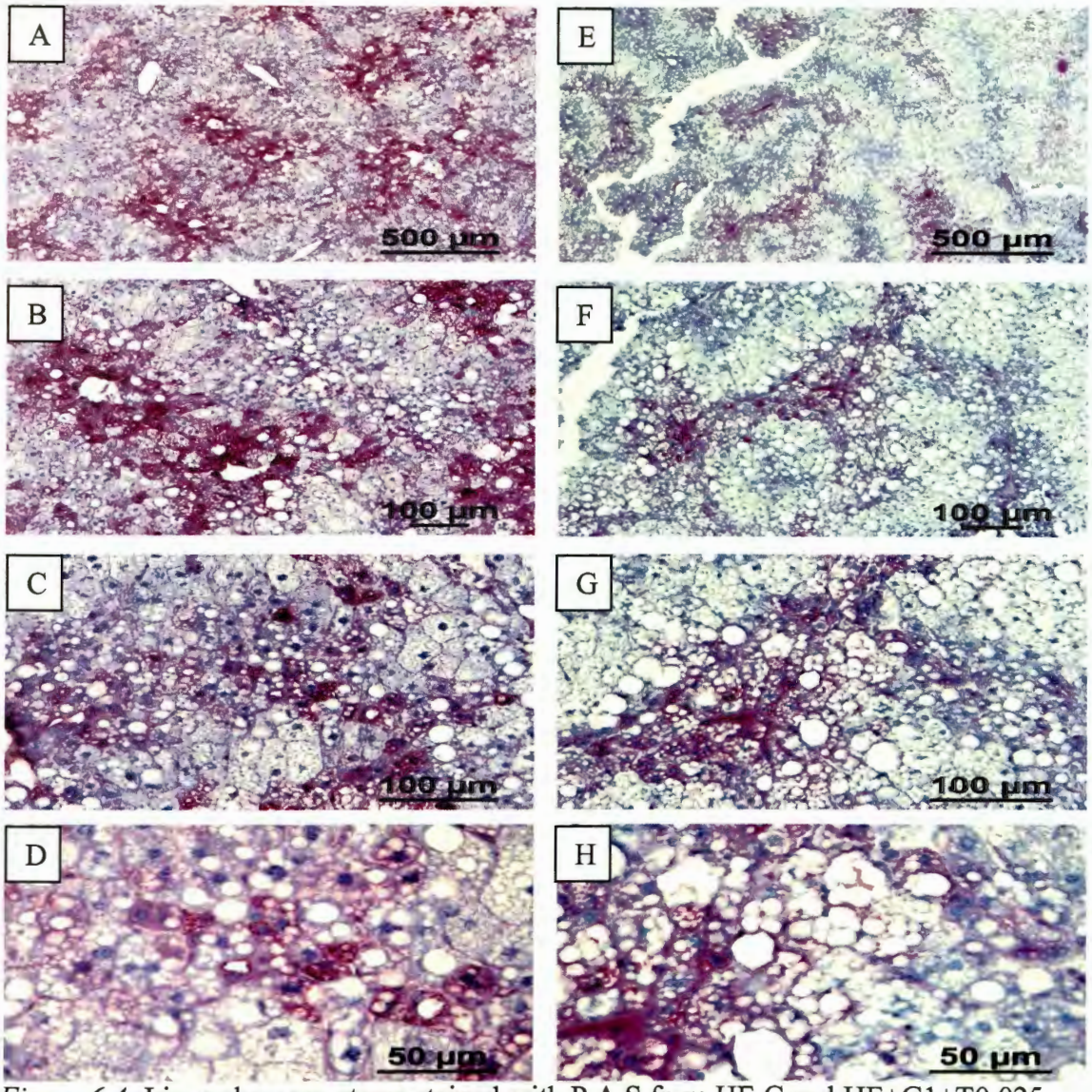


Figure 6.4: Liver glycogen stores stained with P.A.S from HF-C and HF+G1+T0.025 groups. (4X, 10 X, 20X &40X). (A-D) HF-C control group and (E-H) IA- HF-C + 1% GSE +0.025% δ3T group; aggregates of storage form of carbohydrates mostly glycogen (pink) in HF-C group (A-D).Diffused storage form of carbohydrates mostly glycogen (pink) in HF+G1+T0.025 group.

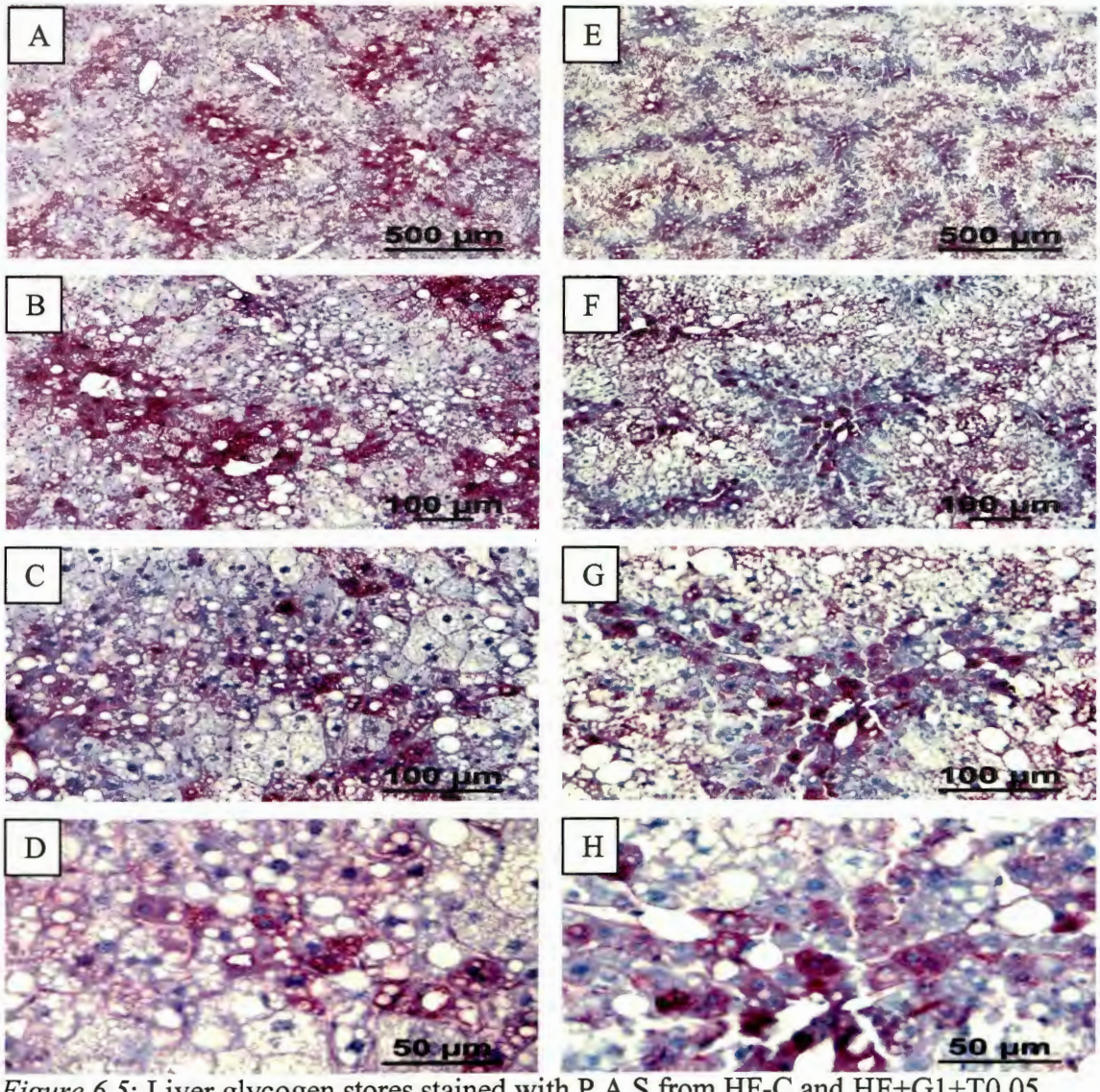


Figure 6.5: Liver glycogen stores stained with P.A.S from HF-C and HF+G1+T0.05 groups. (4X, 10 X, 20X &40X). (A-D) HF-C control group and (E-H) IB- HF-C + 1% GSE +0.05% δ3T group; aggregates of storage form of carbohydrates mostly glycogen (pink) in HF-C group (A-D).Diffused storage form of carbohydrates mostly glycogen (pink) in HF+G1+T0.05 group.

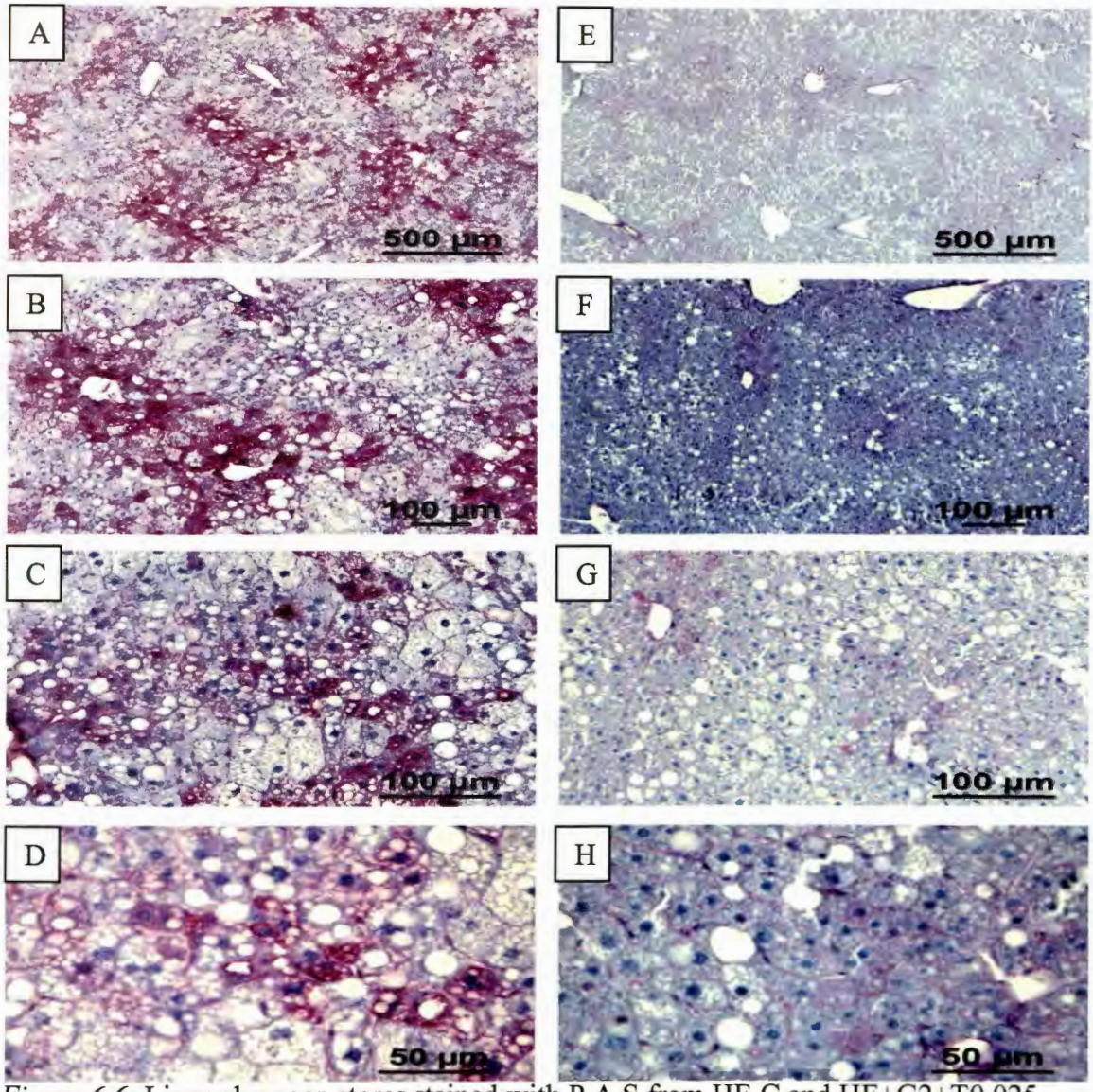


Figure 6.6: Liver glycogen stores stained with P.A.S from HF-C and HF+G2+T0.025 groups. (4X, 10 X, 20X &40X). (A-D) HF-C control group and (E-H) IIA- HF-C + 2% GSE +0.025% δ3T group; aggregates of storage form of carbohydrates mostly glycogen (pink) in HF-C group (A-D).Reduced storage form of carbohydrates mostly glycogen (pink) in HF+G2+T0.025 group.

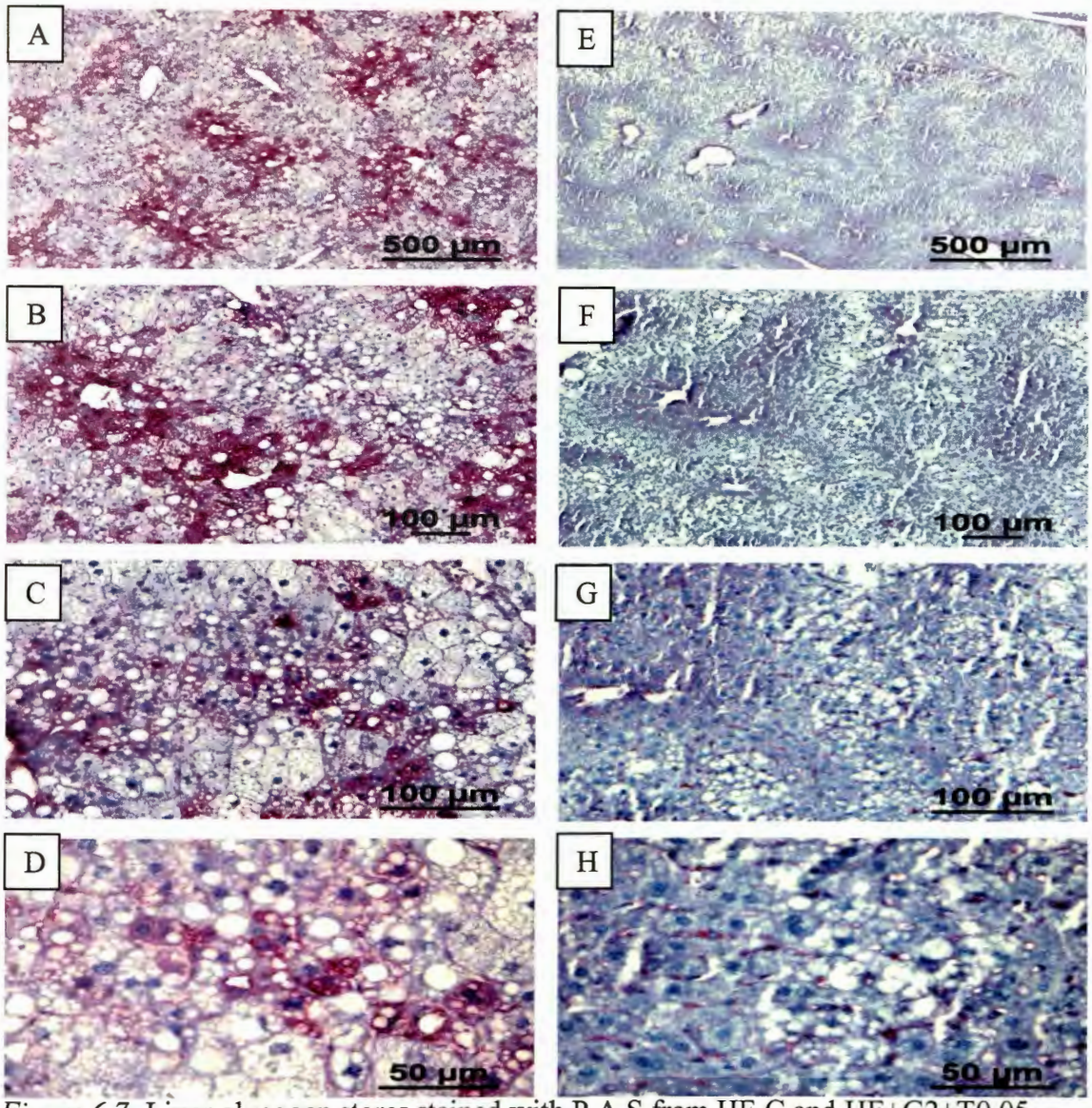


Figure 6.7: Liver glycogen stores stained with P.A.S from HF-C and HF+G2+T0.05 groups. (4X, 10 X, 20X &40X). (A-D) HF-C control group and (E-H) IIB- HF-C + 2% GSE +0.05% δ3T group; aggregates of storage form of carbohydrates mostly glycogen (pink) in HF-C group (A-D).Diffused storage form of carbohydrates mostly glycogen (pink) in HF+G2+T0.05 group.

Masson's Trichrome Stained Photomicrographs

Liver sections stained with Masson's trichrome were used to detect collagen accumulation as filaments of light blue-green color with reddish to magenta colored cytoplasm and heamtoxylin stained bluish-black nuclei (Figure 7.1-7.7).

Morphometric analysis and hepatic fibrosis. All the photomicrographs stained with Masson's trichrome used to calculate and score fibrosis from 0-4 with 0 being absence of fibrosis and 4 as highest degree of fibrosis (Table 3). Hepatic fibrosis was observed by Masson's trichrome stain in HF-C group with collagen deposition which was observed as light blue filaments. Liver sections from HF-C (Figure 7.1, E-H) fed mice demonstrated significant fibrosis. Fibrosis was seen in portal areas; perisinusoidal fibrosis and occasionally perivenular and periportal fibrosis (score 2, Table 3) compared to NF-C (Figure 7.1, A-D) group. No histological signs of hepatic fibrosis were observed in the NF-C group (score 1, Table 3). There was moderate blue staining on the sinusoidal walls of the hepatocytes which were enlarged and had visible lobular degeneration in HF-C group.

The characteristic blue filamentous color was absent in the HF-C supplemented with GSP, the HF-C+G2 group (Figure 7.2, E-H) compared to HF-C group. There was no collagen staining and the tissue architecture was maintained, with sporadic perisinusoidal fibrosis (score 0, Table 3). The HF+T0.05 group (Figure 7.3, E-H) had moderate collagen staining as blue color filaments in the cytoplasm with both perisinusoidal and occasional

periportal fibrosis (score 2, Table 3) compared to HF-C group. The four combination groups (Figure 7.5- 7.8, E-H) liver sections were similar to the single supplemented GSP group with no evidence of collagen accumulation and blue filaments in the cytoplasm and or sinusoidal fibrosis (score 0, Table 3).

Table 3

Results of Histopathological Findings for Liver Fibrosis

Groups	**Liver fibrosis**	**Liver fibrosis**	**Liver fibrosis**
	α- SMA expression	**α- SMA expression**	**α- SMA expression**
	(0-4), Scores	**percent**	**grade**
NF-C, n=7	1.11	>30%	g-1
HF-C, n=7	2.56#	30-60%	g-2
HF+G2, n=8	0.78*	>10%	g-1
HF+T0.05, n=8	2.33	30-60%	g-2
HF+G1+T0.025, n=7	0.56*	>10%	g-1
HF+G1+T0.25, n=8	0.61*	>10%	g-1
HF+G2+T0.025, n=8	0.39*	>10%	g-1
HF+G2+T0.25, n=8	0.22*	>10%	g-1

*P <0.01 is the significant difference from HF-C group, n=8.

