Role of Liver Fatty Acid Binding Protein in Fatty Liver Cell Culture Model

by

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ABSTRACT

Liver fatty acid binding protein has been reported to possess antioxidant properties in the liver. The aim of this study was to investigate the effect of this protein in a nonalcoholic fatty liver disease (NAFLD) cell culture model. Rat hepatoma cells were treated with an oleate:palmitate (2:1) mixture for either 1 and 2 days, or further treated with 500 μM clofibrate to induce L-FABP expression. Intracellular lipid accumulation was quantitated by Nile Red. Lipotoxicity was determined using the WST-1 assay. Dichlorofluorescein (DCF) was utilized to assess intracellular reactive oxidative species (ROS) level. Measurement of lipotoxicity showed statistical decreases in cell viability as lipid concentrations increased in a dose-dependent manner. NAFLD cell cultures showed characteristic cellular damage from increased ROS levels in fatty acid treated cells. All groups treated with clofibrate showed statistically increased intracellular L-FABP levels and reduced ROS levels. The results lead to the conclusion that clofibrate induces L-FABP expression and in this manner suppresses hepatocellular ROS generation.

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ABBREVIATIONS

AAP	Acetaminophen
ATP	Adenosine triphosphate
BCA	bicinchonimic acid
CPT-1a	carnitine palmitoyltransferase-1a
CAT	catalase
cDNA	complementary DNA
CYP2E1	cytochrome P450 enzyme 2E1
CYP1A2	cytochrome P450 enzyme 1A2
Cys	cysteine
DAPI	4’,6-Diamidino-2-phenylindole dihydrochloride
DNA	deoxyribonucleic acid
ECM	extracellular matrix
eIF-2α	eukaryotic translation initiator factor-2alpha
FA	fatty acid
FAT	fatty acid translocase
FABPpm	membrane fatty acid binding protein
FATP	fatty acid transport protein
FFA	free fatty acid
GPx	glutathione peroxidase
GSH	glutathione, reduced form
GSSH	glutathione sulfoxide
H2DCFDA	2,7-dichlorofluorescein diacetate
H2O2	hydrogen peroxide

HNE	4-hydroxy-2nonenal
HSC	hepatic stellate cells
lgG	immunoglobulin
iLBPs	intracellular lipid binding proteins
JNK	c-Jun N-terminal kinases
LCFA	long-chain fatty acid
LDH	lactate dehydrogenase
L-FABP	liver fatty acid binding protein
LPL	lipoprotein lipase
NADPH	nicotinamide adenine dinucleotide phosphate
NAFLD	nonalcoholic fatty liver disease
NAPQI	N-acetyl-p-benzoquinone imine
NASH	nonalcoholic steatohepatitis
MALDI-TOF MS spectrometer	matrix-assisted laser desorption/ionization time-of-flight mass
Met	methionine
MDA	malondialdehyde
Msr	methionine sulfoxide reductase
PPRE	peroxisome proliferator response element
ROS	reactive oxygen species
SOD	superoxide dismutase
TBS	tris buffered saline
TGF-β	transforming growth factor-β
TRAIL	tumour necrosis factor related apoptosis inducing ligand
Tween-20	polyxyethylene-sorbitan monolaurate

UPR	Unfolded Protein Response
VLDL	very-low-density lipoprotein

I. INTRODUCTION

A. Liver, Liver Diseases, Free Radicals and Antioxidant Defense

1. Overview of Liver

The liver is the largest solid organ in the body, constituting approximately 2% of body weight in the adult and 5% in the neonate. In the human, the liver consists of two main lobes (left and right) with the gallbladder positioned under the liver, along with sections of the pancreas and intestines. The liver and these organs work together to digest, absorb, and process food. Although many different types of cells comprise the liver, the major cell type is the hepatocyte, comprising 70-80% of the hepatic mass. As a metabolically highly active cell, the hepatocyte contains numerous organelles: 15% of the cell volume comprises the endoplasmic reticulum; over 1000 mitochondria, about 300 lysosomes, equal numbers of peroxisomes and nearly 50 Golgi complexes are present as well as a typically organized cytoskeleton (Irwin et al., 1994).

The structural organization of the liver reflects its remarkable functions. The liver is a guardian between the digestive tract and the rest of the body. Because of its position, it is provided with a dual blood supply; receiving blood from the portal vein and hepatic artery. Venous and arterial blood mix prior to entering the sinusoids. These channels carry a large variety of endogenous and exogenous substrates that include nutrients as well as toxic substances derived from the digestive system. Hence, one of the major functions of the liver involves the efficient uptake of amino acids, carbohydrates, lipids, vitamins and their subsequent storage, metabolic conversion, and release into the blood or bile. A well known phenomenon of drug uptake is the first-pass effect. After a drug or

nutrient is ingested, it is absorbed by the digestive system and enters the mesenteric vein, which empties into the hepatic portal vein, carrying the drug into the liver before reaching the systemic circulation. The liver extracts and metabolizes many substrates so only a small amount of active substrate may emerge from the liver into the circulatory system depending on its extraction ratio. First-pass metabolism could greatly reduce the bioavailability of substrates and thus could also protect our body from damage induced by the excessive drug levels.