(**α- SMA**, α-smooth muscle actin, **NF-C**, low fat control, **HF-C**, **HF+G2**-HF +2%GSP, **HF+T0.05-** HF +0.05%delta tocotrienol, **HF+G1+T0.025**- HF-C+1% GSE+0.025% delta tocotrienol, - **HF+G1+T0.05**-HF-C+1% GSE+0.05% delta tocotrienol, **HF+G2+T0.025**-HF-C+2% GSE+0.025% delta tocotrienol, **HF+G2+T0.05**-HF-C+2% GSE.05% delta tocotrienol)

Fibrosis scores were calculated as mean value for each group and assigned in a blinded fashion to α-SMA expression by immunohistochemsitry (Batts K.P et al, and Ludwig J.et al, 1995). The criteria for each score were previously described in the materials and methods section.

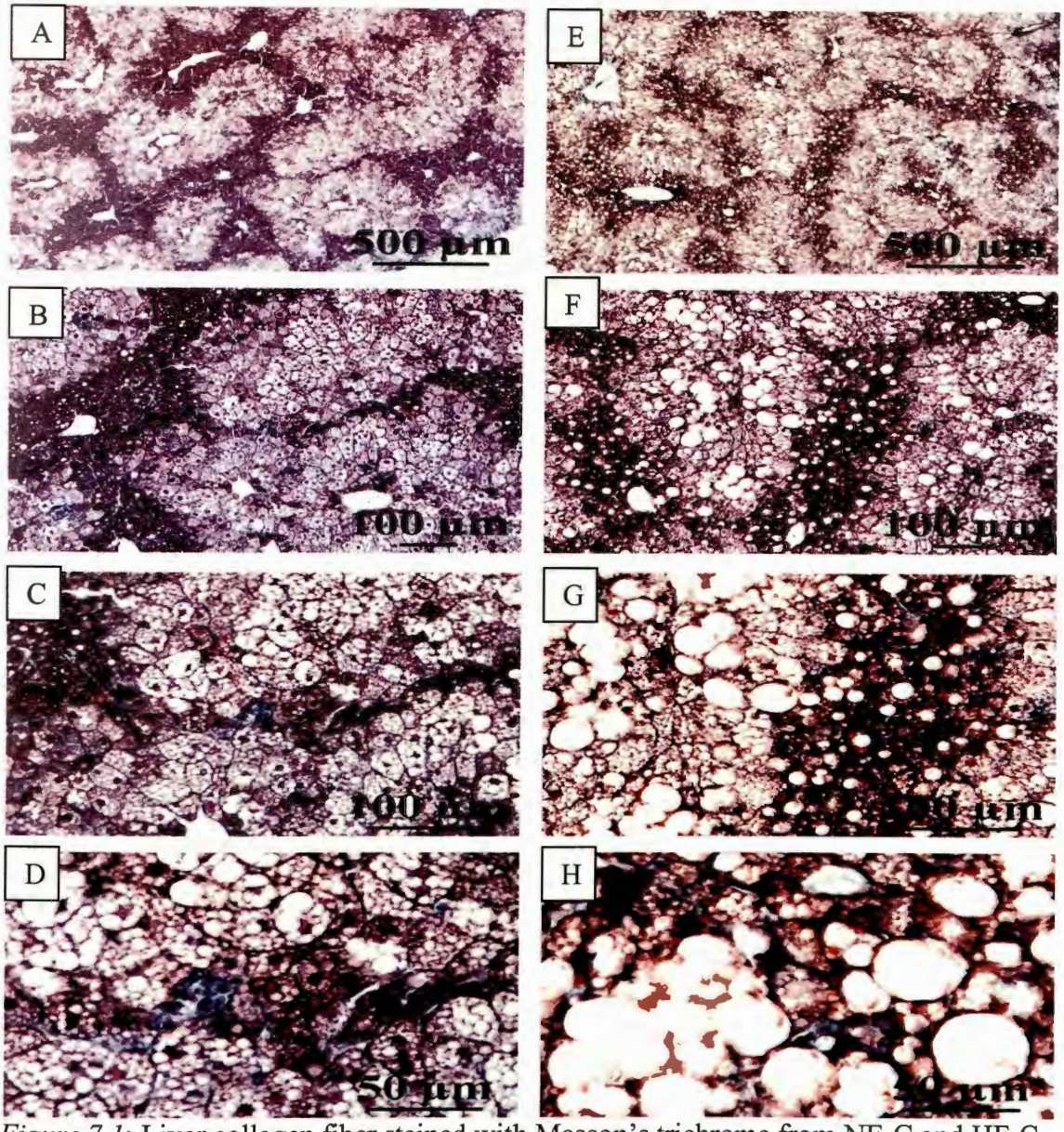


Figure 7.1: Liver collagen fiber stained with Masson's trichrome from NF-C and HF-C groups. (4X, 10 X, 20X &40X)

(A-D) AIN-G control group and (E-H)versus HF-C control group, HF-C group; Normal liver parenchyma with hepatocytes (magenta), sinusoids and portal tracts were observed in control NF-C (A-D).Disorganization of hepatic chords, increased number of degenerated hepatocytes, fibrosis in the form of delicate bands (blue), and inflammatory cell infiltration (E-H)

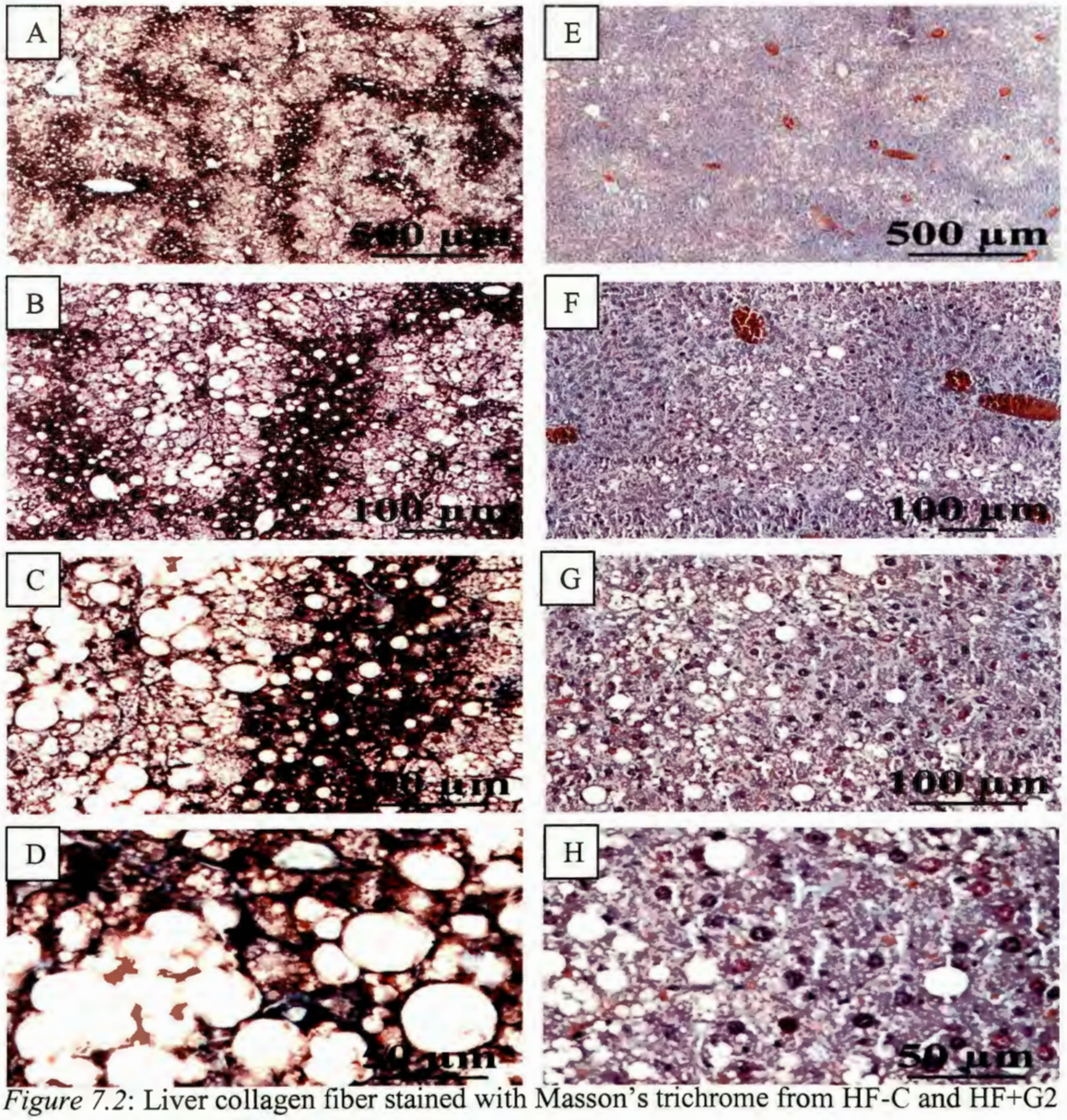


Figure 7.2: Liver collagen fiber stained with Masson's trichrome from HF-C and HF+G2 groups. (4X, 10 X, 20X &40X)

(A-D) HF-C control group and (E-H) versus HF-C+G2 group; Disorganization of hepatic chords, increased number of degenerated hepatocytes, fibrosis in the form of delicate bands (blue), and inflammatory cell infiltration infiltration (A-D). Mild disorganization of hepatic chords, a decrease in degenerated hepatocytes, moderate fibrosis in the form of delicate bands (blue) and inflammatory cell infiltration (E-H)

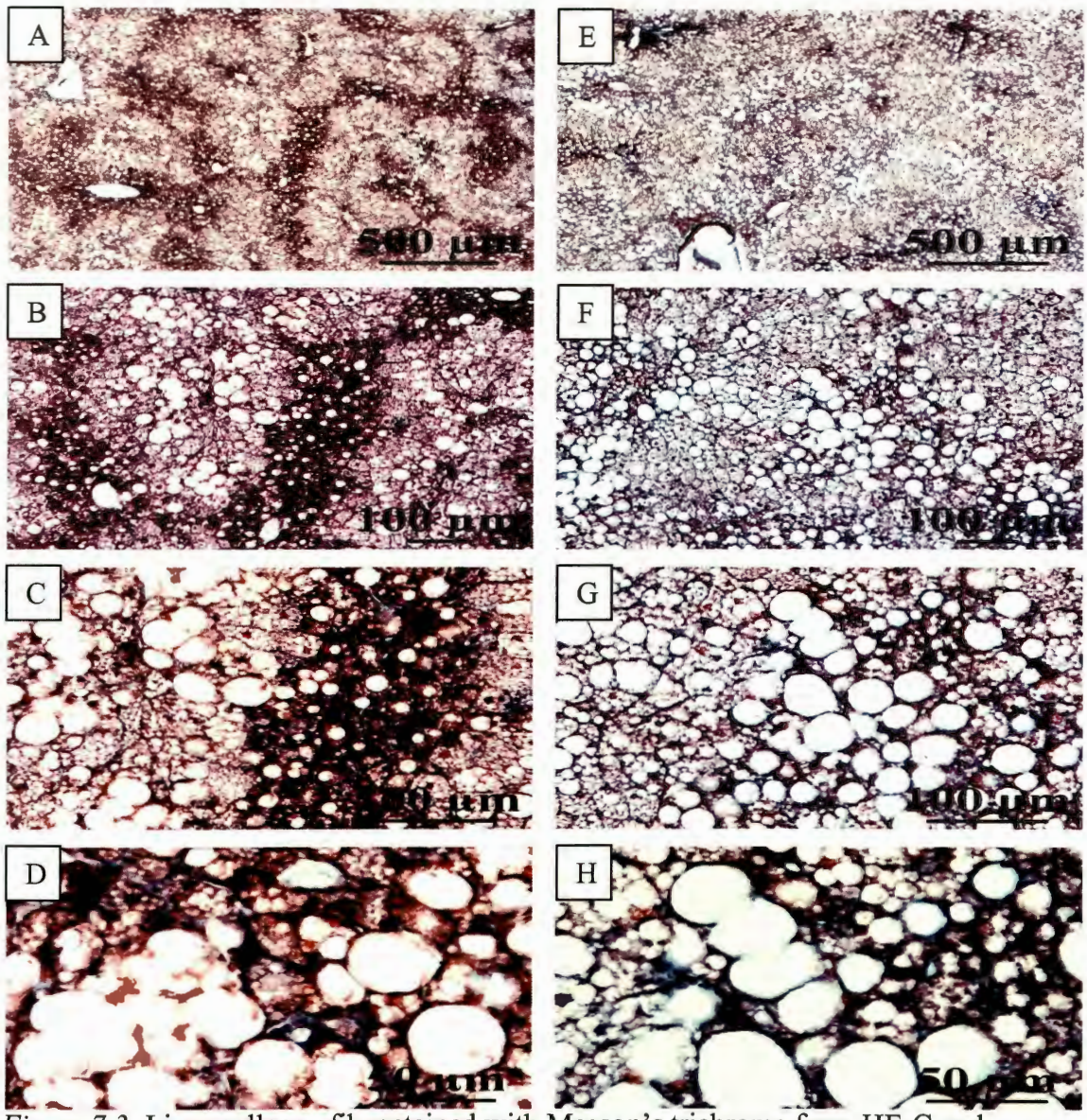


Figure 7.3: Liver collagen fiber stained with Masson's trichrome from HF-C and HF+T0.05 groups. (4X, 10 X, 20X &40X)

(A-D) HF-C control group and (E-H) versus HF+T0.05 group; Disorganization of hepatic chords, increased number of degenerated hepatocytes, fibrosis in the form of delicate bands (blue), and inflammatory cell infiltration (A-D).Moderate disorganization of hepatic chords, increased number of degenerated hepatocytes, moderate fibrosis in the form of delicate bands (blue), and no inflammatory cell infiltration (E-H)

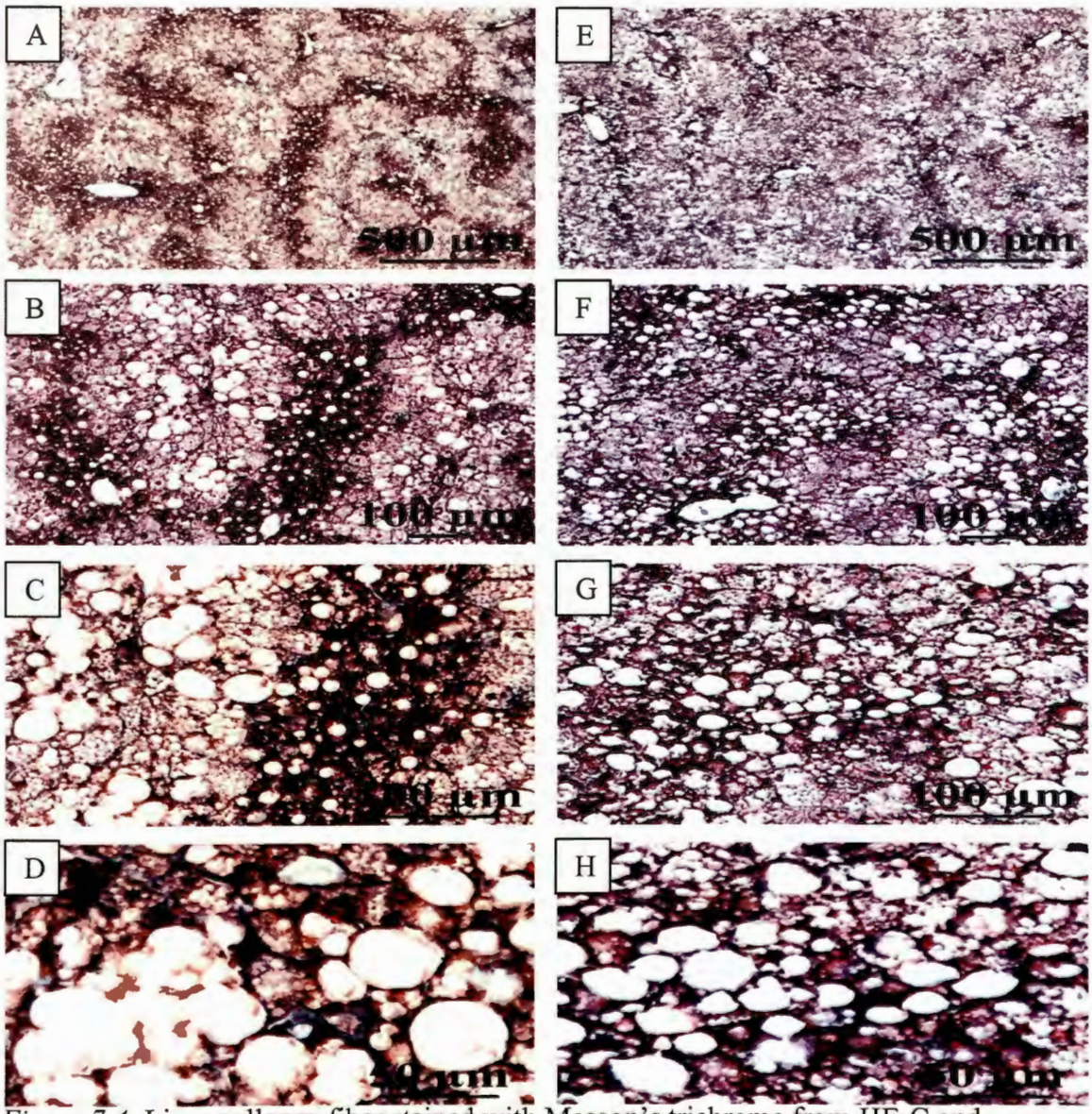


Figure 7.4: Liver collagen fiber stained with Masson's trichrome from HF-C and HF+G1+T0.025 groups. (4X, 10 X, 20X &40X)

(A-D) HF-C control group and (E-H) HF+G1+T0.025 group; Disorganization of hepatic chords, increased number of degenerated hepatocytes, fibrosis in the form of delicate bands (blue), and inflammatory cell infiltration (A-D). Mild disorganization of hepatic chords, a decrease in degenerated hepatocytes, moderate fibrosis in the form of delicate bands (blue) and inflammatory cell infiltration (E-H)

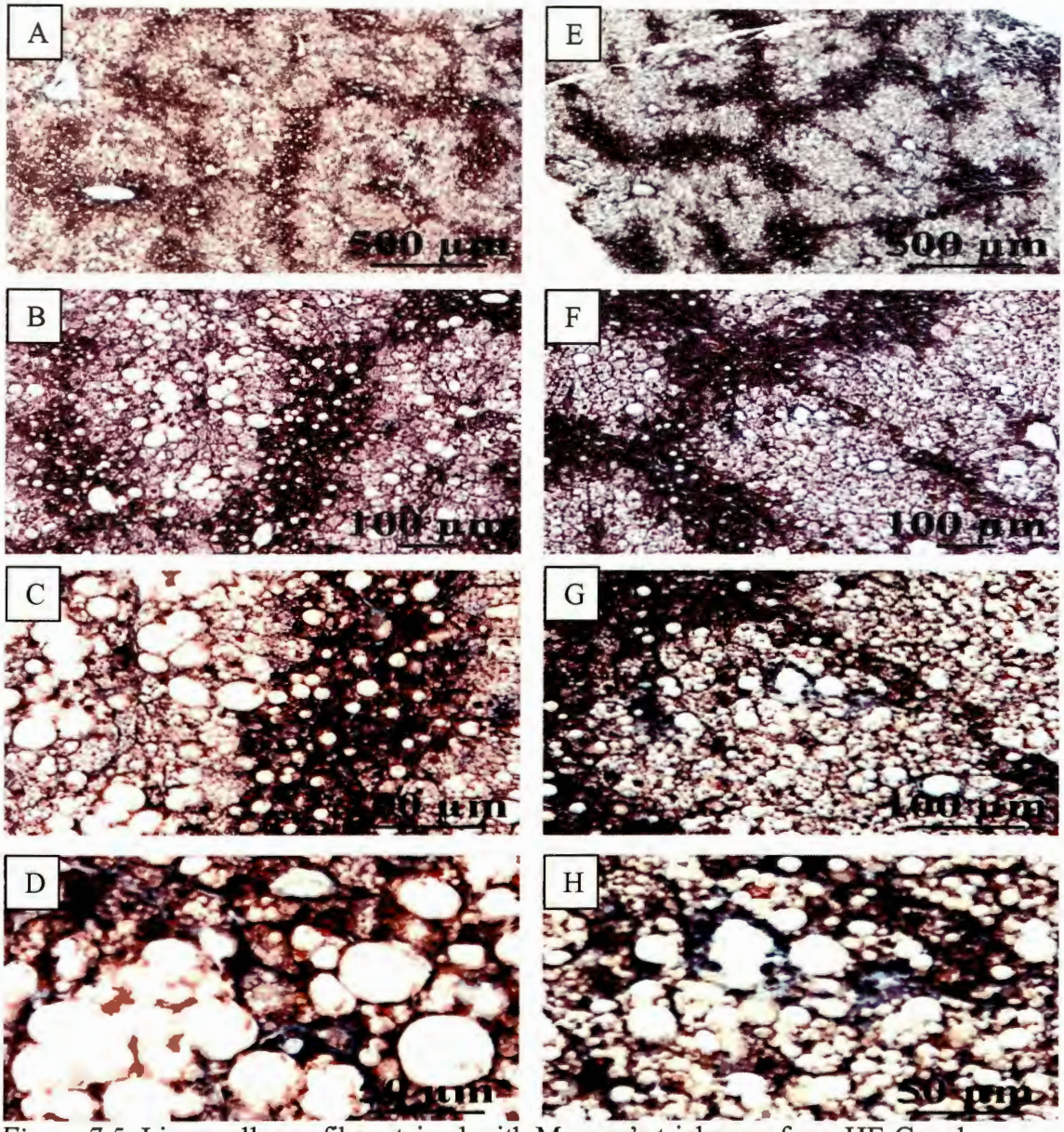


Figure 7.5: Liver collagen fiber stained with Masson's trichrome from HF-C and HF+G1+T0.05 groups. (4X, 10 X, 20X &40X)

(A-D) HF-C control group and (E-H) HF+G1+T0.05 group; Disorganization of hepatic chords, increased number of degenerated hepatocytes, fibrosis in the form of delicate bands (blue), and inflammatory cell infiltration (A-D). Mild disorganization of hepatic chords, a decrease in degenerated hepatocytes, moderate fibrosis in the form of delicate bands (blue) and inflammatory cell infiltration (E-H)

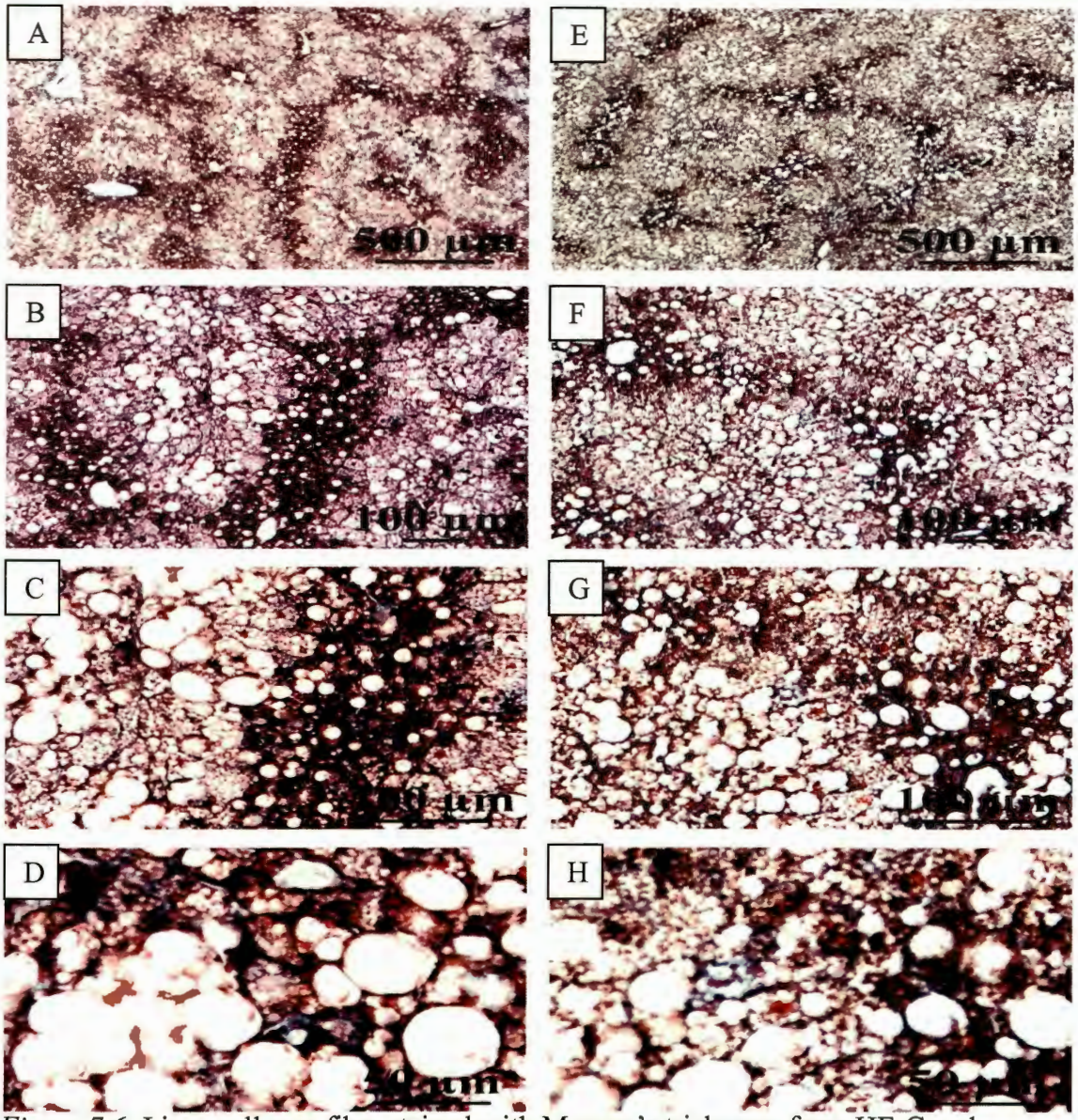


Figure 7.6: Liver collagen fiber stained with Masson's trichrome from HF-C and HF+G2+T0.025 groups. (4X, 10 X, 20X &40X)

(A-D) HF-C control group and (E-H) HF+G2+T0.025 group; Disorganization of hepatic chords, increased number of degenerated hepatocytes, fibrosis in the form of delicate bands (blue), and inflammatory cell infiltration (A-D). Mild disorganization of hepatic chords, a decrease in degenerated hepatocytes, moderate fibrosis in the form of delicate bands (blue) and inflammatory cell infiltration (E-H)

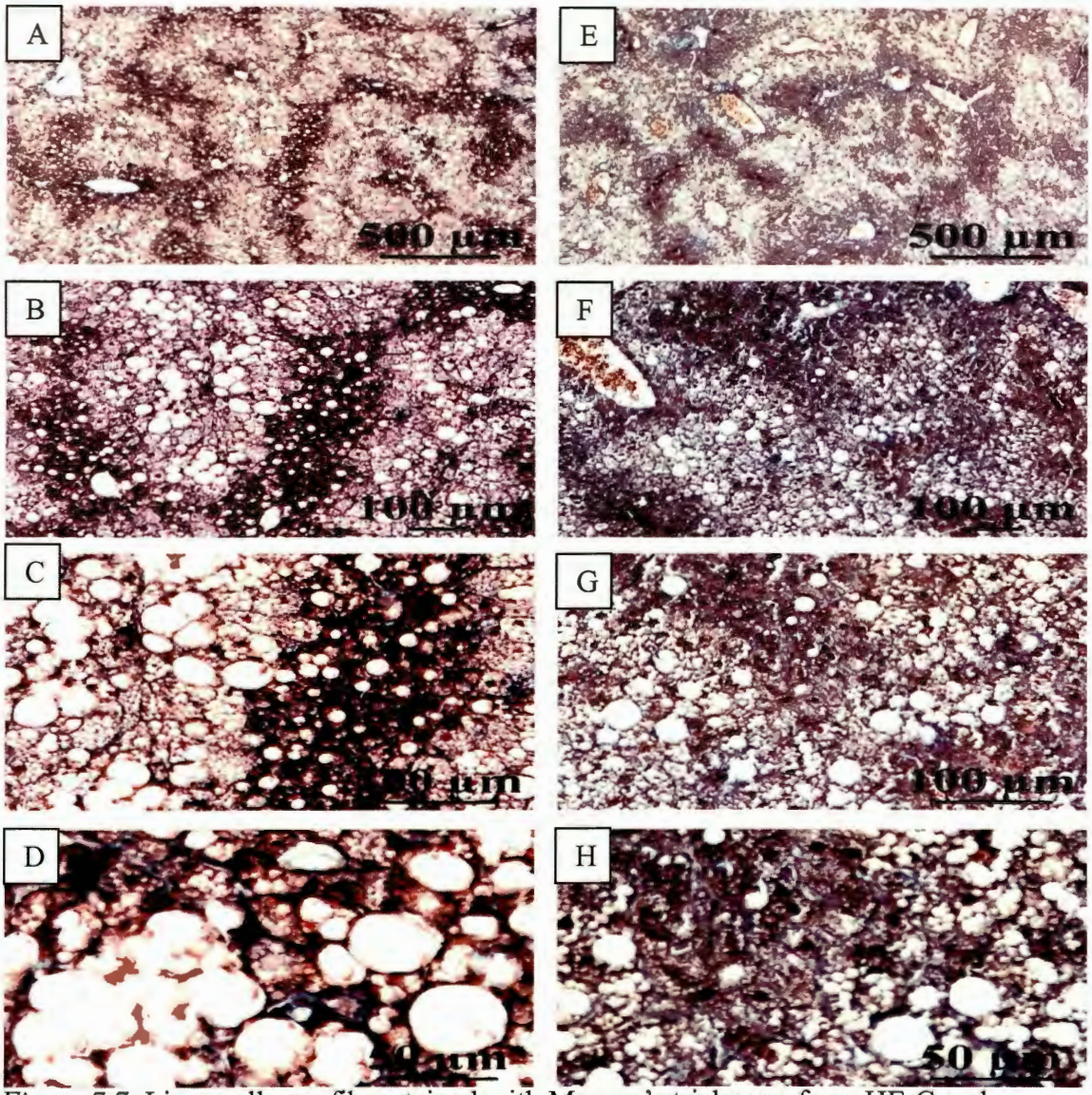


Figure 7.7: Liver collagen fiber stained with Masson's trichrome from HF-C and HF+G2+T0.05 groups. (4X, 10 X, 20X &40X)

(A-D) HF-C control group and (E-H) HF-C+2% GSE+0.05% δ3T(IIB) group; Disorganization of hepatic chords, increased number of degenerated hepatocytes, fibrosis in the form of delicate bands (blue), and inflammatory cell infiltration (A-D). Mild disorganization of hepatic chords, a decrease in degenerated hepatocytes, moderate fibrosis in the form of delicate bands (blue) and inflammatory cell infiltration (E-H)

Immunohistochemical Analysis

Immunohistochemical Analysis for α-SMA Using Confocal Microscopy

Immunohistochemical staining was performed to confirm the presence of fibrosis and evaluate the effect of HF-C supplementation with GSP and δ3T (Figure 8.1-8.8, Table 3, pg # 57) on the secretion of collagenous substances after steatosis. Alpha smooth muscle actin (α-SMA) was used to assess hepatic fibrosis by immunostaining. In the HF-C (Figure 8.1, B) fed control livers, α-SMA was localized along the blood vessels and occasionally on the sinusoidal walls. In the livers of HF-C fed mice, weak α-SMA staining was observed on the sinusoidal wall compared to NF-C group (Figure 8.2, B).

Cells stained positive for α-SMA were largely localized along the sinusoidal wall and in areas with high perisinusoidal fibrosis. The HF-C group liver sections had high intensity of activated hepatic stellate cells with large cytoplasmic processes strongly positive for α-SMA present adjacent to fibrotic areas compared to NF-C and GSP groups (Figures 8.1, B and 8.2 , A). While the HF-C+G2 (Figure 8.3, B) supplemented group showed a significant reduction in the intensity of the immunostain of α-SMA antibody, especially in the hepatocytes in the perivenucular areas with score of 0 (Table 3). The nuclei were well organized and there was no change in shape or size. The HF-C and HF+T0.05 (Figure 8.4, B) were observed to have cytoplasmic processes strongly positive for α-SMA and score 2 (Table 3, pg # 57).

In the four combination supplemented groups (Figure 8.5 to 8.8, B) there was reduction in the intensity of the α-SMA immunostain and the score were 0 (Table 3), which relates to reduction of hepatic steallate cell activation and improvement of liver fibrosis. The extent of fibrosis measured by histological scoring (Table 3) in the HF-C group versus the HF-C+G2 group suggests that decrease in α-SMA correlates to reduction of HSC activation, and therefore reduction in hepatic fibrosis.

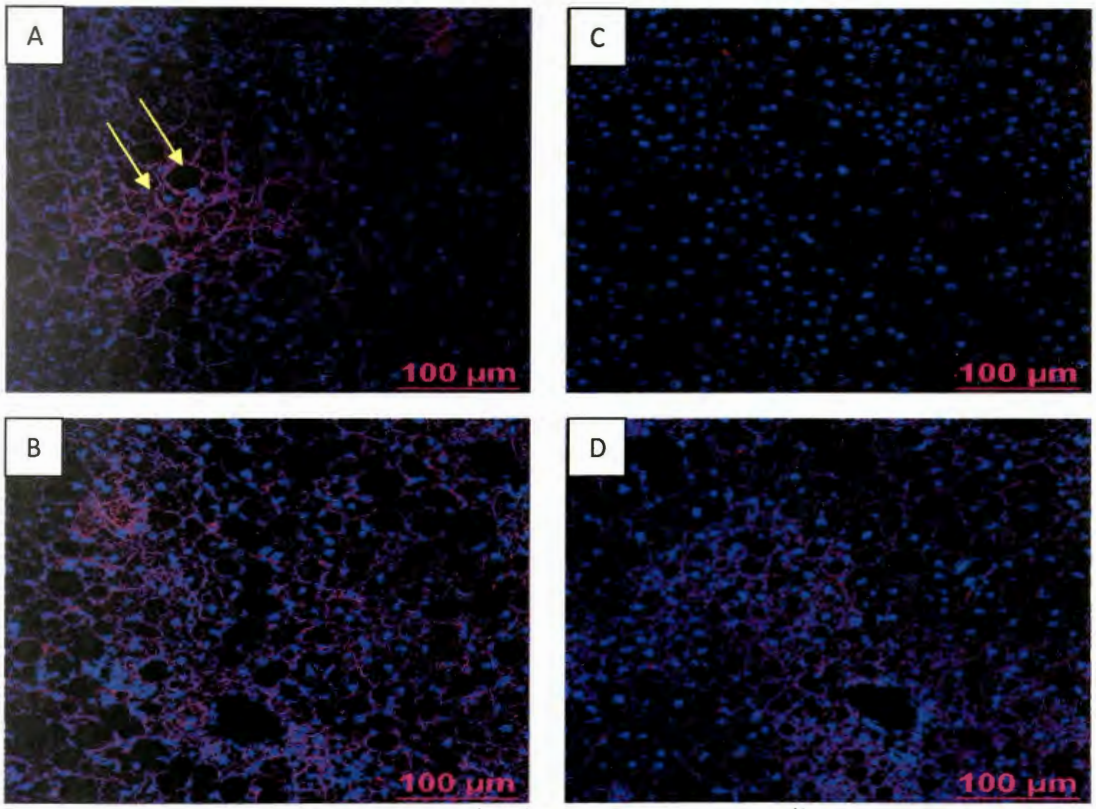


Figure 8.1: Liver α-SMA immunostained with fluorescent antibody (20X) from HF-C and NF-C groups.

Hepatic α-SMA-positive cells (indicated by arrows) were detected by immunohistochemical staining at 20 weeks.The stained photographs show the nuclei as blue dots, and the smooth muscle actin as pink network. (A)HF-C group,(B-D) NF-C group. The photos (B) & (D) are controls with no secondary antibody and no primary antibody respectively of AIN-G group. The original magnification was x100. For α-SMA, anti-mouse primary antibody was used (1:200) dilution and later the secondary antibody with DyLight 594 was used (1:200) and mounting media with DAPI was used to stain the liver sections of C57BL/J6 mice.

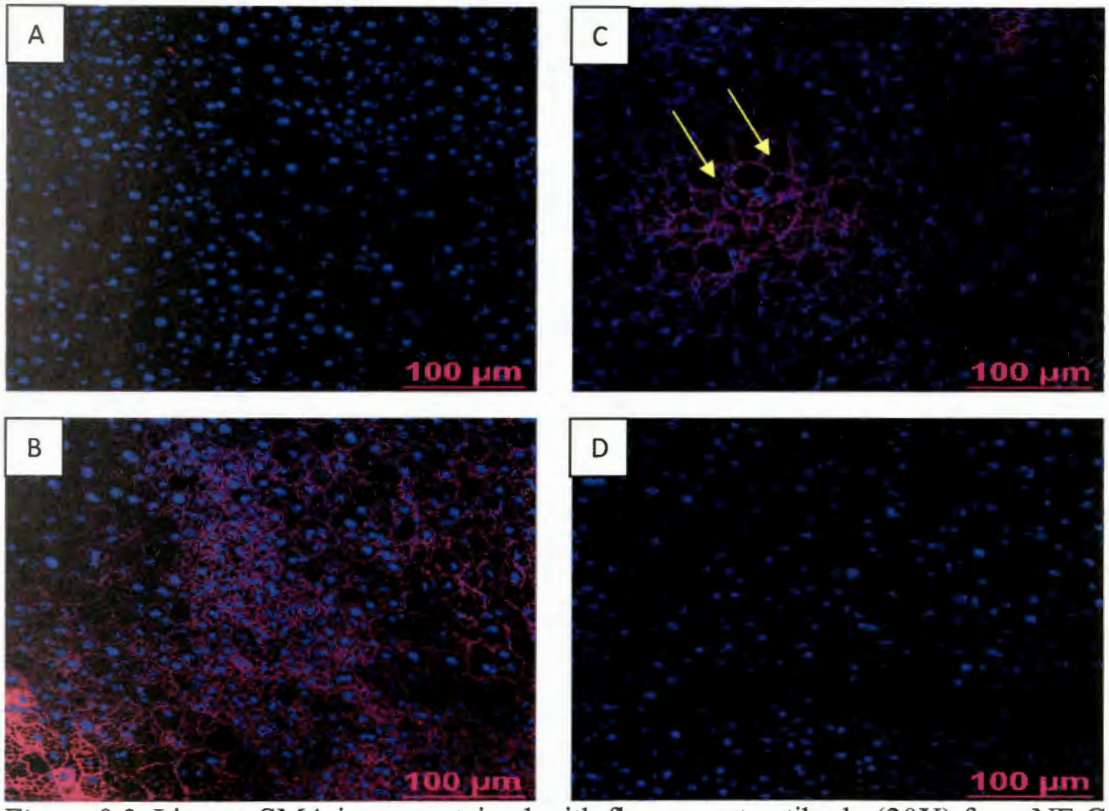


Figure 8.2: Liver α-SMA immunostained with fluorescent antibody (20X) from NF-C and HF-C groups.