2. Common Liver Diseases Associated with Free Radicals

Although liver disease is stereotypically linked to alcohol or drug abuse, there are over 100 known forms of liver diseases caused by a variety of factors and affecting people of all ages. Free radicals are one of the main factors contributing to some forms of liver diseases such as nonalcoholic fatty liver disease, alcoholic liver failure, and drug-induced liver damage.

2.1. Nonalcoholic Fatty Liver Disease (NAFLD)

2.1.1. Introduction

In 1981, Ludwig and his colleagues (Ludwig et al., 1980) published their landmark paper introducing a unique clinical pathologic entity they named nonalcoholic steatohepatitis, abbreviated as NASH. The features traditionally recognized as components of nonalcoholic fatty liver disease (NAFLD) involve steatosis, acute and chronic inflammation that are usually most severe in the lobular parenchyma, cytologic ballooning of hepatocytes (Straub and Schirmacher, 2010), perisinusoidal fibrosis, and Mallory hyaline. This more embracing term, NAFLD, has been adopted to cover the full

spectrum of metabolic fatty liver disorders (Angulo, 2002; Clark et al., 2002). During the last decade there has been an explosion of interest in this disorder. Several single topic conferences focusing on NAFLD have been convened by the National Institutes of Health, the Falk Foundation, the American Association for the Study of Liver Diseases, the European Association for Study of the Liver and other major hepatology organizations worldwide (Farrell and Larter, 2006). The first book on NAFLD was published in 2005 (Farrell et al., 2005). Although the interrelationships are still unclear, obesity (Moore, 2010), cardiovascular disease (Hamaguchi et al., 2007), hyperlipidemia (Al-Gayyar et al., 2012) and type-2 diabetes mellitus (Ismail, 2011) are frequently recognized as comorbidities with NAFLD. As a disease usually recognized with the Western lifestyle, factors such as food intake, food composition, and physical activity play important roles in the epidemiology of NAFLD. The high intake of saturated fat was thought to be one of the major factors contributing to NAFLD (Musso et al., 2003). A correlation between higher carbohydrate intake and liver inflammation was also noted by other researchers (Solga et al., 2004). A histological scoring system for identification the degree of nonalcoholic fatty liver disease in adults and children was proposed by the Nonalcoholic Steatohepatitis Clinical Research Network in 2005 (Kleiner et al., 2005).

2.1.2. Terminology and Epidemiology

Defined by the accumulation of liver fat > 5% per liver weight in the presence of < 10 g of daily alcohol consumption (Byrne et al., 2009), NAFLD has been regarded as a manifestation of the metabolic syndrome (Farrell and Larter, 2006). Nonalcoholic steatohepatitis (NASH) is the progressive form of NAFLD.

NAFLD has a very high prevalence in North and South America, much of Asia-Pacific, and Europe (Clark et al., 2002; Neuschwander-Tetri and Caldwell, 2003; Salt, 2004). However, accurate estimates of its incidence as well as historical evidence are lacking (McCullough, 2005). Evidence from autopsy (Wanless and Lentz, 1990) and imaging studies (Bellentani et al., 2000; Dassanayake et al., 2009) showed NAFLD was present in 20-30% of the population worldwide, with 10% of these cases being NASH. Prevalence is much higher among obese patients (Dixon et al., 2001; Gholam et al., 2007) and patients with type-2 diabetes where 25-70% of these patients have advanced disease encompassing NASH and fibrosis (Gupte et al., 2004; Targher et al., 2007; Leite et al., 2011).

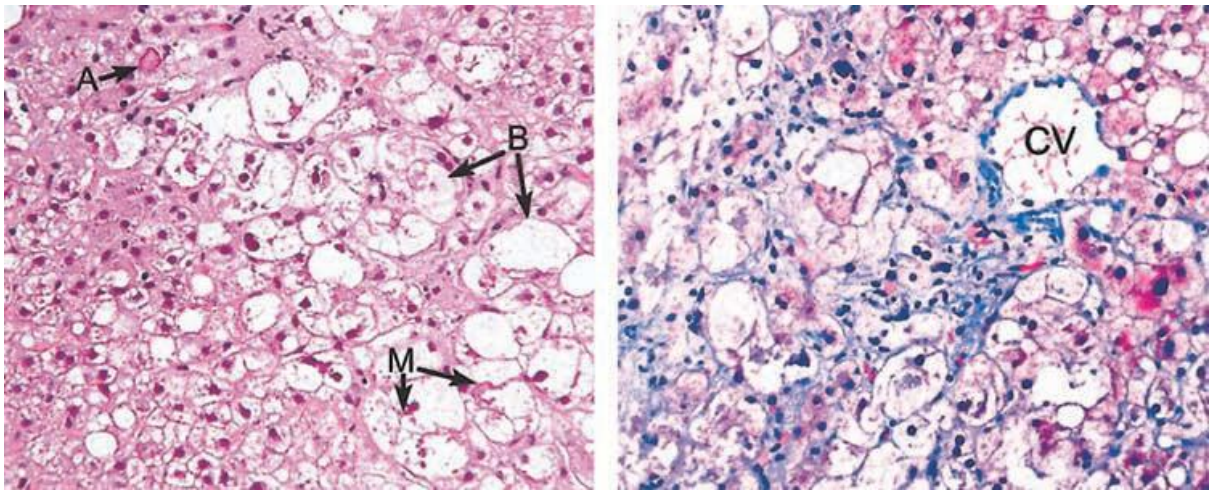
NAFLD is becoming the most common cause of liver disease in children. Incidence of paediatric NAFLD rises rapidly, corresponding with the increase in childhood obesity worldwide (Moore, 2010). A retrospective review of paediatric autopsy reports in the USA found fatty liver in 13% of children and 38% of obese children between 2 and 19 years of age (Schwimmer et al., 2006). With the help of ultrasonography, the estimates of NAFLD prevalence in obese children were found to range 45% to 60% (Chan et al., 2004; Sagi et al., 2007; Sartorio et al., 2007). The International Obesity Task Force has concluded that the lowest estimated prevalence of hepatic steatosis is 28% among obese children in Europe (Lobstein and Jackson-Leach, 2006). Since the increasing rate of obesity in children and occurrence of paediatric NAFLD warrants considerable clinical and research attention.