Hepatic α-SMA-positive cells (indicated by arrows) were detected by immunohistochemical staining at 20 weeks. The stained photographs show the nuclei as blue dots, and the smooth muscle actin as pink network. (A)AIN-G group, (B-D) AIN-G group. The photos (B) & (D) are controls with no secondary antibody and no primary antibody respectively in HF-C group. The original magnification was x100. For α-SMA, anti-mouse primary antibody was used (1:200) dilution and later the secondary antibody with DyLight 594 was used (1:200) and mounting media with DAPI was used to stain the liver sections of C57BL/J6 mice.

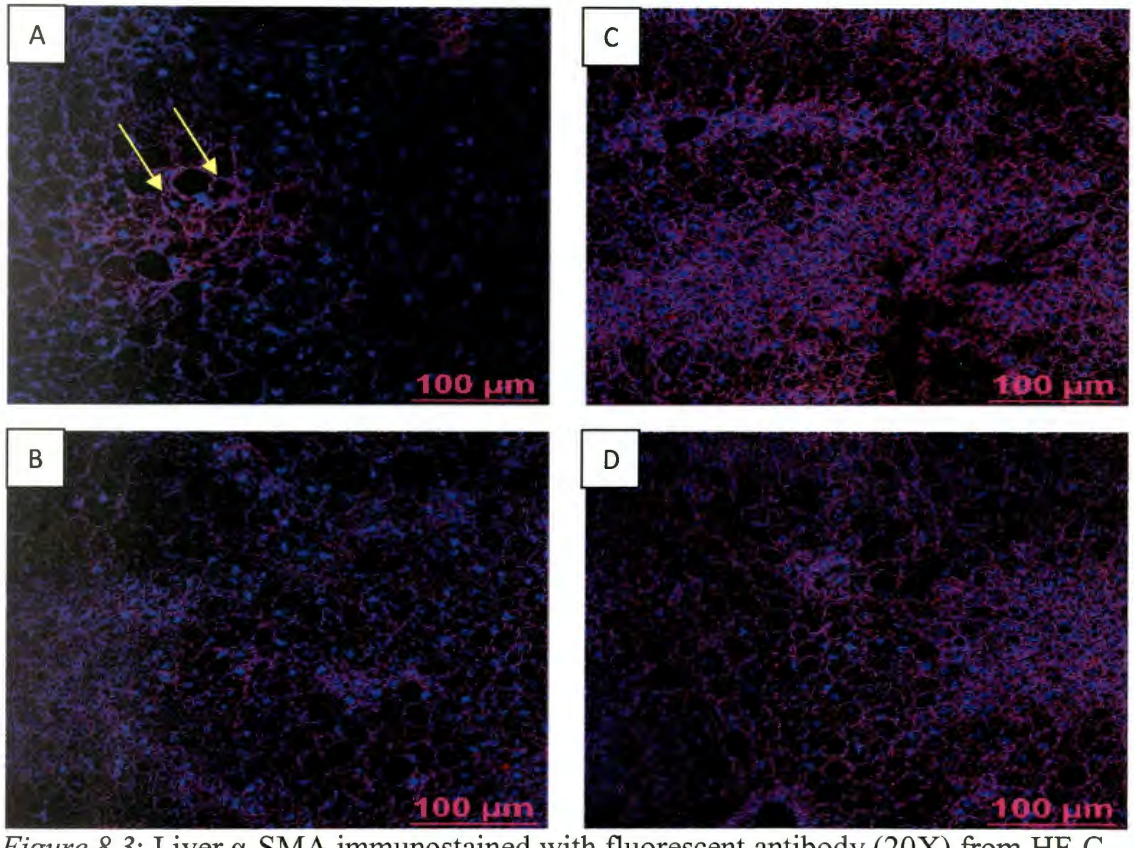


Figure 8.3: Liver α-SMA immunostained with fluorescent antibody (20X) from HF-C and HF+G2 groups.

Hepatic α-SMA-positive cells (indicated by arrows) were detected by immunohistochemical staining at 20 weeks. The stained photographs show the nuclei as blue dots, and the smooth muscle actin as pink network. (A)HF-C group (B-D) HF-C+ 2% GSE group. The photos (B) & (D) are controls with no secondary antibody and no primary antibody respectively in HF-C+2% GSE group. The original magnification was x100. For α-SMA, anti-mouse primary antibody was used (1:200) dilution and later the secondary antibody with DyLight 594 was used (1:200) and mounting media with DAPI was used to stain the liver sections of C57BL/J6 mice.

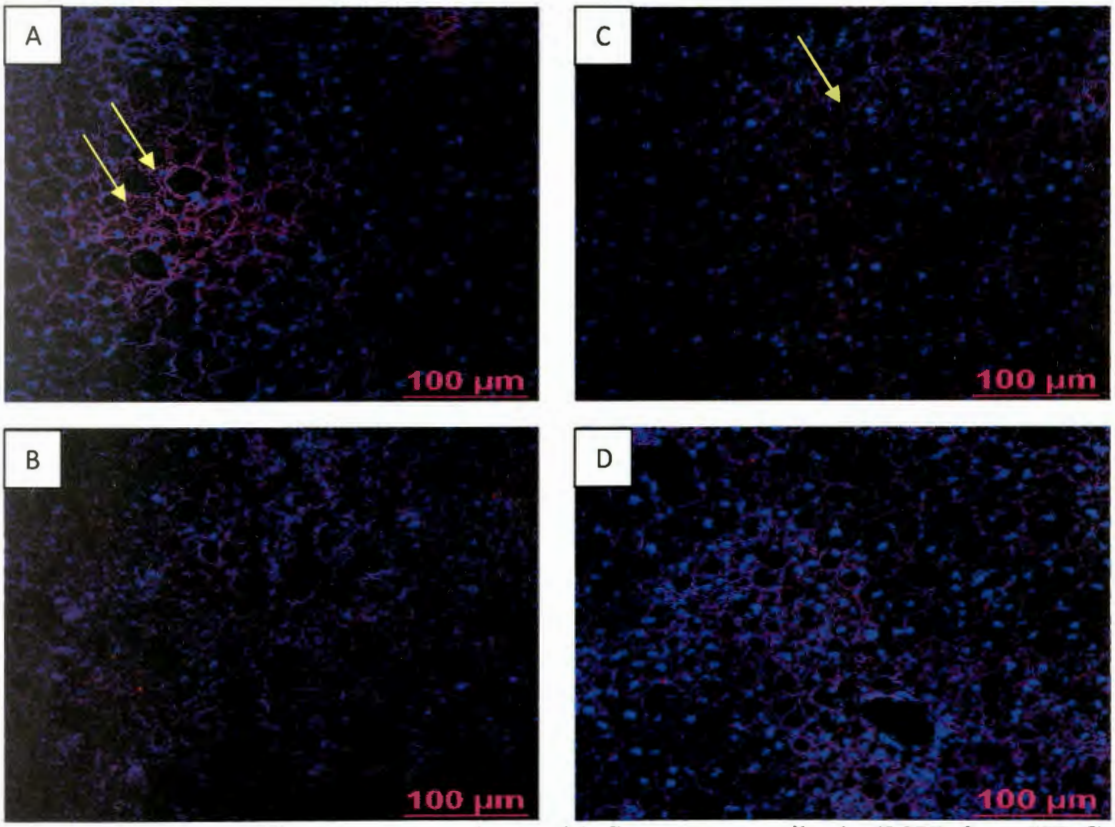


Figure 8.4: Liver α-SMA immunostained with fluorescent antibody (20X) from HF-C and HF+T0.05 groups.

Hepatic α-SMA-positive cells (indicated by arrows) were detected by immunohistochemical staining at 20 weeks. The stained photographs show the nuclei as blue dots, and the smooth muscle actin as pink network. (A)HF-C group, (B-D) HF+T0.05 group. The photos (B) & (D) are controls with no secondary antibody and no primary antibody respectively in HF-C+0.05% δ3T group. The original magnification was x100. For α-SMA, anti-mouse primary antibody was used (1:200) dilution and later the secondary antibody with DyLight 594 was used (1:200) and mounting media with DAPI was used to stain the liver sections of C57BL/J6 mice.

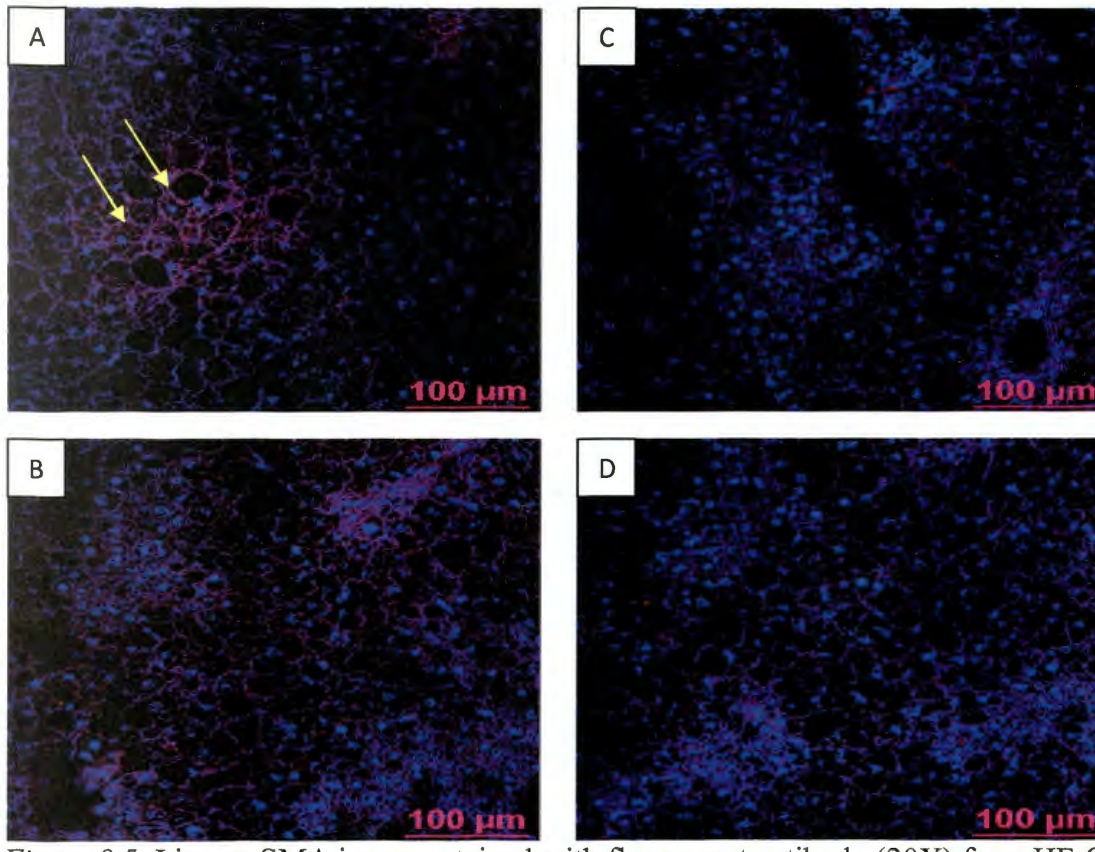


Figure 8.5: Liver α-SMA immunostained with fluorescent antibody (20X) from HF-C and HF+G1+T0.025 groups.

Hepatic α-SMA-positive cells (indicated by arrows) were detected by immunohistochemical staining at 20 weeks. The stained photographs show the nuclei as blue dots, and the smooth muscle actin as pink network. (A)HF-C group, (B-D) HF+G1+T0.025 group. The photos (B) & (D) are controls with no secondary antibody and no primary antibody respectively in HF+G1+T0.025 group. The original magnification was x100. For α-SMA, anti-mouse primary antibody was used (1:200) dilution and later the secondary antibody with DyLight 594 was used (1:200) and mounting media with DAPI was used to stain the liver sections of C57BL/J6 mice.

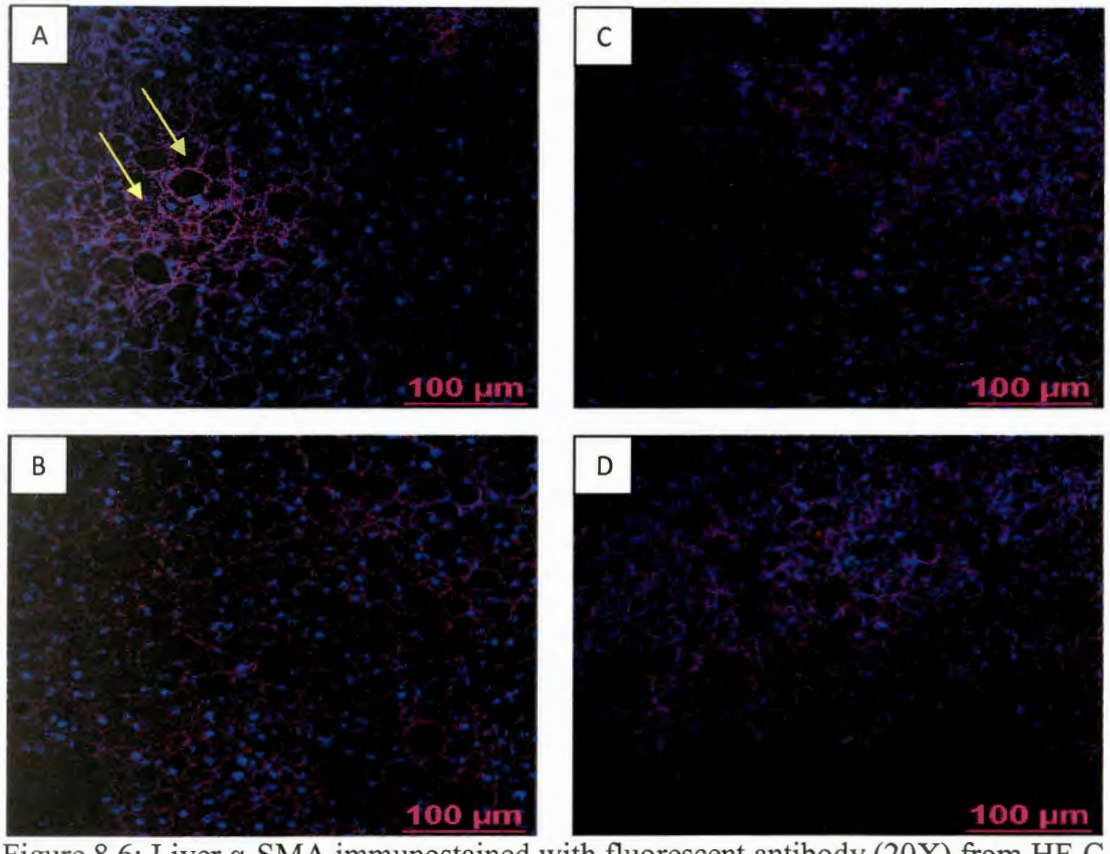


Figure 8.6: Liver α-SMA immunostained with fluorescent antibody (20X) from HF-C and HF+G1+T0.05 groups.

Hepatic α-SMA-positive cells (indicated by arrows) were detected by immunohistochemical staining at 20 weeks. The stained photographs show the nuclei as blue dots, and the smooth muscle actin as pink network. (A)HF-C group (B-D) HF+G1+T0.05 group. The photos (B) & (D) are controls with no secondary antibody and no primary antibody respectively in HF+G1+T0.05 group. The original magnification was x100. For α-SMA, anti-mouse primary antibody was used (1:200) dilution and later the secondary antibody with DyLight 594 was used (1:200) and mounting media with DAPI was used to stain the liver sections of C57BL/J6 mice.

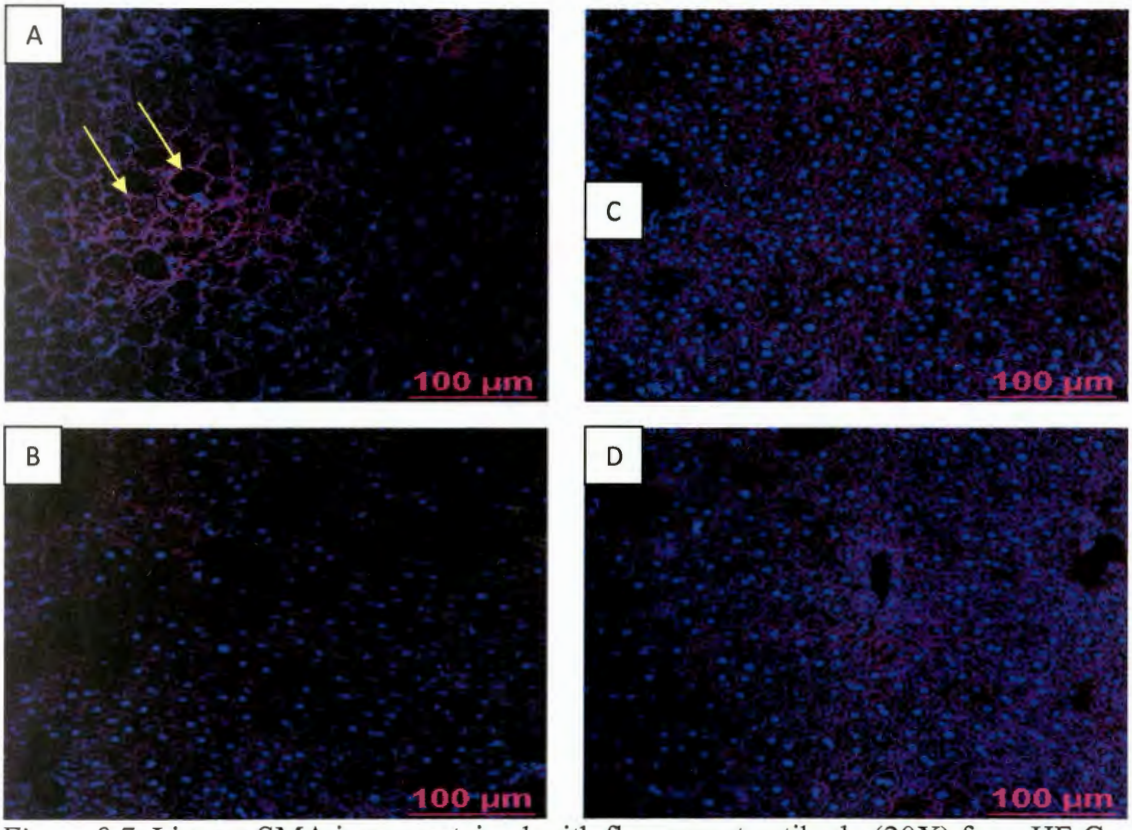


Figure 8.7: Liver α-SMA immunostained with fluorescent antibody (20X) from HF-C and HF+G2+T0.025 groups.

Hepatic α-SMA-positive cells (indicated by arrows) were detected by immunohistochemical staining at 20 weeks. The stained photographs show the nuclei as blue dots, and the smooth muscle actin as pink network. (A)HF-C group (B-D) HF+G2+T0.025 group. The photos (B) & (D) are controls with no secondary antibody and no primary antibody respectively in HF+G2+T0.025 group. The original magnification was x100. For α-SMA, anti-mouse primary antibody was used (1:200) dilution and later the secondary antibody with DyLight 594 was used (1:200) and mounting media with DAPI was used to stain the liver sections of C57BL/J6 mice.

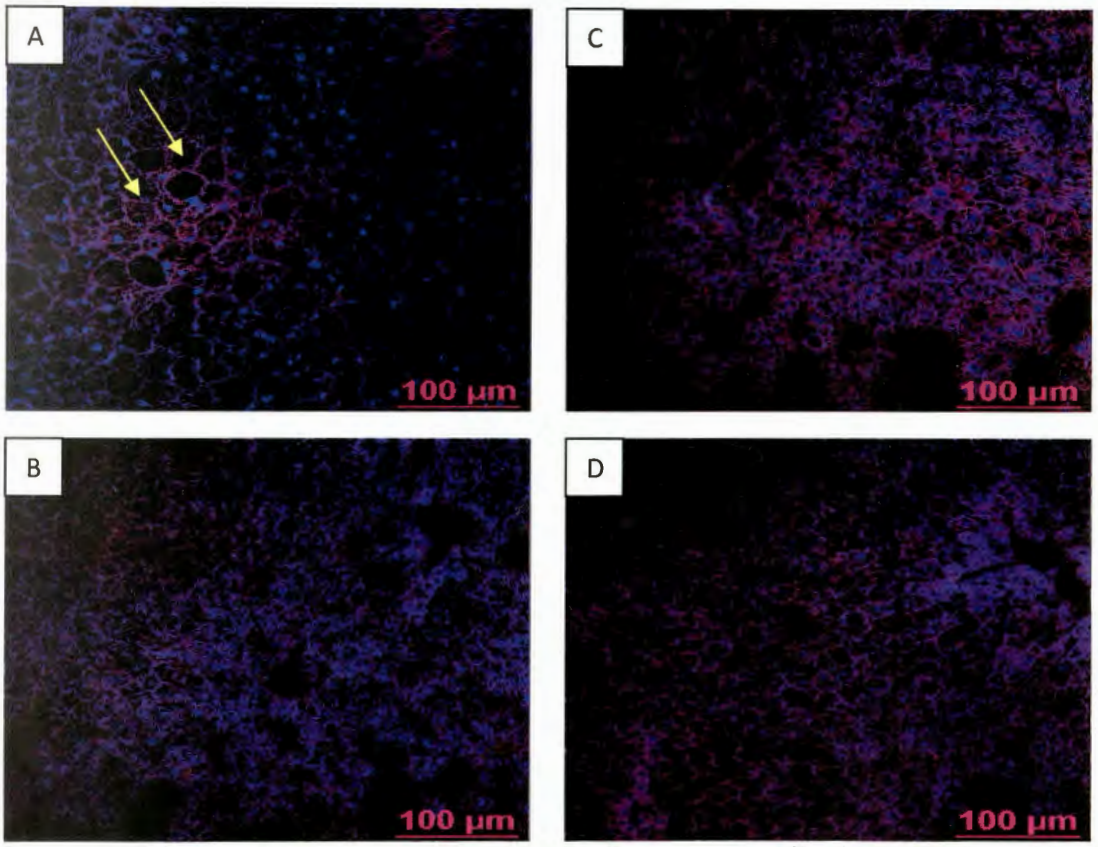


Figure 8.8: Liver α-SMA immunostained with fluorescent antibody (20X) from HF-C and HF+G2+T0.05 groups.

Hepatic α-SMA-positive cells (indicated by arrows) were detected by immunohistochemical staining at 20 weeks. The stained photographs show the nuclei as blue dots, and the smooth muscle actin as pink network. (A)HF-C group (B-D) HF+G2+T0.05 group. The photos (B) & (D) are controls with no secondary antibody and no primary antibody respectively in HF+G2+T0.05 group. The original magnification was x100. For α-SMA, anti-mouse primary antibody was used (1:200) dilution and later the secondary antibody with DyLight 594 was used (1:200) and mounting media with DAPI was used to stain the liver sections of C57BL/J6 mice

CHAPTER V

DISCUSSION

This study induced steatohepatitis in C57BLJ/6 mouse model using a high fat (HF) diet and observed beneficial effect of supplementation of dietary grape seed polyphenols (GSP) and delta tocotrienols (δ3T) on tissue weights and hitopathological changes characteristic of human NASH.

The mean liver weights were significantly different in HF control (HF-C) group than the normal fat control (NF-C) group. This study is similar with the earlier study with C57BL/J6 mouse model where high fat diet was used to induce NASH-like characteristic features (Sundaresan S et al, 2010). The changes in the liver weight can be attributed to various known mechanisms, the primary one is the increased delivery of free fatty acids to the liver via diet which may lead to the accumulation of fat mainly triglycerides and therefore cause steatosis in HF-C fed mice (Wanless I.R et al, 2004 and Fan J.G et al, 2008). There was a significant effect of supplementation with dietary GSP in (HF+G2) group, mice had reduced liver weight and adipose tissue weights followed by all the combination groups with GSP and δ3T. This significant change in liver and adipose tissue weights with GSP supplementation alone and with delta tocotrienol shows the beneficial effects and the use of GSP as a treatment agent in NASH. The earlier studies with resveratrol, a bioactive compound found in grapes show similar results (Ahn. J et al, 2008)

On histopathological analysis the HF diet fed mice seem to have developed steatosis (at least 33%) at the end of 20 weeks. Results from the three histology stains of liver (H&E, PAS and Masson's trichrome) suggest a possible link between GSP alone and with δ3T as a supplement in HF diet, and the reduction of histopathological signs of NASH. There is observable reduction of lipid droplets as fractional area (% /total area per mm2) in HF+G2 fed liver sections followed by the four combination groups whose mice liver sections also showed reduction in the lipid droplet area. The HF+G2 fed mice demonstrated marked reduction of microvesicular and macrovesicular steatosis. This positive action of GSP may be attributed to either anti-lipogenic action or increased lipid oxidation or excretion (Osada et al, 2006 and Ahn. J et al, 2006) which is the primary effect of GSP supplementation in liver. This might be indirectly supported by the reduction the liver glycolipid storage as observed by PAS stained sections. Thus the present data is in agreement with the earlier *in vivo* and *in vitro* reports showing that grape seed polyphenols have a beneficial effect in reducing steatosis (Pinent M et al, 2005; Quesada. H et al, 2009 and Baiges I et al, 2010)

The relation between liver fibrosis and steatosis is not clearly known, there is an indirect correlation which connects the hepatic stellate cells (HSC) activation and increase in secretion of α-SMA after steatosis occurs. An increase in α-SMA concentration is a key factor in the histopathological analysis to assess the progression of fibrotic changes after steatosis. The present study suggests a decrease in steatosis with improved fibrotic modulation of HSC by reduction of α-SMA antibody concentration as

observed by immunohistochemistry in the mice liver supplemented with GSP. The GSP supplement suppressed α -SMA accumulation which is similar to other studies which used α –SMA as one of the biomarkers in the onset of liver fibrosis (Ahn J et al, 2006; Lee E.S et al, 2010 and Hong S. W, et al 2010). Therefore liver fibrosis related to NASH can be reduced by supplementation of dietary GSP suggesting a protective function against steatohepatitis. There were improvement in characteristic histopathological features and initiation of fibrosis observed with the reduction of α-SMA seen in HF-C supplemented with GSP liver sections with reduction of fibrosis either in zone 1 or perisinusoidal.

Tocopherols have been used as treatment for NAFLD and documented by several studies with inconsistent effects in NAFLD (Lavine J et al, 2000; Kugelmas M et al 2003 and Sanayal A J 2004 & 2010). In the present study the δ3T group showed fat vesicular droplets similar to HF-C but was found to have mild fibrosis. On the contrary, GSP alone may enhamce the anti-lipogenic function in liver. There were no adverse effects observed with GSP via the histology. This study, for the first time, demonstrates that GSP as a dietary supplement which is known to be excellent primary antioxidant may be used for the amelioration of similar NASH-like features in humans. This study highlights the role of histological and immunohistochemical methods in the identification of NASH. Histology and immunohistochemistry helped to identify the changes in characteristic features of NASH like reduction of steatosis, hepatocyte degeneration and liver fibrosis with detection of the biomarker α-SMA and onset of fibrosis. This study helped to use

immunohistochemistry as a confirmative diagnostic tool for detection and distinction of NASH and fibrosis from NAFLD or simple steatosis (Oh M.K et al, 2008 and Straub B.K et al, 2010). The use of confocal microscopy helps avoid or reduce human biases or errors and provides a possible solution for the need of a standardized method for early detection of NASH.

One of the limitations is the available histological grading of NASH for classification may not be applicable in relation to the clinical and histopathological background and changes in the morphology caused by severity of this chronic disease. To overcome this simple histology and immunohistochemical approach was used for the identification of characteristic features of NASH and onset of fibrosis.

Overall, this study shows the use of GSP and δ-tocorienol supplements may be used as part of the diet therapy to reduce liver steatosis and fibrogenesis. The study shows that GSP is hepatoprotective as anti-lipogenic and or reduce lipid droplets, reduce the initial fibrogenic process as observed by immunostaining of α-SMA. While δ3T, with GSP may act as an effective hepatoprotective agent against NASH, detailed observation(s) with only δ3T is still required. For future, intensive studies to investigate the molecular mechanisms of these supplements and their efficacy in reversal of NASH are required.

CHAPTER VI

SUMMARY AND CONCLUSION

The present study focused on the effect the two dietary supplements; grape seed polyphenols (GSP) and delta tocotrienols (δ3T) as dietary supplements with high fat (HF)diet in amelioration of NASH in C57BLJ6 mice model. In this study, NASH-like characteristic features were successfully induced using a high-fat diet in C57BL/J6 mice. The HF diet supplementation with dietary GSP reduced NASH features like hepatocye ballooning, steatosis and initiation of liver fibrosis. With 10 weeks of GSP feeding, presence of steatosis was reduced and as a result liver tissue architecture was partially restored. This proves that there is a link between hepatic steatosis and hepatic steallate cell activation which leads to development of liver fibrosis which may be improved by dietary supplementation specifically with GSP. A decrease in α -SMA biomarker in the initial stages of hepatic fibrosis was immunohistochemically observed.

While the goals of this study is to evaluate the effect of diet supplemented with GSP and δ3T, alone and in combination, in reducing HF diet induced NASH features our finding in the δ3T supplemented groups did not yield any positive effects in reducing steatosis while it did not exacerbate liver fibrosis. Future studies are warranted to understand the metabolic mechanism and interaction of dietary GSP and δ3T separately and in combination as supplements using animal models of NASH and NAFLD.

REFERENCES

Abdelmalek, M. F., Angulo, P., Jorgensen, R. A., Sylvestre, P. B., & Lindor, K. D. (2001). Betaine, a promising new agent for patients with nonalcoholic steatohepatitis: Results of a pilot study. *The American Journal of Gastroenterology, 96*(9), 2711-2717. doi:10.1111/j.1572-0241.2001.04129.x

Adams, L. A., & Angulo, P. (2005). Recent concepts in non-alcoholic fatty liver disease. *Diabetic Medicine : A Journal of the British Diabetic Association, 22*(9), 1129-1133. doi:10.1111/j.1464-5491.2005.01748.x

Adams, L. A., Angulo, P., & Lindor, K. D. (2005). Nonalcoholic fatty liver disease. *CMAJ : Canadian Medical Association Journal = Journal De l'Association Medicale Canadienne, 172*(7), 899-905. doi:10.1503/cmaj.045232

Adams, L. A., Lymp, J. F., St Sauver, J., Sanderson, S. O., Lindor, K. D., Feldstein, A., & Angulo, P. (2005). The natural history of nonalcoholic fatty liver disease: A population-based cohort study. *Gastroenterology, 129*(1), 113-121.

Ahn, J., Cho, I., Kim, S., Kwon, D., & Ha, T. (2008). Dietary resveratrol alters lipid metabolism-related gene expression of mice on an atherogenic diet. *Journal of Hepatology, 49*(6), 1019-1028. doi:10.1016/j.jhep.2008.08.012

Allard, J. P., Aghdassi, E., Mohammed, S., Raman, M., Avand, G., Arendt, B. M., . . . Heathcote, J. E. (2008). Nutritional assessment and hepatic fatty acid composition in non-alcoholic fatty liver disease (NAFLD): A cross-sectional study. *Journal of Hepatology, 48*(2), 300-307. doi:10.1016/j.jhep.2007.09.009

Angulo, P. (2002). Treatment of nonalcoholic fatty liver disease. *Annals of Hepatology : Official Journal of the Mexican Association of Hepatology, 1*(1), 12-19.

Angulo, P. (2007). Obesity and nonalcoholic fatty liver disease. *Nutrition Reviews, 65*(6 Pt 2), S57-63.

Aoun, M., Michel, F., Fouret, G., Casas, F., Jullien, M., Wrutniak-Cabello, C., . . . Feillet-Coudray, C. (2010). A polyphenol extract modifies quantity but not quality of liver fatty acid content in high-fat-high-sucrose diet-fed rats: Possible implication of the sirtuin pathway. *The British Journal of Nutrition, 104*(12), 1760-1770. doi:10.1017/S0007114510002850

Aron, M., Nguyen, M. M., Stein, R. J., & Gill, I. S. (2008). Impact of gender in renal cell carcinoma: An analysis of the SEER database. *European Urology, 54*(1), 133-140. doi:10.1016/j.eururo.2007.12.001

Baiges, I., Palmfeldt, J., Blade, C., Gregersen, N., & Arola, L. (2010). Lipogenesis is decreased by grape seed proanthocyanidins according to liver proteomics of rats fed a high fat diet. *Molecular & Cellular Proteomics : MCP, 9*(7), 1499-1513. doi:10.1074/mcp.M000055-MCP201

Batts, K. P., & Ludwig, J. (1995). Chronic hepatitis. an update on terminology and reporting. *The American Journal of Surgical Pathology, 19*(12), 1409-1417.

Baumgardner, J. N., Shankar, K., Hennings, L., Badger, T. M., & Ronis, M. J. (2008). A new model for nonalcoholic steatohepatitis in the rat utilizing total enteral nutrition to overfeed a high-polyunsaturated fat diet. *American Journal of Physiology.Gastrointestinal and Liver Physiology, 294*(1), G27-38. doi:10.1152/ajpgi.00296.2007

Boden, G., & Zhang, M. (2006). Recent findings concerning thiazolidinediones in the treatment of diabetes. *Expert Opinion on Investigational Drugs, 15*(3), 243-250. doi:10.1517/13543784.15.3.243

Browning, J. D., Szczepaniak, L. S., Dobbins, R., Nuremberg, P., Horton, J. D., Cohen, J. C., . . . Hobbs, H. H. (2004). Prevalence of hepatic steatosis in an urban population in the united states: Impact of ethnicity. *Hepatology (Baltimore, Md.), 40*(6), 1387-1395. doi:10.1002/hep.20466

Brunt, E. M. (2001). Nonalcoholic steatohepatitis: Definition and pathology. *Seminars in Liver Disease, 21*(1), 3-16.

Brunt, E. M. (2007). Pathology of fatty liver disease. *Modern Pathology : An Official Journal of the United States and Canadian Academy of Pathology, Inc, 20 Suppl 1*, S40-8. doi:10.1038/modpathol.3800680

Brunt, E. M., Janney, C. G., Di Bisceglie, A. M., Neuschwander-Tetri, B. A., & Bacon, B. R. (1999). Nonalcoholic steatohepatitis: A proposal for grading and staging the histological lesions. *The American Journal of Gastroenterology, 94*(9), 2467-2474. doi:10.1111/j.1572-0241.1999.01377.x

Brunt, E. M., Neuschwander-Tetri, B. A., Oliver, D., Wehmeier, K. R., & Bacon, B. R. (2004). Nonalcoholic steatohepatitis: Histologic features and clinical correlations with 30 blinded biopsy specimens. *Human Pathology, 35*(9), 1070-1082.

Brunt, E. M., & Tiniakos, D. G. (2005). Pathological features of NASH. *Frontiers in Bioscience : A Journal and Virtual Library, 10*, 1475-1484.

Budin, S. B., Othman, F., Louis, S. R., Bakar, M. A., Das, S., & Mohamed, J. (2009). The effects of palm oil tocotrienol-rich fraction supplementation on biochemical parameters, oxidative stress and the vascular wall of streptozotocin-induced diabetic rats. *Clinics (Sao Paulo, Brazil), 64*(3), 235-244.

Bugianesi, E. (2007). Non-alcoholic steatohepatitis and cancer. *Clinics in Liver Disease, 11*(1), 191-207, x-xi. doi:10.1016/j.cld.2007.02.006

Bugianesi, E., Gentilcore, E., Manini, R., Natale, S., Vanni, E., Villanova, N., . . . Marchesini, G. (2005). A randomized controlled trial of metformin versus vitamin E or prescriptive diet in nonalcoholic fatty liver disease. *The American Journal of Gastroenterology, 100*(5), 1082-1090. doi:10.1111/j.1572-0241.2005.41583.x

Bugianesi, E., McCullough, A. J., & Marchesini, G. (2005). Insulin resistance: A metabolic pathway to chronic liver disease. *Hepatology (Baltimore, Md.), 42*(5), 987-1000. doi:10.1002/hep.20920

Bugianesi, E., Vanni, E., & Marchesini, G. (2007). NASH and the risk of cirrhosis and hepatocellular carcinoma in type 2 diabetes. *Current Diabetes Reports, 7*(3), 175-180.

Burton, G. W., & Traber, M. G. (1990). Vitamin E: Antioxidant activity, biokinetics, and bioavailability. *Annual Review of Nutrition, 10*, 357-382. doi:10.1146/annurev.nu.10.070190.002041

Carmiel-Haggai, M., Cederbaum, A. I., & Nieto, N. (2005). A high-fat diet leads to the progression of non-alcoholic fatty liver disease in obese rats. *The FASEB Journal : Official Publication of the Federation of American Societies for Experimental Biology, 19*(1), 136-138. doi:10.1096/fj.04-2291fje

Cave, M., Deaciuc, I., Mendez, C., Song, Z., Joshi-Barve, S., Barve, S., & McClain, C. (2007). Nonalcoholic fatty liver disease: Predisposing factors and the role of nutrition. *The Journal of Nutritional Biochemistry, 18*(3), 184-195. doi:10.1016/j.jnutbio.2006.12.006

Chang, C. T., Chen, Y. C., Fang, J. T., & Huang, C. C. (2002). Metformin-associated lactic acidosis: Case reports and literature review. *Journal of Nephrology, 15*(4), 398-402.

Chavez-Tapia, N. C., Tellez-Avila, F. I., Barrientos-Gutierrez, T., Mendez-Sanchez, N., Lizardi-Cervera, J., & Uribe, M. (2010). Bariatric surgery for non-alcoholic steatohepatitis in obese patients. *Cochrane Database of Systematic Reviews (Online), (1)*(1), CD007340. doi:10.1002/14651858.CD007340.pub2

Chavez-Tapia, N. N., Uribe, M., Ponciano-Rodriguez, G., Medina-Santillan, R., & Mendez-Sanchez, N. (2009). New insights into the pathophysiology of nonalcoholic fatty liver disease. *Annals of Hepatology : Official Journal of the Mexican Association of Hepatology, 8 Suppl 1*, S9-17.