2.1.3. Histologic abnormalities, Diagnosis and Grading

The spectrum of histologic abnormalities defined by NAFLD includes simple steatosis and its more extreme form, NASH. Common as well as unusual components present in NAFLD were summarized during the American Association for the Study of Liver Diseases Clinical Single Topic Conference on Nonalcoholic Steatohepatitis (NASH) held in Atlanta, 2002. Components include steatosis, scattered polymorphonuclear leukocytes as well as mononuclear cells, and hepatocellular ballooning (Neuschwander-Tetri and Caldwell, 2003). Although there is a diversity of opinion among expert pathologists about the definition of NAFLD, the increased amount and type of fat (both macrovesicular and microvesicular) in hepatocytes, lobular inflammation, and ballooning hepatocyte degeneration comprise the most remarkable histological characteristics of NAFLD and NASH (Figure 1). Other features such as Mallory hyaline, mitochondrial abnormalities and pericellular fibrosis are also usually seen in NAFLD patients (Neuschwander-Tetri and Caldwell, 2003).

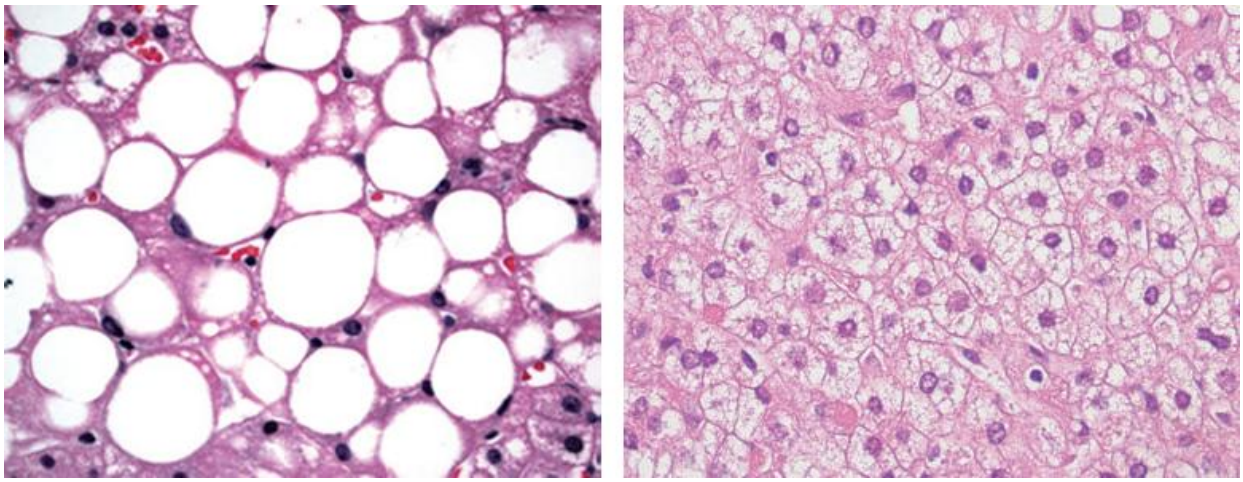
As the major histological feature of NAFLD, the presence of steatosis is necessary for diagnosis. A minimum of 5% steatosis is used for the operational minimal definition of histological NAFLD in biopsy specimens from adults and children. (Kleiner et al., 2005). Traditionally, steatosis is classified as macro- or microvesicular (see Figure 2). Macrovesicular is characterized by the presence of only one or a few well-demarcated fat droplets in the hepatocyte cytoplasm. Numerous tiny lipid droplets (less than 1 μm) are often observed in microvesicular, occupying the hepatocyte cytoplasm. These lipid droplets surround rather than displace the nucleus. The enlarged nucleus maintains its

Figure 1. Characteristic Hepatocellular Abnormalities of NAFLD.