Chiquette, E., Ramirez, G., & Defronzo, R. (2004). A meta-analysis comparing the effect of thiazolidinediones on cardiovascular risk factors. *Archives of Internal Medicine, 164*(19), 2097-2104. doi:10.1001/archinte.164.19.2097

Chitturi, S., Abeygunasekera, S., Farrell, G. C., Holmes-Walker, J., Hui, J. M., Fung, C., . . . George, J. (2002). NASH and insulin resistance: Insulin hypersecretion and specific association with the insulin resistance syndrome. *Hepatology (Baltimore, Md.), 35*(2), 373-379. doi:10.1053/jhep.2002.30692

Chou, T. W., Ma, C. Y., Cheng, H. H., Chen, Y. Y., & Lai, M. H. (2009). A rice bran oil diet improves lipid abnormalities and suppress hyperinsulinemic responses in rats with streptozotocin/nicotinamide-induced type 2 diabetes. *Journal of Clinical Biochemistry and Nutrition, 45*(1), 29-36. doi:10.3164/jcbn.08-257

Clark, J. M. (2006). The epidemiology of nonalcoholic fatty liver disease in adults. *Journal of Clinical Gastroenterology, 40 Suppl 1*, S5-10. doi:10.1097/01.mcg.0000168638.84840.ff

Clark, J. M., & Diehl, A. M. (2003). Nonalcoholic fatty liver disease: An underrecognized cause of cryptogenic cirrhosis. *JAMA : The Journal of the American Medical Association, 289*(22), 3000-3004. doi:10.1001/jama.289.22.3000

Cohen, P. A., Mak, K. M., Rosman, A. S., Kessova, I., Mishin, V. M., Koivisto, T., & Lieber, C. S. (1997). Immunohistochemical determination of hepatic cytochrome P-4502E1 in formalin-fixed, paraffin-embedded sections. *Alcoholism, Clinical and Experimental Research, 21*(6), 1057-1062.

Cong, W. N., Tao, R. Y., Tian, J. Y., Liu, G. T., & Ye, F. (2008). The establishment of a novel non-alcoholic steatohepatitis model accompanied with obesity and insulin resistance in mice. *Life Sciences, 82*(19-20), 983-990. doi:10.1016/j.lfs.2008.01.022

Contos, M. J., Choudhury, J., Mills, A. S., & Sanyal, A. J. (2004). The histologic spectrum of nonalcoholic fatty liver disease. *Clinics in Liver Disease, 8*(3), 481-500, vii. doi:10.1016/j.cld.2004.04.013

Cortez-Pinto, H., Baptista, A., Camilo, M. E., & de Moura, M. C. (2001). Hepatic stellate cell activation occurs in nonalcoholic steatohepatitis. *Hepato-Gastroenterology, 48*(37), 87-90.

Day, C. P. (2006). Genes or environment to determine alcoholic liver disease and non-alcoholic fatty liver disease. *Liver International : Official Journal of the International Association for the Study of the Liver, 26*(9), 1021-1028. doi:10.1111/j.1478-3231.2006.01323.x

Del Bas, J. M., Fernandez-Larrea, J., Blay, M., Ardevol, A., Salvado, M. J., Arola, L., & Blade, C. (2005). Grape seed procyanidins improve atherosclerotic risk index and induce liver CYP7A1 and SHP expression in healthy rats. *The FASEB Journal : Official Publication of the Federation of American Societies for Experimental Biology, 19*(3), 479-481. doi:10.1096/fj.04-3095fje

Deng, Q. G., She, H., Cheng, J. H., French, S. W., Koop, D. R., Xiong, S., & Tsukamoto, H. (2005). Steatohepatitis induced by intragastric overfeeding in mice. *Hepatology (Baltimore, Md.), 42*(4), 905-914. doi:10.1002/hep.20877

Diehl, A. M. (2004). Obesity and alcoholic liver disease. *Alcohol (Fayetteville, N.Y.), 34*(1), 81-87. doi:10.1016/j.alcohol.2004.07.010

Dixon, J. B., Bhathal, P. S., Hughes, N. R., & O'Brien, P. E. (2004). Nonalcoholic fatty liver disease: Improvement in liver histological analysis with weight loss. *Hepatology (Baltimore, Md.), 39*(6), 1647-1654. doi:10.1002/hep.20251

Dormandy, J. A., Charbonnel, B., Eckland, D. J., Erdmann, E., Massi-Benedetti, M., Moules, I. K., . . . PROactive investigators. (2005). Secondary prevention of macrovascular events in patients with type 2 diabetes in the PROactive study (PROspective pioglitAzone clinical trial in macroVascular events): A randomised controlled trial. *Lancet, 366*(9493), 1279-1289. doi:10.1016/S0140-6736(05)67528-9

DREAM (Diabetes REduction Assessment with ramipril and rosiglitazone Medication) Trial Investigators, Gerstein, H. C., Yusuf, S., Bosch, J., Pogue, J., Sheridan, P., . . . Holman, R. R. (2006). Effect of rosiglitazone on the frequency of diabetes in patients with impaired glucose tolerance or impaired fasting glucose: A randomised controlled trial. *Lancet, 368*(9541), 1096-1105. doi:10.1016/S0140-6736(06)69420-8

Ekstedt, M., Franzen, L. E., Mathiesen, U. L., Thorelius, L., Holmqvist, M., Bodemar, G., & Kechagias, S. (2006). Long-term follow-up of patients with NAFLD and elevated liver enzymes. *Hepatology (Baltimore, Md.), 44*(4), 865-873. doi:10.1002/hep.21327

Fabbrini, E., Sullivan, S., & Klein, S. (2010). Obesity and nonalcoholic fatty liver disease: Biochemical, metabolic, and clinical implications. *Hepatology (Baltimore, Md.), 51*(2), 679-689. doi:10.1002/hep.23280

Fan, J. G., & Farrell, G. C. (2009). Epidemiology of non-alcoholic fatty liver disease in china. *Journal of Hepatology, 50*(1), 204-210. doi:10.1016/j.jhep.2008.10.010

Fan, J. G., & Qiao, L. (2009). Commonly used animal models of non-alcoholic steatohepatitis. *Hepatobiliary & Pancreatic Diseases International : HBPD INT, 8*(3), 233-240.

Fang, F., Kang, Z., & Wong, C. (2010). Vitamin E tocotrienols improve insulin sensitivity through activating peroxisome proliferator-activated receptors. *Molecular Nutrition & Food Research, 54*(3), 345-352. doi:10.1002/mnfr.200900119

Farrell, G. C., & Larter, C. Z. (2006). Nonalcoholic fatty liver disease: From steatosis to cirrhosis. *Hepatology (Baltimore, Md.), 43*(2 Suppl 1), S99-S112. doi:10.1002/hep.20973

Fassio, E., Alvarez, E., Dominguez, N., Landeira, G., & Longo, C. (2004). Natural history of nonalcoholic steatohepatitis: A longitudinal study of repeat liver biopsies. *Hepatology (Baltimore, Md.), 40*(4), 820-826. doi:10.1002/hep.20410

Feldstein, A. E., Papouchado, B. G., Angulo, P., Sanderson, S., Adams, L., & Gores, G. J. (2005). Hepatic stellate cells and fibrosis progression in patients with nonalcoholic fatty liver disease. *Clinical Gastroenterology and Hepatology, 3*(4), 384-389.

Feng, Q., Torii, Y., Uchida, K., Nakamura, Y., Hara, Y., & Osawa, T. (2002). Black tea polyphenols, theaflavins, prevent cellular DNA damage by inhibiting oxidative stress and suppressing cytochrome P450 1A1 in cell cultures. *Journal of Agricultural and Food Chemistry, 50*(1), 213-220.

Fitzgerald, E., Mathieu, S., & Ball, A. (2009). Metformin associated lactic acidosis. *BMJ (Clinical Research Ed.), 339*, b3660. doi:10.1136/bmj.b3660

Fraulob, J. C., Ogg-Diamantino, R., Fernandes-Santos, C., Aguila, M. B., & Mandarim-de-Lacerda, C. A. (2010). A mouse model of metabolic syndrome: Insulin resistance, fatty liver and non-alcoholic fatty pancreas disease (NAFPD) in C57BL/6 mice fed a high fat diet. *Journal of Clinical Biochemistry and Nutrition, 46*(3), 212-223. doi:10.3164/jcbn.09-83

Friedman, S. L. (2008). Hepatic stellate cells: Protean, multifunctional, and enigmatic cells of the liver. *Physiological Reviews, 88*(1), 125-172. doi:10.1152/physrev.00013.2007

Gallou-Kabani, C., Vige, A., Gross, M. S., Rabes, J. P., Boileau, C., Larue-Achagiotis, C., . . . Junien, C. (2007). C57BL/6J and A/J mice fed a high-fat diet delineate components of metabolic syndrome. *Obesity (Silver Spring, Md.), 15*(8), 1996-2005. doi:10.1038/oby.2007.238

Gauthier, M. S., Couturier, K., Latour, J. G., & Lavoie, J. M. (2003). Concurrent exercise prevents high-fat-diet-induced macrovesicular hepatic steatosis. *Journal of Applied Physiology (Bethesda, Md.: 1985), 94*(6), 2127-2134. doi:10.1152/japplphysiol.01164.2002

Gruzman, A., Babai, G., & Sasson, S. (2009). Adenosine monophosphate-activated protein kinase (AMPK) as a new target for antidiabetic drugs: A review on metabolic, pharmacological and chemical considerations. *The Review of Diabetic Studies : RDS, 6*(1), 13-36. doi:10.1900/RDS.2009.6.13

Harmon, A. W., & Harp, J. B. (2001). Differential effects of flavonoids on 3T3-L1 adipogenesis and lipolysis. *American Journal of Physiology.Cell Physiology, 280*(4), C807-13.

Hasegawa, T., Yoneda, M., Nakamura, K., Makino, I., & Terano, A. (2001). Plasma transforming growth factor-beta1 level and efficacy of alpha-tocopherol in patients with non-alcoholic steatohepatitis: A pilot study. *Alimentary Pharmacology & Therapeutics, 15*(10), 1667-1672.

Hilden, M., Christoffersen, P., Juhl, E., & Dalgaard, J. B. (1977). Liver histology in a 'normal' population--examinations of 503 consecutive fatal traffic casualties. *Scandinavian Journal of Gastroenterology, 12*(5), 593-597.

Hong, S. W., Jung, K. H., Zheng, H. M., Lee, H. S., Suh, J. K., Park, I. S., . . . Hong, S. S. (2010). The protective effect of resveratrol on dimethylnitrosamine-induced liver fibrosis in rats. *Archives of Pharmacal Research, 33*(4), 601-609. doi:10.1007/s12272-010-0415-y

Hübscher, S. G. (2006). Histological assessment of non-alcoholic fatty liver disease. *Histopathology, 49*(5), 450-465. doi:10.1111/j.1365-2559.2006.02416.x

Inokuchi, H., Hirokane, H., Tsuzuki, T., Nakagawa, K., Igarashi, M., & Miyazawa, T. (2003). Anti-angiogenic activity of tocotrienol. *Bioscience, Biotechnology, and Biochemistry, 67*(7), 1623-1627.

Iqbal, J., Minhajuddin, M., & Beg, Z. H. (2003). Suppression of 7,12-dimethylbenz[alpha]anthracene-induced carcinogenesis and hypercholesterolaemia in rats by tocotrienol-rich fraction isolated from rice bran oil. *European Journal of Cancer Prevention : The Official Journal of the European Cancer Prevention Organisation (ECP), 12*(6), 447-453. doi:10.1097/01.cej.0000102802.33147.4b

Kamal-Eldin, A., & Appelqvist, L. A. (1996). The chemistry and antioxidant properties of tocopherols and tocotrienols. *Lipids, 31*(7), 671-701.

Kashireddy, P. V., & Rao, M. S. (2004). Lack of peroxisome proliferator-activated receptor α in mice enhances methionine and choline deficient diet-induced steatohepatitis. *Hepatology Research, 30*(2), 104-110.

Kechagias, S., Ernersson, A., Dahlqvist, O., Lundberg, P., Lindstrom, T., Nystrom, F. H., & Fast Food Study Group. (2008). Fast-food-based hyper-alimentation can induce rapid and profound elevation of serum alanine aminotransferase in healthy subjects. *Gut, 57*(5), 649-654. doi:10.1136/gut.2007.131797

Khoshbaten, M., Aliasgarzadeh, A., Masnadi, K., Farhang, S., Tarzamani, M. K., Babaei, H., . . . Najafipoor, F. (2010). Grape seed extract to improve liver function in patients with nonalcoholic fatty liver change. *Saudi Journal of Gastroenterology : Official Journal of the Saudi Gastroenterology Association, 16*(3), 194-197. doi:10.4103/1319-3767.65197

Kim, B. J., Hood, B. L., Aragon, R. A., Hardwick, J. P., Conrads, T. P., Veenstra, T. D., & Song, B. J. (2006). Increased oxidation and degradation of cytosolic proteins in alcohol-exposed mouse liver and hepatoma cells. *Proteomics, 6*(4), 1250-1260. doi:10.1002/pmic.200500447

Klein, S., Mittendorfer, B., Eagon, J. C., Patterson, B., Grant, L., Feirt, N., . . . McCrea, J. (2006). Gastric bypass surgery improves metabolic and hepatic abnormalities associated with nonalcoholic fatty liver disease. *Gastroenterology, 130*(6), 1564-1572. doi:10.1053/j.gastro.2006.01.042

Kleiner, D. E., Brunt, E. M., Van Natta, M., Behling, C., Contos, M. J., Cummings, O. W., . . . Nonalcoholic Steatohepatitis Clinical Research Network. (2005). Design and validation of a histological scoring system for nonalcoholic fatty liver disease. *Hepatology (Baltimore, Md.), 41*(6), 1313-1321. doi:10.1002/hep.20701

Ko, S. H., Choi, S. W., Ye, S. K., Cho, B. L., Kim, H. S., & Chung, M. H. (2005). Comparison of the antioxidant activities of nine different fruits in human plasma. *Journal of Medicinal Food, 8*(1), 41-46. doi:10.1089/jmf.2005.8.41

Koga, T., Moro, K., Nakamori, K., Yamakoshi, J., Hosoyama, H., Kataoka, S., & Ariga, T. (1999). Increase of antioxidative potential of rat plasma by oral administration of proanthocyanidin-rich extract from grape seeds. *Journal of Agricultural and Food Chemistry, 47*(5), 1892-1897.

Kruse, J. A. (2001). Metformin-associated lactic acidosis. *The Journal of Emergency Medicine, 20*(3), 267-272.

Kugelmas, M., Hill, D. B., Vivian, B., Marsano, L., & McClain, C. J. (2003). Cytokines and NASH: A pilot study of the effects of lifestyle modification and vitamin E. *Hepatology (Baltimore, Md.), 38*(2), 413-419. doi:10.1053/jhep.2003.50316

Kuhad, A., Bishnoi, M., Tiwari, V., & Chopra, K. (2009). Suppression of NF-kappabeta signaling pathway by tocotrienol can prevent diabetes associated cognitive deficits. *Pharmacology, Biochemistry, and Behavior, 92*(2), 251-259. doi:10.1016/j.pbb.2008.12.012

Kuhad, A., & Chopra, K. (2009). Tocotrienol attenuates oxidative-nitrosative stress and inflammatory cascade in experimental model of diabetic neuropathy. *Neuropharmacology, 57*(4), 456-462. doi:10.1016/j.neuropharm.2009.06.013

Lalau, J. D., Lacroix, C., Compagnon, P., de Cagny, B., Rigaud, J. P., Bleichner, G., . . . Haegy, J. M. (1995). Role of metformin accumulation in metformin-associated lactic acidosis. *Diabetes Care, 18*(6), 779-784.

Latendresse, J. R., Warbrittion, A. R., Jonassen, H., & Creasy, D. M. (2002). Fixation of testes and eyes using a modified davidson's fluid: Comparison with bouin's fluid and conventional davidson's fluid. *Toxicologic Pathology, 30*(4), 524-533.

Lau, D. T., Luxon, B. A., Xiao, S. Y., Beard, M. R., & Lemon, S. M. (2005). Intrahepatic gene expression profiles and alpha-smooth muscle actin patterns in hepatitis C virus induced fibrosis. *Hepatology (Baltimore, Md.), 42*(2), 273-281. doi:10.1002/hep.20767

Lavine, J. E. (2000). Vitamin E treatment of nonalcoholic steatohepatitis in children: A pilot study. *The Journal of Pediatrics, 136*(6), 734-738.

Lavine, J. E., Schwimmer, J. B., Van Natta, M. L., Molleston, J. P., Murray, K. F., Rosenthal, P., . . . Nonalcoholic Steatohepatitis Clinical Research Network. (2011). Effect of vitamin E or metformin for treatment of nonalcoholic fatty liver disease in children and adolescents: The TONIC randomized controlled trial. *JAMA : The Journal of the American Medical Association, 305*(16), 1659-1668. doi:10.1001/jama.2011.520

Le, T. H., Caldwell, S. H., Redick, J. A., Sheppard, B. L., Davis, C. A., Arseneau, K. O., . . . Peterson, T. C. (2004). The zonal distribution of megamitochondria with crystalline inclusions in nonalcoholic steatohepatitis. *Hepatology, 39*(5), 1423-1429. doi:10.1002/hep.20202

Lee, E. S., Shin, M. O., Yoon, S., & Moon, J. O. (2010). Resveratrol inhibits dimethylnitrosamine-induced hepatic fibrosis in rats. *Archives of Pharmacal Research, 33*(6), 925-932. doi:10.1007/s12272-010-0616-4

Li, Z., Soloski, M. J., & Diehl, A. M. (2005). Dietary factors alter hepatic innate immune system in mice with nonalcoholic fatty liver disease. *Hepatology (Baltimore, Md.), 42*(4), 880-885. doi:10.1002/hep.20826

Lindor, K. D., Kowdley, K. V., Heathcote, E. J., Harrison, M. E., Jorgensen, R., Angulo, P., . . . Colin, P. (2004). Ursodeoxycholic acid for treatment of nonalcoholic steatohepatitis: Results of a randomized trial. *Hepatology, 39*(3), 770-778. doi:10.1002/hep.20092

London, R. M., & George, J. (2007). Pathogenesis of NASH: Animal models. *Clinics in Liver Disease, 11*(1), 55-74, viii. doi:10.1016/j.cld.2007.02.010

Longerich, T., Flechtenmacher, C., & Schirmacher, P. (2008). Quality and quantity in hepatopathology. diagnostic and clinically relevant grading for non-tumourous liver diseases. [Mass und Zahl in der Hepatopathologie: Diagnostisch und klinisch relevante Graduierungen bei nichttumorosen Lebererkrankungen] *Der Pathologe, 29*(1), 15-26. doi:10.1007/s00292-007-0963-1

Ludwig, J., Viggiano, T. R., McGill, D. B., & Oh, B. J. (1980). Nonalcoholic steatohepatitis: Mayo clinic experiences with a hitherto unnamed disease. *Mayo Clinic Proceedings.Mayo Clinic, 55*(7), 434-438.

Machado, M., & Cortez-Pinto, H. (2006). Non-alcoholic steatohepatitis and metabolic syndrome. *Current Opinion in Clinical Nutrition and Metabolic Care, 9*(5), 637-642. doi:10.1097/01.mco.0000241677.40170.17

Mannucci, E., Monami, M., Cresci, B., Pala, L., Bardini, G., Petracca, M. G., . . . Rotella, C. M. (2008). National cholesterol education program and international diabetes federation definitions of metabolic syndrome in the prediction of diabetes. results from the FIrenze-bagno A ripoli study. *Diabetes, Obesity & Metabolism, 10*(5), 430-435. doi:10.1111/j.1463-1326.2007.00724.x

Marchesini, G., Moscatiello, S., Di Domizio, S., & Forlani, G. (2008). Obesity-associated liver disease. *The Journal of Clinical Endocrinology and Metabolism, 93*(11 Suppl 1), S74-80. doi:10.1210/jc.2008-1399

Matteoni, C. A., Younossi, Z. M., Gramlich, T., Boparai, N., Liu, Y. C., & McCullough, A. J. (1999). Nonalcoholic fatty liver disease: A spectrum of clinical and pathological severity. *Gastroenterology, 116*(6), 1413-1419.