(**Left panel**) Mixed micro- and macrovesicular steatosis is present and examples of acidophil bodies (A), ballooning (B), and Mallory's hyaline (M) are identified by arrows. (**Right panel**) The characteristic initial pattern of fibrosis in steatohepatitis is the perisinusoidal collagen deposition in zone 3 around the central vein (CV), as identified by blue staining. Figure adopted from Neuschwander-Tetri and Caldwell, 2003 on November 5, 2011 with the permission from © John Wiley and Sons.

Figure 2. Macrovesicular and Microvesicular Steatosis.



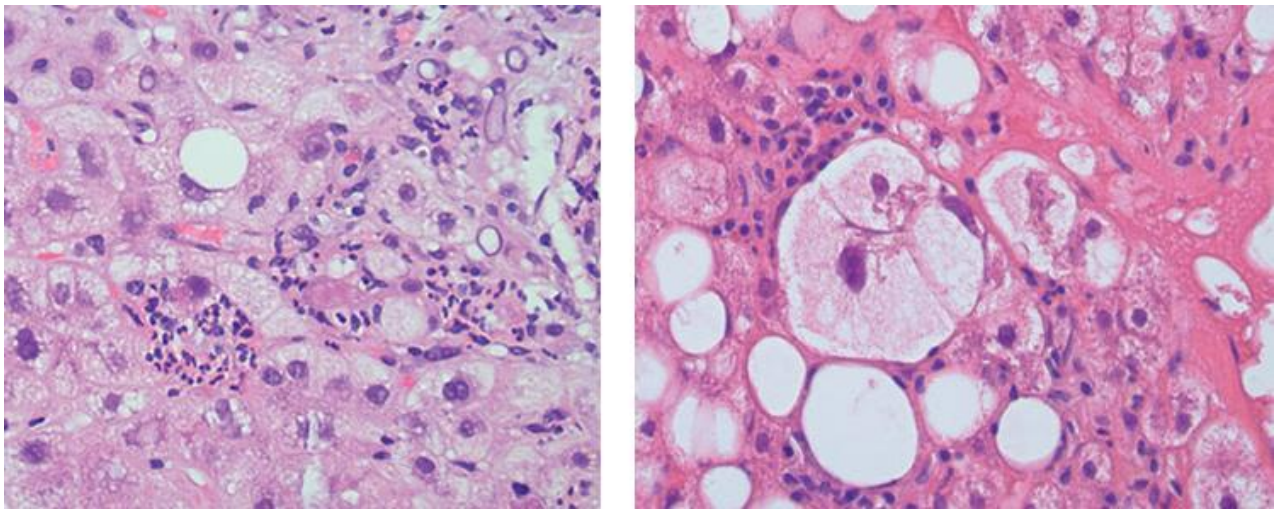
(**Left panel**) Macrovesicular steatosis. **(Right panel)** Microvesicular steatosis. Figure adopted from Yerian, 2011 on November 4, 2011 with the permission from © John Wiley and Sons.

central position in the cells. Although the steatosis is exclusively or predominantly macrovesicular, contiguous patches of microvesicular steatosis can be seen. These two types of steatosis need to be distinguished because they are associated with very different etiologies, pathogeneses and prognoses. Whereas macrovesicular or mixed steatosis is a common finding most often seen in ambulatory patients, pure microvesicular steatosis is rare and is associated with severe mitochondrial injury and acute hepatic dysfunction, typically such as Reye's syndrome (Bove et al., 1975), acute fatty liver of pregnancy (Minakami et al., 1988), Jamaican vomiting disease (Tanaka et al., 1976), alcohol-induced microvesicular liver steatosis (Uchida et al., 1983), and other diseases associated with hepatic injury (Hautekeete et al., 1990). A predominantly macrovesicular, ranging from less than 33% to up to 66% of the lobules, is often seen in a mild steatosis. However, mixed steatosis is more commonly seen in the moderate or severe stages of NAFLD and accompanied with lobular inflammation (Neuschwander-Tetri and Caldwell, 2003).

In histologic studies of NAFLD, lobular inflammation was identified in most patients and was characterized by mixed infiltrates of acute and chronic inflammatory cells (Contos et al., 2004). A mixture of neutrophils, lymphocytes, and macrophages are involved (Itoh et al., 1987; Powell et al., 1990) (Figure 3, left panel). The inflammation is usually present in scattered lobular clusters of inflammatory cells and may also be present in portal tracts.

An important feature required for a histological diagnosis of steatohepatitis is evidence of hepatocyte injury termed ballooning hepatocyte degeneration (Figure 3, right panel). This is probably the most challenging and subjective feature for the pathologist. Ballooned hepatocytes are recognized as those that stand out from background hepatocytes based on their large size and pale, irregular cobweb like cytoplasm. The

Figure 3. Lobular Inflammation and Ballooning Hepatocyte Degeneration.