McCullough, A. J. (2004). The clinical features, diagnosis and natural history of nonalcoholic fatty liver disease. *Clinics in Liver Disease, 8*(3), 521-33, viii. doi:10.1016/j.cld.2004.04.004

McCullough, A. J. (2006). Pathophysiology of nonalcoholic steatohepatitis. *Journal of Clinical Gastroenterology, 40 Suppl 1*, S17-29. doi:DOI: 10.1097/01.mcg.0000168645.86658.22

McIntyre, B. S., Briski, K. P., Tirmenstein, M. A., Fariss, M. W., Gapor, A., & Sylvester, P. W. (2000). Antiproliferative and apoptotic effects of tocopherols and tocotrienols on normal mouse mammary epithelial cells. *Lipids, 35*(2), 171-180.

Miele, L., Forgione, A., Gasbarrini, G., & Grieco, A. (2007). Noninvasive assessment of fibrosis in non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH). *Translational Research : The Journal of Laboratory and Clinical Medicine, 149*(3), 114-125. doi:10.1016/j.trsl.2006.11.011

Montagut, G., Blade, C., Blay, M., Fernandez-Larrea, J., Pujadas, G., Salvado, M. J., . . . Ardevol, A. (2010). Effects of a grapeseed procyanidin extract (GSPE) on insulin resistance. *The Journal of Nutritional Biochemistry, 21*(10), 961-967. doi:10.1016/j.jnutbio.2009.08.001

Montagut, G., Onnockx, S., Vaque, M., Blade, C., Blay, M., Fernandez-Larrea, J., . . . Pinent, M. (2010). Oligomers of grape-seed procyanidin extract activate the insulin receptor and key targets of the insulin signaling pathway differently from insulin. *The Journal of Nutritional Biochemistry, 21*(6), 476-481. doi:10.1016/j.jnutbio.2009.02.003

Musso, G., Gambino, R., De Michieli, F., Cassader, M., Rizzetto, M., Durazzo, M., . . . Pagano, G. (2003). Dietary habits and their relations to insulin resistance and postprandial lipemia in nonalcoholic steatohepatitis. *Hepatology (Baltimore, Md.), 37*(4), 909-916. doi:10.1053/jhep.2003.50132

Neuschwander-Tetri, B. A., Brunt, E. M., Wehmeier, K. R., Sponseller, C. A., Hampton, K., & Bacon, B. R. (2003). Interim results of a pilot study demonstrating the early effects of the PPAR-gamma ligand rosiglitazone on insulin sensitivity, aminotransferases, hepatic steatosis and body weight in patients with non-alcoholic steatohepatitis. *Journal of Hepatology, 38*(4), 434-440.

Niki, E., Kawakami, A., Saito, M., Yamamoto, Y., Tsuchiya, J., & Kamiya, Y. (1985). Effect of phytyl side chain of vitamin E on its antioxidant activity. *The Journal of Biological Chemistry, 260*(4), 2191-2196.

Ogden, C. L., Carroll, M. D., Curtin, L. R., McDowell, M. A., Tabak, C. J., & Flegal, K. M. (2006). Prevalence of overweight and obesity in the united states, 1999-2004. *JAMA : The Journal of the American Medical Association, 295*(13), 1549-1555. doi:10.1001/jama.295.13.1549

Oh, M. K., Winn, J., & Poordad, F. (2008). Review article: Diagnosis and treatment of non-alcoholic fatty liver disease. *Alimentary Pharmacology & Therapeutics, 28*(5), 503-522. doi:10.1111/j.1365-2036.2008.03752.x

O'Leary, V. B., Marchetti, C. M., Krishnan, R. K., Stetzer, B. P., Gonzalez, F., & Kirwan, J. P. (2006). Exercise-induced reversal of insulin resistance in obese elderly is associated with reduced visceral fat. *Journal of Applied Physiology (Bethesda, Md.: 1985), 100*(5), 1584-1589. doi:10.1152/japplphysiol.01336.2005

Ong, J. P., & Younossi, Z. M. (2007). Epidemiology and natural history of NAFLD and NASH. *Clinics in Liver Disease, 11*(1), 1-16, vii. doi:10.1016/j.cld.2007.02.009

Osada, K., Suzuki, T., Kawakami, Y., Senda, M., Kasai, A., Sami, M., . . . Ikeda, M. (2006). Dose-dependent hypocholesterolemic actions of dietary apple polyphenol in rats fed cholesterol. *Lipids, 41*(2), 133-139.

Park, S. H. (2008). Nonalcoholic steatohepatitis: Pathogenesis and treatment. *The Korean Journal of Hepatology, 14*(1), 12-27. doi:10.3350/kjhep.2008.14.1.12

Paschos, P., & Paletas, K. (2009). Non alcoholic fatty liver disease and metabolic syndrome. *Hippokratia, 13*(1), 9-19.

Pearce, B. C., Parker, R. A., Deason, M. E., Qureshi, A. A., & Wright, J. J. (1992). Hypocholesterolemic activity of synthetic and natural tocotrienols. *Journal of Medicinal Chemistry, 35*(20), 3595-3606.

Pinent, M., Blade, C., Salvado, M. J., Blay, M., Pujadas, G., Fernandez-Larrea, J., . . . Ardevol, A. (2006). Procyanidin effects on adipocyte-related pathologies. *Critical Reviews in Food Science and Nutrition, 46*(7), 543-550. doi:10.1080/10408390500354537

Pinent, M., Blade, M. C., Salvado, M. J., Arola, L., & Ardevol, A. (2005). Intracellular mediators of procyanidin-induced lipolysis in 3T3-L1 adipocytes. *Journal of Agricultural and Food Chemistry, 53*(2), 262-266. doi:10.1021/jf048947y

Pinent, M., Blay, M., Blade, M. C., Salvado, M. J., Arola, L., & Ardevol, A. (2004). Grape seed-derived procyanidins have an antihyperglycemic effect in streptozotocin-induced diabetic rats and insulinomimetic activity in insulin-sensitive cell lines. *Endocrinology, 145*(11), 4985-4990. doi:10.1210/en.2004-0764

Portincasa, P., Grattagliano, I., Palmieri, V. O., & Palasciano, G. (2005). Nonalcoholic steatohepatitis: Recent advances from experimental models to clinical management. *Clinical Biochemistry, 38*(3), 203-217. doi:10.1016/j.clinbiochem.2004.10.014

Powell, E. E., Cooksley, W. G., Hanson, R., Searle, J., Halliday, J. W., & Powell, L. W. (1990). The natural history of nonalcoholic steatohepatitis: A follow-up study of forty-two patients for up to 21 years. *Hepatology (Baltimore, Md.), 11*(1), 74-80.

Powell, E. E., Jonsson, J. R., & Clouston, A. D. (2005). Dangerous liaisons: The metabolic syndrome and nonalcoholic fatty liver disease. *Annals of Internal Medicine, 143*(10), 753-754.

Promrat, K., Kleiner, D. E., Niemeier, H. M., Jackvony, E., Kearns, M., Wands, J. R., . . . Wing, R. R. (2010). Randomized controlled trial testing the effects of weight loss on nonalcoholic steatohepatitis. *Hepatology (Baltimore, Md.), 51*(1), 121-129. doi:10.1002/hep.23276

Puiggros, F., Llopiz, N., Ardevol, A., Blade, C., Arola, L., & Salvado, M. J. (2005). Grape seed procyanidins prevent oxidative injury by modulating the expression of antioxidant enzyme systems. *Journal of Agricultural and Food Chemistry, 53*(15), 6080-6086. doi:10.1021/jf050343m

Quesada, H., del Bas, J. M., Pajuelo, D., Diaz, S., Fernandez-Larrea, J., Pinent, M., . . . Blade, C. (2009). Grape seed proanthocyanidins correct dyslipidemia associated with a high-fat diet in rats and repress genes controlling lipogenesis and VLDL assembling in liver. *International Journal of Obesity (2005), 33*(9), 1007-1012. doi:10.1038/ijo.2009.136

Qureshi, A. A., Salser, W. A., Parmar, R., & Emeson, E. E. (2001). Novel tocotrienols of rice bran inhibit atherosclerotic lesions in C57BL/6 ApoE-deficient mice. *The Journal of Nutrition, 131*(10), 2606-2618.

Qureshi, A. A., Sami, S. A., Salser, W. A., & Khan, F. A. (2002). Dose-dependent suppression of serum cholesterol by tocotrienol-rich fraction (TRF25) of rice bran in hypercholesterolemic humans. *Atherosclerosis, 161*(1), 199-207.

Qureshi, K., & Abrams, G. A. (2007). Metabolic liver disease of obesity and role of adipose tissue in the pathogenesis of nonalcoholic fatty liver disease. *World Journal of Gastroenterology : WJG, 13*(26), 3540-3553.

Rayalam, S., Yang, J. Y., Ambati, S., Della-Fera, M. A., & Baile, C. A. (2008). Resveratrol induces apoptosis and inhibits adipogenesis in 3T3-L1 adipocytes. *Phytotherapy Research : PTR, 22*(10), 1367-1371. doi:10.1002/ptr.2503

Reddy, J. K., & Rao, M. S. (2006). Lipid metabolism and liver inflammation. II. fatty liver disease and fatty acid oxidation. *American Journal of Physiology.Gastrointestinal and Liver Physiology, 290*(5), G852-8. doi:10.1152/ajpgi.00521.2005

Reeves, H. L., & Friedman, S. L. (2002). Activation of hepatic stellate cells--a key issue in liver fibrosis. *Frontiers in Bioscience : A Journal and Virtual Library, 7*, d808-26.

Rinella, M. E., Elias, M. S., Smolak, R. R., Fu, T., Borensztajn, J., & Green, R. M. (2008). Mechanisms of hepatic steatosis in mice fed a lipogenic methionine choline-deficient diet. *Journal of Lipid Research, 49*(5), 1068-1076. doi:10.1194/jlr.M800042-JLR200

Rinella, M. E., & Green, R. M. (2004). The methionine-choline deficient dietary model of steatohepatitis does not exhibit insulin resistance. *Journal of Hepatology, 40*(1), 47-51.

Romestaing, C., Piquet, M. A., Bedu, E., Rouleau, V., Dautresme, M., Hourmand-Ollivier, I., . . . Sibille, B. (2007). Long term highly saturated fat diet does not induce NASH in wistar rats. *Nutrition & Metabolism, 4*, 4. doi:10.1186/1743-7075-4-4

Roy, S., Khanna, S., Alessio, H. M., Vider, J., Bagchi, D., Bagchi, M., & Sen, C. K. (2002). Anti-angiogenic property of edible berries. *Free Radical Research, 36*(9), 1023-1031.

Rubenstrunk, A., Hanf, R., Hum, D. W., Fruchart, J. C., & Staels, B. (2007). Safety issues and prospects for future generations of PPAR modulators. *Biochimica Et Biophysica Acta, 1771*(8), 1065-1081. doi:10.1016/j.bbalip.2007.02.003

Ruhl, C. E., & Everhart, J. E. (2003). Determinants of the association of overweight with elevated serum alanine aminotransferase activity in the united states. *Gastroenterology, 124*(1), 71-79. doi:10.1053/gast.2003.50004

Sahai, A., Malladi, P., Pan, X., Paul, R., Melin-Aldana, H., Green, R. M., & Whitington, P. F. (2004). Obese and diabetic db/db mice develop marked liver fibrosis in a model of nonalcoholic steatohepatitis: Role of short-form leptin receptors and osteopontin. *American Journal of Physiology.Gastrointestinal and Liver Physiology, 287*(5), G1035-43. doi:10.1152/ajpgi.00199.2004

Saito, Y., Yoshida, Y., Nishio, K., Hayakawa, M., & Niki, E. (2004). Characterization of cellular uptake and distribution of vitamin E. *Annals of the New York Academy of Sciences, 1031*, 368-375. doi:10.1196/annals.1331.047

Sanyal, A. J. (2010). ACP journal club: Vitamin E, but not pioglitazone, improved nonalcoholic steatohepatitis in nondiabetic patients. *Annals of Internal Medicine, 153*(6), JC3-12. doi:10.1059/0003-4819-153-6-201009210-02012

Sanyal, A. J., Mofrad, P. S., Contos, M. J., Sargeant, C., Luketic, V. A., Sterling, R. K., . . . Mills, A. S. (2004). A pilot study of vitamin E versus vitamin E and pioglitazone for the treatment of nonalcoholic steatohepatitis. *Clinical Gastroenterology and Hepatology : The Official Clinical Practice Journal of the American Gastroenterological Association, 2*(12), 1107-1115.

Sato, F., Tamura, Y., Watada, H., Kumashiro, N., Igarashi, Y., Uchino, H., . . . Kawamori, R. (2007). Effects of diet-induced moderate weight reduction on intrahepatic and intramyocellular triglycerides and glucose metabolism in obese subjects. *The Journal of Clinical Endocrinology and Metabolism, 92*(8), 3326-3329. doi:10.1210/jc.2006-2384

Sen, C. K., Khanna, S., & Roy, S. (2006). Tocotrienols: Vitamin E beyond tocopherols. *Life Sciences, 78*(18), 2088-2098.

Serbinova, E., Kagan, V., Han, D., & Packer, L. (1991). Free radical recycling and intramembrane mobility in the antioxidant properties of alpha-tocopherol and alpha-tocotrienol. *Free Radical Biology & Medicine, 10*(5), 263-275.

Sheth, S. G., Gordon, F. D., & Chopra, S. (1997). Nonalcoholic steatohepatitis. *Annals of Internal Medicine, 126*(2), 137-145.

Shi, J., Yu, J., Pohorly, J. E., & Kakuda, Y. (2003). Polyphenolics in grape seeds-biochemistry and functionality. *Journal of Medicinal Food, 6*(4), 291-299. doi:10.1089/109662003772519831

Shoelson, S. E., Herrero, L., & Naaz, A. (2007). Obesity, inflammation, and insulin resistance. *Gastroenterology, 132*(6), 2169-2180.

Solga, S., Alkhuraishe, A. R., Clark, J. M., Torbenson, M., Greenwald, A., Diehl, A. M., & Magnuson, T. (2004). Dietary composition and nonalcoholic fatty liver disease. *Digestive Diseases and Sciences, 49*(10), 1578-1583.

Song, B. L., & DeBose-Boyd, R. A. (2006). Insig-dependent ubiquitination and degradation of 3-hydroxy-3-methylglutaryl coenzyme a reductase stimulated by delta- and gamma-tocotrienols. *The Journal of Biological Chemistry, 281*(35), 25054-25061. doi:10.1074/jbc.M605575200

Steiner, M., Glantz, M., & Lekos, A. (1995). Vitamin E plus aspirin compared with aspirin alone in patients with transient ischemic attacks. *The American Journal of Clinical Nutrition, 62*(6 Suppl), 1381S-1384S.

Straub, B. K., & Schirmacher, P. (2010). Pathology and biopsy assessment of non-alcoholic fatty liver disease. *Digestive Diseases (Basel, Switzerland), 28*(1), 197-202. doi:10.1159/000282086

Suarna, C., Hood, R. L., Dean, R. T., & Stocker, R. (1993). Comparative antioxidant activity of tocotrienols and other natural lipid-soluble antioxidants in a homogeneous system, and in rat and human lipoproteins. *Biochimica Et Biophysica Acta, 1166*(2-3), 163-170.

Sun, G. Y., Xia, J., Xu, J., Allenbrand, B., Simonyi, A., Rudeen, P. K., & Sun, A. Y. (1999). Dietary supplementation of grape polyphenols to rats ameliorates chronic ethanol-induced changes in hepatic morphology without altering changes in hepatic lipids. *The Journal of Nutrition, 129*(10), 1814-1819.

Sundaram, S. S., Whitington, P. F., & Green, R. M. (2005). Steatohepatitis develops rapidly in transgenic mice overexpressing Abcb11 and fed a methionine-choline-deficient diet. *American Journal of Physiology.Gastrointestinal and Liver Physiology, 288*(6), G1321-7. doi:10.1152/ajpgi.00455.2004

Sundaresan, S., Vijayagopal, P., Mills, N., Imrhan, V., & Prasad, C. (2011). A mouse model for nonalcoholic steatohepatitis. *The Journal of Nutritional Biochemistry, 22*(10), 979-984. doi:10.1016/j.jnutbio.2010.08.011; 10.1016/j.jnutbio.2010.08.011

Suzuki, A., Lindor, K., St Saver, J., Lymp, J., Mendes, F., Muto, A., . . . Angulo, P. (2005). Effect of changes on body weight and lifestyle in nonalcoholic fatty liver disease. *Journal of Hepatology, 43*(6), 1060-1066. doi:10.1016/j.jhep.2005.06.008

Suzuki, Y. J., Tsuchiya, M., Wassall, S. R., Choo, Y. M., Govil, G., Kagan, V. E., & Packer, L. (1993). Structural and dynamic membrane properties of alpha-tocopherol and alpha-tocotrienol: Implication to the molecular mechanism of their antioxidant potency. *Biochemistry, 32*(40), 10692-10699.

Svegliati-Baroni, G., Candelaresi, C., Saccomanno, S., Ferretti, G., Bachetti, T., Marzioni, M., . . . Casini, A. (2006). A model of insulin resistance and nonalcoholic steatohepatitis in rats: Role of peroxisome proliferator-activated receptor-alpha and n-3 polyunsaturated fatty acid treatment on liver injury. *The American Journal of Pathology, 169*(3), 846-860.

Targher, G., Bertolini, L., Padovani, R., Rodella, S., Tessari, R., Zenari, L., . . . Arcaro, G. (2007). Prevalence of nonalcoholic fatty liver disease and its association with cardiovascular disease among type 2 diabetic patients. *Diabetes Care, 30*(5), 1212-1218. doi:10.2337/dc06-2247

Terra, X., Montagut, G., Bustos, M., Llopiz, N., Ardevol, A., Blade, C., . . . Blay, M. (2009). Grape-seed procyanidins prevent low-grade inflammation by modulating cytokine expression in rats fed a high-fat diet. *The Journal of Nutritional Biochemistry, 20*(3), 210-218. doi:10.1016/j.jnutbio.2008.02.005

Terra, X., Valls, J., Vitrac, X., Merrillon, J. M., Arola, L., Ardevol, A., . . . Blay, M. (2007). Grape-seed procyanidins act as antiinflammatory agents in endotoxin-stimulated RAW 264.7 macrophages by inhibiting NFkB signaling pathway. *Journal of Agricultural and Food Chemistry, 55*(11), 4357-4365. doi:10.1021/jf0633185

Tiniakos, D. G. (2009). Liver biopsy in alcoholic and non-alcoholic steatohepatitis patients. *Gastroenterologie Clinique Et Biologique, 33*(10-11), 930-939. doi:10.1016/j.gcb.2009.05.009

Tsang, C., Auger, C., Mullen, W., Bornet, A., Rouanet, J. M., Crozier, A., & Teissedre, P. L. (2005). The absorption, metabolism and excretion of flavan-3-ols and procyanidins following the ingestion of a grape seed extract by rats. *The British Journal of Nutrition, 94*(2), 170-181.