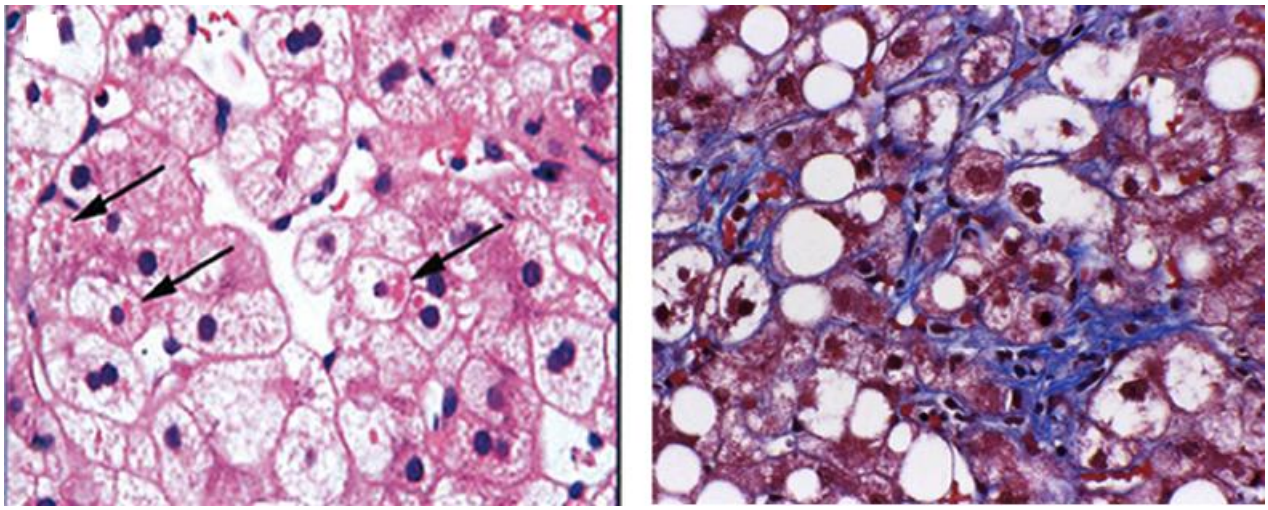


(Left panel) Lobular inflammation in HE-stained slide shows clusters of lobular inflammatory cells including lymphocytes, Kupffer cells and neutrophils. **(Right panel)** Ballooning hepatocyte degeneration in HE-stained slide demonstrates enlarged hepatocytes with pale staining wispy cytoplasm. Figure adopted from Yerian, 2011 on November 4, 2011 with the permission from © John Wiley and Sons.

affected cells tend to be most prominent in zone 3 where they are associated with perisinusoidal fibrosis and steatotic hepatocytes. As an important feature in adult NAFLD patients, ballooning hepatocyte degeneration is notably absent in many cases of pediatric NAFLD (Schwimmer et al., 2005; Carter-Kent et al., 2009).

Mitochondrial abnormalities are seen frequently in subjects with NAFLD. These include megamitochondria, development of multi-lamellar mitochondria, loss of cristae, and presence of intramitochondrial paracrystalline inclusion bodies (Caldwell et al., 1999; Sanyal et al., 2001) (Figure 4). Paracrystalline inclusions have been described in association with conditions where there are abnormalities of the mitochondrial DNA resulting in impaired function of the electron transport chain enzymes in the mitochondria (Marin-Garcia et al., 2000), release of free radicals and eventually the induction of oxidative stress (Heddi et al., 1999). Another hallmark of NAFLD is the development of a specific form of fibrosis where a collagenous matrix is deposited along the hepatocytes in the space of Disse (Figure 4). However, evaluating severity of fibrosis is difficult, and the amount of fibrotic tissue identified is dependent on the quality of the collagen stain (Contos et al., 2004). The majority of cases of NAFLD are identified after an incidental finding of either elevated liver enzymes on routine blood test or suspected fatty liver on abdominal imaging in patients consuming little or no alcohol (Vuppalanchi and Chalasani, 2009). While liver enzymes such as alanine aminotransferase (ALT) and γ-glutamyl transpeptidase levels may be found to be elevated, they are not specific to NAFLD and the full histological spectrum of NAFLD has been observed in patients with normal ALT levels (Mofrad et al., 2003). The grade of the individual histologic parameters of NAFLD was similar to those with an elevated ALT. It has been shown that NAFLD can be

Figure 4. Megamitochondria and Pericellular Fibrosis.



(Left panel) Photomicrograph of cells with easily identified megamitochondria **(arrows)**. This case would be scored positive for this feature, although it is not necessary to see so many positive cells in a single field. Figure adopted from Kleiner et al., 2005 on November 5, 2011 with the permission from © John Wiley and Sons. **(Right panel)** Sinusoidal (chicken wire) fibrosis is highlighted blue in a Masson Trichrome. Figure adopted from Contos et al., 2004 on November 5, 2011 with the permission from © John Wiley and Sons.

diagnosed with approximately 50% accuracy based on clinical parameters (Van Ness and Diehl, 1989). Consideration of all these findings, for diagnosing and assessing the stage of NAFLD a liver biopsy remains the "gold standard". Although several other methods such as ultrasound-based transient elastography and hepatic magnetic resonance imaging are currently being investigated and developed to predict steatosis (Poynard et al., 2005), steatohepatitis (Poynard et al., 2006), and fibrosis (Guha et al., 2008) in NAFLD, they require independent population validation and still lack sensitivity and specificity for widespread use (Foucher et al., 2006; Salameh et al., 2009). However, liver biopsy is invasive and associated with morbidities (Cadranel et al., 2000) and rare cases of mortality (Gilmore et al., 1995), precluding its routine use in screening for NAFLD or for repeated assessment of either disease progression or response to therapy. Furthermore, as histological lesions are not evenly distributed in the liver, considerable sampling error and misdiagnosis exists (Ratziu et al., 2005). The development of the non-invasive techniques capable of diagnosing the stage of NAFLD is a research priority (Wieckowska et al., 2007).

Difficulty in diagnosing the NAFLD also makes it not a simple task to establish an easy to use and reproducible system to grade the stages of this disease in the patients. A well-designed grading system should be one that is without ceiling or floor effects and able to detect and correlate change with clinical outcomes (Contos et al., 2004). The NASH clinical research network of NIH attempted to develop an independent scoring method for histologically grading and staging steatosis (Kleiner et al., 2005). Parameters include individual grades for steatosis, lobular inflammation, and cytologic ballooning (Table 1). This system is simple and requires only routine histochemical stains, so that it

Table 1. Nonalcoholic Fatty Liver Disease (NAFLD activity score)

Activity score Presumes overall histological pattern is that of nonalcoholic fatty liver disease	**Fibrosis score** Fibrosis is assessed using Masson's trichrome stain
Steatosis (%)	1a: Zone 3 perisinusoidal fibrosis trichrome required to see
0: <5	1b: Zone 3 perisinusoidal fibrosis evident on HE-stained slide
1: 5–33	1c: Portal fibrosis only
2: >33–<66	
3: >66	
Lobular inflammation (foci per 20x field):	2: zone 3 + portal/periportal fibrosis
0: none	
1: <2	
2: 2–4	
3: >4 foci per 20x field	
Ballooning hepatocyte degeneration:	3: Bridging fibrosis
0: None	
1: Few ballooned hepatocytes	
2: Many or prominent hepatocyte ballooning	
Total: Sum of steatosis, lobular inflammation, and ballooning score noted above. Maximum score is 8.	4: Cirrhosis

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can be used in clinical trials to standardize reporting, establish minimal histological criteria for inclusion in trials and assess changes in the component features that might occur as a treatment response (Yerian, 2011).

2.1.4. Oxidative Stress and NAFLD

Although the exact pathogenesis of NAFLD remains poorly understood, the prevailing "two-hit hypothesis" is that several insults are involved in causing the inflammatory reactions and loss of metabolic competency and finally triggering simple fatty liver toward NASH (Day and James, 1998). Insulin resistance most likely plays a central role in the "first hit" of NAFLD. Insulin resistance activates the lipoprotein lipase (LPL) in adipocytes and skeletal muscle cells, triggering the processes of triglyceride hydrolysis and fatty acid (FA) release into blood from fatty tissue (Anthonsen et al., 1998). As a result, large amounts of FA are released into the circulation (Solis Herruzo et al., 2006). Almost two thirds of liver fat deposits in NAFLD patients have been seen to be derived from circulating FA (Donnelly et al., 2005). Dietary fat is another source contributing to the hepatic intracellular lipid pool. Liver insulin resistance gives rise to the retention of lipids in the liver (Krawczyk et al., 2010), which has been found to be associated with the excessive influx of FA from adipose tissue (Donnelly et al., 2005), diminished hepatic export of FA due to, for instance, decreased very-low-density lipoprotein (VLDL) secretion (Browning and Horton, 2004), decreased disposal of fatty acids due to impaired mitochondrial β-oxidation (Reid, 2001) and increased hepatic de novo lipogenesis caused triglyceride overproduction (Smith and Adams, 2011). The "second-hit" is generally attributed to oxidative stress (Perlemuter et al., 2005), which causes peroxidation of lipids in the hepatocyte membrane (Day and James, 1998), over-

production (Kern et al., 1995), Fas ligand induction (Pessayre et al., 2001), increased intracellular lipid peroxidation (Madan et al., 2006), decreased hepatic antioxidant capacity (Saricam et al., 2005), and impaired function of antioxidant enzymes in the liver (Hardwick et al., 2010). Several lines of evidence suggest that chronic oxidative stress is concomitant in human NAFLD (Cortez-Pinto et al., 1999; Loguercio et al., 2001; Videla et al., 2004). As shown in Table 2, the liver protein oxidation index is markedly enhanced in NAFLD patients with steatosis compared with controls, whereas reduced form glutathione (GSH) content, superoxide dismutase (SOD) activity, and the antioxidant capacity of plasma are significantly lower (Videla et al., 2004). Hepatic malondialdehyde (MDA) levels are increased in steatosis (Cortez-Pinto et al., 1999). Such data point to an enhancement in the oxidative stress status with steatosis, indicating a high free radical activity in the liver of NAFLD patients.