Tsuzuki, W., Yunoki, R., & Yoshimura, H. (2007). Intestinal epithelial cells absorb gamma-tocotrienol faster than alpha-tocopherol. *Lipids, 42*(2), 163-170. doi:10.1007/s11745-007-3021-0

Ueno, T., Sugawara, H., Sujaku, K., Hashimoto, O., Tsuji, R., Tamaki, S., . . . Tanikawa, K. (1997). Therapeutic effects of restricted diet and exercise in obese patients with fatty liver. *Journal of Hepatology, 27*(1), 103-107.

Vuppalanchi, R., Ünalp, A., Van Natta, M. L., Cummings, O. W., Sandrasegaran, K. E., Hameed, T., . . . Chalasani, N. (2009). Effects of liver biopsy sample length and number of readings on sampling variability in nonalcoholic fatty liver disease. *Clinical Gastroenterology and Hepatology, 7*(4), 481-486.

Wanless, I. R., & Lentz, J. S. (1990). Fatty liver hepatitis (steatohepatitis) and obesity: An autopsy study with analysis of risk factors. *Hepatology (Baltimore, Md.), 12*(5), 1106-1110.

Wanless, I. R., & Shiota, K. (2004). The pathogenesis of nonalcoholic steatohepatitis and other fatty liver diseases: A four-step model including the role of lipid release and hepatic venular obstruction in the progression to cirrhosis. *Seminars in Liver Disease, 24*(1), 99-106. doi:10.1055/s-2004-823104

Washington, K., Wright, K., Shyr, Y., Hunter, E. B., Olson, S., & Raiford, D. S. (2000). Hepatic stellate cell activation in nonalcoholic steatohepatitis and fatty liver. *Human Pathology, 31*(7), 822-828.

Williams, R. (2006). Global challenges in liver disease. *Hepatology (Baltimore, Md.), 44*(3), 521-526. doi:10.1002/hep.21347

Yakaryilmaz, F., Guliter, S., Savas, B., Erdem, O., Ersoy, R., Erden, E., . . . Ozenirler, S. (2007). Effects of vitamin E treatment on peroxisome proliferator-activated receptor-alpha expression and insulin resistance in patients with non-alcoholic steatohepatitis: Results of a pilot study. *Internal Medicine Journal, 37*(4), 229-235. doi:10.1111/j.1445-5994.2006.01295.x

Yeh, M. M., & Brunt, E. M. (2007). Pathology of nonalcoholic fatty liver disease. *American Journal of Clinical Pathology, 128*(5), 837-847. doi:10.1309/RTPM1PY6YGBL2G2R

Yoshida, Y., Niki, E., & Noguchi, N. (2003). Comparative study on the action of tocopherols and tocotrienols as antioxidant: Chemical and physical effects. *Chemistry and Physics of Lipids, 123*(1), 63-75.

Yu, S. G., Thomas, A. M., Gapor, A., Tan, B., Qureshi, N., & Qureshi, A. A. (2006). Dose-response impact of various tocotrienols on serum lipid parameters in 5-week-old female chickens. *Lipids, 41*(5), 453-461.

Zaiden, N., Yap, W. N., Ong, S., Xu, C. H., Teo, V. H., Chang, C. P., . . . Yap, Y. L. (2010). Gamma delta tocotrienols reduce hepatic triglyceride synthesis and VLDL secretion. *Journal of Atherosclerosis and Thrombosis, 17*(10), 1019-1032.

Zatloukal, K., French, S. W., Stumptner, C., Strnad, P., Harada, M., Toivola, D. M., . . . Omary, M. B. (2007). From mallory to mallory-denk bodies: What, how and why? *Experimental Cell Research, 313*(10), 2033-2049. doi:10.1016/j.yexcr.2007.04.024

Zern, T. L., Wood, R. J., Greene, C., West, K. L., Liu, Y., Aggarwal, D., . . . Fernandez, M. L. (2005). Grape polyphenols exert a cardioprotective effect in pre- and postmenopausal women by lowering plasma lipids and reducing oxidative stress. *The Journal of Nutrition, 135*(8), 1911-1917.

Zou, Y., Li, J., Lu, C., Wang, J., Ge, J., Huang, Y., . . . Wang, Y. (2006). High-fat emulsion-induced rat model of nonalcoholic steatohepatitis. *Life Sciences, 79*(11), 1100-1107. doi:10.1016/j.lfs.2006.03.021

APPENDIX A

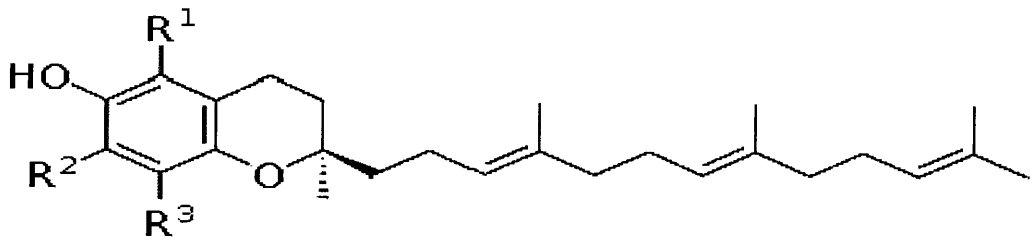
Chemical Stucture

APPENDIX – A

Chemical Structure

Figure 1:

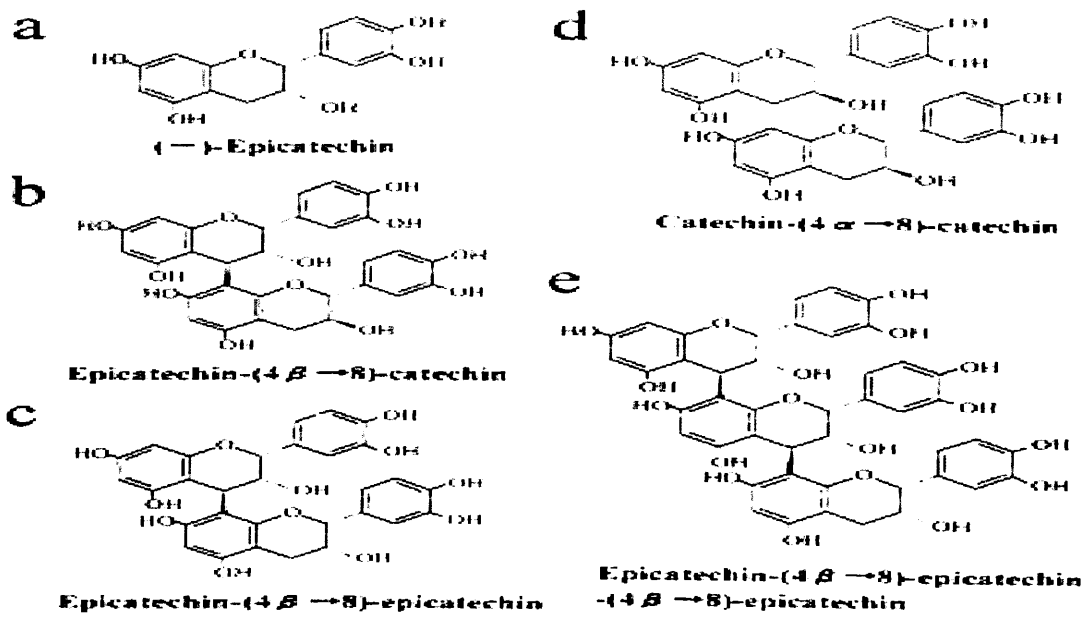
(d) delta tocotrienol



(delta) δ: $R^1 = R^2 = H$, $R^3 = CH_3$

Figure 2:

Grape seed procyanidins



APPENDIX B

Diet Composition

APPENDIX B

Diet Composition

HarlanTeklad	**TD#09752**	**TD#88137**	**(TD#88137 + GSP)**	**(TD#88137 + δ 3T)**
Control diets for C57LB/J6 mice	Low fat AIN 93 G, (7%)	High fat (42%)	GSP 1% and 2%	δ tocotrienol 0.025% and 0.5%
Ingredients	**g/kg**	**g/kg**	**g/kg**	**g/kg**
Casein	**195.0**	**195.0**	**195.0**	**195.0**
DL Methionine	**3.0**	**3.0**	**3.0**	**3.0**
Sucrose	**321.0**	**341.46**	**341.46**	**341.46**
Corn Starch	**211.99**	**150.0**	**150.0**	**150.0**
Maltodextrin	**100.0**	**0 ***	**0 ***	**0 ***
Milkfat	**60.0**	**210.0***	**210.0***	**210.0***
Soybean Oil	**10.0**	**1.5 ***	**1.5 ***	**1.5 ***
Cellulose	**50.0**	**50.0**	**50.0**	**50.0**
Mineral Mix,				
AIN76 (170915)	**35.0**	**35.0**	**35.0**	**35.0**
Calcium				
Carbonate	**4.0**	**4.0**	**4.0**	**4.0**
Vitamin Mix,				
Teklad (40060)	**10.0**	**10.0**	**10.0**	**10.0**
Ethoxyquin,				
Antioxidant	**0.01**	**0.01**	**0.01**	**0.01**
1. GSP *2. δ tocotrienol*	0	0	Level 1: 10 g for 10% in 1kg of TD#88137 Level 2: 20 g for 20% in 1 kg TD#88137	Level 1: 330 μg in 1 kg of TD#88137 Level 2: 760 μg in 1 kg of TD#88137

APPENDIX C

Histology Staining Protocol

APPENDIX C

Histology Techniques and Protocols for liver tissue staining

I.Processing of liver tissue samples:-

Wash fresh liver samples in saline solution and later with PBS buffer.

1. Wash in 70% Ethanol
2. Wash in 90% Ethanol
3. Wash in 100% Ethanol

II. Fixation:

Modified Davidson's Fixative used to fix approx 200mg of fresh liver samples at necropsy.

Reagents required:

Formalin – 30 %

Acetic acid – 5%

Ethanol/Methanol – 15%

Mix properly to get the final volume of 300ml. Measure the pH between 3-4.Store at room temperature.For approx. 200mg tissue sample (2mmx 4mm) sections use 10ml fixative solution.

Change the modified Davidson's solution with 70% ethanol until ready for further processing (To avoid any microbial growth and degradation).

Change with 90% Alcohol; incubate for 2 hours, than to 100% alcohol twicw for 2 hours each. Finally 50-50 Ethanol –Xylene solutions for 2 hours each, further with 100% Xylene for 2 hours and then 50-50 Xylene-wax solution. These steps need to be done slowly for the tissue samples to transition from polar to less polar solutions. Removal of water, as Wax / Xylene does not get along with small percent of water.

III. Embedding: Reagents required are Paraplast Plus (wax) and Base mold (metal)

Embedding is at 60° C . The soft wax needs to be melted, the base mold kept on hot plate to keep them warm. Use label either laser printed or pencil lead for each sample (coding) if required. Care need to be taken, to maintain temperatures of the samples to be embedded throughout. No bubbles should be formed while pouring the molten wax in the base mold with the samples in it. Leave the samples with the labels to cool, solidify and ready to be sectioned into small sections of 6-7 microns using the microtome.

IV. Slide preparation: To make a thin layer with the mixture of gelatins and chromate (Dr. Mill's recipe) to improve the attachment of the thin sections of the liver tissue by using microtome.

V. Stains:
(A) **H&E staining** of the liver samples later stained by hematoxylin and Eosin (H&E) staining. This is a standard histology stain.
(B) **Periodic Acid Schiff stain:**
(C)**The Masson's Trichome stain:** A trichrome histology stain is a mixture of three dyes. This histology stain can be helpful for differentiating cellular from extracellular items. This histology stain uses light green, iron hematoxylin, and acid fuchsin. It is useful on connective tissue. Collagen fibers stain green or blue with Masson's trichrome stain. Muscle and keratin will be red. Cytoplasm will be pink to red. Nuclei will be black.

V. Stains
Preparation for
(A) **H&E staining** of the liver samples later stained by hematoxylin and Eosin (H&E) staining. This is a standard histology stain.
(B)**The Masson's Trichome stain:** A trichrome histology stain is a mixture of three dyes. This histology stain can be helpful for differentiating cellular from extracellular items. This histology stain uses light green, iron hematoxylin, and acid fuchsin. It is useful on connective tissue. Collagen fibers stain green or blue with Masson's trichrome stain. Muscle and keratin will be red. Cytoplasm will be pink to red. Nuclei will be black.

Solutions and Reagents:
Bouin's Solution:

Picric acid (saturated) ---------- 75 ml
Formaldehyde (37-40%) ---------- 25 ml
Glacial acetic acid ---------- 5 ml
Mix well. This solution will improve Masson Trichrome staining quality.

Weigert's Iron Hematoxylin Solution:

Stock Solution A:
Hematoxylin ---------- 1 g
95% Alcohol ---------- 100 ml
Stock Solution B:
29% Ferric chloride in water --------- 4 ml
Distilled water ---------- 95 ml
Hydrochloric acid, concentrated ---- 1ml

Weigert's Iron Hematoxylin Working Solution:

Mix equal parts of stock solution A and B. This working solution is stable for 3 months (no good after 4 months)

Biebrich Scarlet-Acid Fuchsin Solution:

Biebrich scarlet, 1% aqueous --------- 90 ml
Acid fuchsin, 1% aqueous ----------10 ml
Acetic acid, glacial ---------- 1 ml

Phosphomolybdic-Phosphotungstic Acid Solution:

5% Phosphomolybdic acid ---------- 25 ml
5% Phosphotungstic acid ---------- 25 ml

Aniline Blue Solution:

Aniline blue ---------- 2.5 g
Acetic acide, glacial ---------- 2 ml
Distilled water ---------- 100 ml

1% Acetic Acid Solution:

Acetic acid, glacial ---------- 1 ml
Distilled water ---------- 99 ml

(B) **The Periodic Schiff's stain (PAS):** This histology stain is particularly useful for staining glycogen and other carbohydrates, but is useful for many things. It is often used to show glomeruli, basement membranes, and glycogen in the liver. PAS stains glycogen, mucin, mucoprotein, and glycoproteins magenta. The nuclei will stain blue. Collagen will stain pink.

Solutions and Reagents:

0.5% Periodic Acid Solution:

Periodic acid ---------- 0.5 g
Distilled water ---------- 100 ml

Schiff Reagent:

Test for Schiff reagent:

Pour 10 ml of 37% formalin into a watch glass. To this add a few drops of the Schiff reagent to be tested. A good Schiff reagent will rapidly turn a red-purple color. A deteriorating schiff reagent will give a delayed reaction and the color produced will be a deep blue-purple.

Mayer's Hematoxylin Solution:

Procedure:

1. Deparaffinize and hydrate to water.
2. Oxidize in 0.5% periodic acid solution for 5 minutes.
3. Rinse in distilled water.

4. Place in Schiff reagent for 15 minutes (Sections become light pink color during this step).
5. Wash in lukewarm tap water for 5 minutes (Immediately sections turn dark pink color).
6. Counterstain in Mayer's hematoxylin for 1 minute.
7. Wash in tap water for 5 minutes.
8. Dehydrate and coverslip using a synthetic mounting medium.

(C) Masson's Trichrome staining

Procedure:

1. Deparaffinize and rehydrate through 100% alcohol, 95% alcohol 70% alcohol.
2. Wash in distilled water.
3. For Formalin fixed tissue, re-fix in Bouin's solution for 1 hour at 56 C to improve staining quality although this step is not absolutely necessary.
4. Rinse running tap water for 5-10 minutes to remove the yellow color.
3. Stain in Weigert's iron hematoxylin working solution for 10 minutes.
4. Rinse in running warm tap water for 10 minutes.
5. Wash in distilled water.
6. Stain in Biebrich scarlet-acid fuchsin solution for 10-15 minutes. Solution can be saved for future use.
7. Wash in distilled water.
8. Differentiate in phosphomolybdic-phosphotungstic acid solution for 10-15 minutes or until collagen is not red.
9. Transfer sections directly (without rinse) to aniline blue solution and stain for 5-10 minutes. Rinse briefly in distilled water and differentiate in 1% acetic acid solution for 2-5 minutes.
10. Wash in distilled water.
11. Dehydrate very quickly through 95% ethyl alcohol, absolute ethyl alcohol (these step will wipe off Biebrich scarlet-acid fuchsin staining) and clear in xylene.
12. Mount with resinous mounting medium.

APPENDIX D

Histopathological Grading

APPENDIX D

The following **grading scale** was used and a mean and standard error was determined for each of the treatment groups:

1. Hepatic lobules are observed, demonstrating a radial arrangement of hepatic plates from the central vein with nominal branching. Sinusoids are regular between the hepatic plates. Hepatocytes show centrally located nuclei and no evidence of steatosis.
2. Hepatic lobules are observed, but the radial arrangement of the hepatic plates from the central vein is less distinct. Branching of the hepatic plates is more apparent and sinusoids may show dilation. Hepatocytes stained lightly with large central nuclei. No evidence of steatosis.
3. Hepatic lobules are apparent, but the radial arrangement of hepatic plates from the central vein is disordered with extensive branching of the hepatic plates. Sinusoids appear dilated but rarely vesicular. Small lipid droplets are present, occasionally in isolated groups of the liver acini, and usually limited to Zone 3.
4. Some organized hepatic lobules are apparent, but many show disorganization of the radial arrangement of the hepatic plates due to branching. Moderate steatosis is present in at least 50% of the liver acini and extends throughout Zone 3. The steatosis appears as small lipid inclusions. Zone 1 is intact and generally free of steatosis. Sinusoids appear dilated and occasionally vesicular. Some hepatocytes appear hypertrophied.
5. Disruption of the hepatic lobule organization is observed. Extensive steatosis is present in most of the liver acini, extending throughout Zone 3 as large lipid inclusions. Zone 1 may be affected in some lobules. Sinusoids are dilated and frequently vesicular. Hepatocytes are hypertrophied with large, pale-staining nuclei.

APPENDIX E

Immunohistochemistry Protocol

APPENDIX E

Immunohistochemistry with fluorescent probes

1. dewax the slides with tissue section in xylene for 15 minutes
2. dewax the slides with tissue section in xylene for 15 minutes
3. air dry the slides with tissue section for 10 minutes
4. dewax the slides with tissue section in xylene for 15 minutes
5. air dry the slides with tissue section for 10 minutes
6. Immerse the slides with tissue section in100% ethanol 15 minutes
7. air dry the slides with tissue section for 10 minutes
8. Immerse the slides with tissue section in 100% ethanol 15 minutes
9. air dry the slides with tissue section for 10 minutes
10. Immerse the slides with tissue section in100% ethanol 15 minutes
11. air dry for 10 minutes
12. Immerse the slides with tissue section in100% ethanol 15 minutes
13. air dry for 10 minutes
14. Immerse the slides with tissue section in100% ethanol 15 minutes
15. air dry for 10 minutes
16. Immerse the slides with tissue section in 90% ethanol 15 minutes
17. Immerse the slides with tissue section in 70% ethanol 15 minutes
18. Immerse the slides with tissue section in 1X-PBS 10 minutes
19. Boil the slides with tissue section in 0.1 mM EDTA for 12 minutes
20. Cool down the slides with tissue section for 45 minutes at room temperature
21. Wash the slides with tissue section in 1X-PBS 10 minutes
22. Incubate sections in 10% donkey serum made in PBS @ 37 °C for 1 hour
23. Add primary antibody made in 10% donkey serum (dilution to be determined) @ 4 ° C refrigerate for overnight
24. Wash the slides with tissue section in for PBS 5 minutes 5x
25. Add secondary antibody made in 10% donkey serum (dilution to be determined) @ 37 °C for 2 hours
26. Wash the slides with tissue section in PBS 5 minutes 5x
27. Mount sections in mounting media with DAPI.