Increased lipid peroxidation products have been observed in patients with nonalcoholic steatohepatitis due to the imbalance between hepatic pro-oxidant and antioxidant chemical species (Yesilova et al., 2005; Madan et al., 2006). Lipid peroxidation is referred as the oxidative degradation of lipids proceeding by a free radical chain reaction mechanism. The significant increase in hepatic malondialdehyde, the main lipid peroxidation product, was demonstrated in animal models of fatty liver (Sexena et al., 2001) and patients with nonalcoholic fatty liver disease (Garg, 2001). Other lipid peroxidation markers like thiobarbituric acid reactive substances (Madan et al., 2006) and hepatic peroxides (Allard et al., 2008) were also present in elevated levels in human NAFLD patients. In NAFLD patients, lipid peroxidation is mainly caused by free radicals

Table 2. Oxidative Stress Related Parameters the Patients with NAFLD.

Parameter	**Controls (%)**	**Steatosis (% of control)**	**Steatohepatitis (% of control)**
In Liver			
Superoxide dismutase activity	100 ± 10	52 ± 5	36 ± 7
Catalase activity	100 ± 11	90 ± 14	52 ± 10
Glutathione (reduced form) content	100 ± 10	43 ± 8	73 ± 13
Malondialdehyde content	100 ± 5	281 ± 9	290 ± 13
Protein oxidation index	100 ± 26	403 ± 103	174 ± 32
In Plasma			
Antioxidant capacity	100 ± 3	79 ± 2	67 ± 2
Malondialdehyde levels	100	124	133

Values shown correspond to means ± SEM and are expressed as a percentage of control value. All the values are taken and calculated from Videla et al., 2004 and Cortez-Pinto et al., 1999. Control values are: superoxide dismutase activity, 12.6 ± 1.2 U/mg protein; catalase activity, 0.55 ± 0.06 k/mg protein; reduced glutathione (GSH) content, 23.8 ± 2.4 nmol/mg protein; liver malondialdehyde content, 2.25 ± 0.11 nmol/mg protein; protein oxidation index, 0.98 ± 0.26 nmol carbonyls/mg protein; antioxidant capacity of plasma, 390 ± 11 µM iron; plasma malondialdehyde levels, 0.3 nmol/ml.

(Kaikaus et al., 1993b). As a catalyst of the Fenton reaction, excessive free iron (Mitsuyoshi et al., 2009) in the liver contributes to redox activation and reactive oxygen species formation, in turn resulting in further lipid peroxidation (Videla et al., 2003).

2.1.5. NAFLD Disease Models

To better understand the pathogenesis and to identify the potential therapeutic targets of NAFLD, several animal models focusing on impaired hepatic lipogenesis and abnormal fatty acid uptake and oxidation have been proposed and tested. These models mainly include dietary models (Romestaing et al., 2007; Baumgardner et al., 2008), genetic models (Baffy et al., 2002; Sahai et al., 2004) and models combining oxidative stress, nutritional factors and drugs (Ito et al., 2006; Matsuzawa et al., 2007; Wouters et al., 2008). One of the classical dietary models is the methionine and choline deficient model. Methionine and choline are essential factors for hepatic β-oxidation. Markedly enhanced oxidative stress can be observed from 3 weeks after intake of the methionine/choline deficient diet (Anstee and Goldin, 2006). Genetic models usually focus on defective genes that associate with lipid metabolism resulting in severe lipid peroxidation. The most common ones are mouse models, involving leptin-deficient obese mice (Brix et al., 2002), acyl-coenzyme A oxidase null mice (Cook et al., 2001), and mice lacking the gene encoding peroxisome proliferator-activated receptor-α (Kashireddy and Rao, 2004). Db/db mice fed with a methionine and choline deficient diet is one of the combination models. Not only large amount of lipid accumulation and hepatic insulin resistance are observed in those mice (Sahai et al., 2004), but they readily develop symptoms of NASH like inflammation and fibrosis (Wortham et al., 2008). There are also *in vitro* hepatocellular NAFLD models, utilizing either human and rat hepatoma cell

lines (Ji et al., 2005; Gomez-Lechon et al., 2007; Garcia et al., 2011) or primary culture of human and mouse hepatocytes isolated from the liver tissues with steatosis (Malhi et al., 2006). These *in vitro* models demonstrate the major characteristics of NAFLD in terms of the elevated lipid accumulation and severe lipotoxicity. They are developed to investigate either the mechanism of FA mediated lipoapoptosis (Malhi et al., 2006) or the role of FA in oxidative stress associated NAFLD progression (Garcia et al., 2011).

2.2. Alcohol Induced Liver Injury

Another common hepatic failure related to free radical damage is alcoholic liver disease. The involvement of oxidative injury in ethanol toxicity was initially proposed by Di Luzio and Hartman almost half century ago in studies showing antioxidants could prevent acute ethanol-induced fatty liver (Di Luzio, 1963; Di Luzio and Hartman, 1967). Many studies have demonstrated that alcohol increases lipid peroxidation as well as the oxidative modification of proteins (Nordmann, 1994). Numerous investigations have found that administering antioxidants, agents that reduce the levels of free iron, or agents that replenish glutathione levels can prevent or ameliorate the toxic actions of alcohol. One of the examples was shown in the intragastric infusion model where alcoholic liver disease was associated with enhanced lipid peroxidation, protein modification, formation of the 1-hydroxyethl radical and lipid radicals, and decreases in the hepatic antioxidant defense, particularly glutathione levels (Li et al., 2001; Tokars et al., 2001; Smith et al., 2004). Studies with rat liver cells grown in culture also showed that alcohol metabolism via the enzyme alcohol dehydrogenase leads to oxidative stress and hepatocyte toxicity, which was associated with elevated ROS generation in both cytosol and mitochondria (Abbadi et al., 2001; Buffenstein et al., 2001), accompanied with the consumption of

glutathione in the intracellular compartments (Garg and Kumar, 2001), and increased lipid peroxidation (Sharma et al., 2001) leading to mitochondria dysfunction and finally cell apoptosis (Giusti et al., 2001).

2.3. Drug-Induced Liver Damage

The liver is vulnerable to acute or chronic failure caused by various chemicals that enter the body. One example is acetaminophen induced liver damage. Acetaminophen (Paracetamol, 4-hydroxyacetanilide, AAP) is a safe and effective analgesic and antipyretic drug when used at therapeutic doses (500 to 1000 mg, three to four times per day). However, overdoses (more than 150 mg/kg) can cause acute liver injury both in experimental animals and humans (Indurwede et al., 2001). Research into the toxicity of acetaminophen has focused on metabolic activation of excessive drug levels by the cytochrome P-450 enzyme system (primarily by two isoenzymes of cytochrome P-450: CYP2E1 and CYP1A2). At clinical doses, the toxic metabolite NAPQ1 is quickly detoxified by glutathione via conjugation with the thiol group of glutathione. However, overdose of acetaminophen leads to the accumulation of N-acetyl-p-benzoquinone imine (NAPQI) and generation of free radicals like superoxide anions and hydrogen peroxide, which deplete cytosolic and mitochondrial glutathione and results in mitochondria dysfunction. This will trigger a cascade resulting in decreased production of mitochondrial ATP, formation of mitochondrial membrane permeability transition pore, loss of mitochondrial membrane potential, and release of proteins such as cytochrome C which initiates caspase 9-dependent apoptosis (Favale et al., 2001). This mechanism is the basis for the rational clinical use of N-acetylcysteine, a glutathione precursor, as an antidote against acetaminophen toxicity (Panagariya et al., 2001).

3. Free Radicals in Liver

3.1. Reactive Oxygen Species (ROS)

Free radicals are highly reactive molecules, ions or atoms having unpaired electrons. Then have a tendency to pair up those electrons, forming a stable compound. Reactive oxygen species (ROS) are highly reactive molecules containing reactive oxygen. ROS are natural by-products of normal oxygen metabolism.

$$O_2 \xrightarrow{e^-} O_2^{-}\cdot \xrightarrow{e^-} O_2^{=} \xrightarrow{2H^+} H_2O_2 \xrightarrow[H^+]{e^-} H_2O + \cdot OH \xrightarrow[H^+]{e^-} H_2O$$

Molecular oxygen can accept four electrons and corresponding number of protons to generate two molecules of water. During this process, different oxygen radicals are formed as intermediate products including superoxide (O_2^-) and peroxide ($O_2^{=}$), which normally exist in cells as hydrogen peroxide (H_2O_2) and hydroxyl radical (·OH). These radicals are considered as primary ROS. Because they are unstable and rapidly react with additional electrons and protons, most of these ROS are converted to water before they can damage cells. The major source of ROS production in the cell is the mitochondrial respiratory chain, which utilizes ~80 to 90% of the O_2 it consumes (Tomar et al., 2003). The over production of highly reactive species will break down lipids, inactivate enzymes, introduce changes in DNAs (i.e., mutations) and destroy cell membranes, and eventually could lead to serious cellular and tissue damage. This cellular state is called oxidative stress.

In the healthy liver, hepatocytes produce low amounts of ROS, which are normally inactivated by various cellular antioxidant systems. If the liver becomes

inflamed or infected, its ability to suppress ROS production may become impaired. In chronic liver diseases there is an over production of ROS resulting in oxidative stress, such that the normal antioxidant capacity of the cell becomes overwhelmed. In mammalian hepatic cells large amounts of ROS are generated through various metabolic and chemical reactions, of which the well-known sources include the drug detoxification reactions carried out by the cytochrome P-450 system (Schlezinger et al., 2006); electron leakage through electron transport systems such as mitochondrial respiratory chain reactions (Begriche et al., 2006); redox cycling of xenobiotic compounds, as in the case of quinones (Brunmark and Cadenas, 1989), azo compounds (Kappus, 1986), and furans (Garcia Martinez et al., 1995). ROS induce hepatocyte necrosis and triggers pro-inflammatory cytokines (i.e. IL-1α) release, mediating carcinogen-induced increased proliferation, a response that maintains liver mass but may also be the main driver of hepatocarcinogenesis (Sakurai et al., 2008). Palmitate-induced ROS generation at 100 μM was also found to be associated with an increased cell proliferation in QZG cell, a human hepatocyte cell line, which may be due to the ROS-activated p38 MAPK/ERK-Akt cascade (Wang et al., 2011). CYP2E1-derived ROS were also able to stimulate hepatic stellate cell (HSC) activation and collagen synthesis (Nieto et al., 2002). The possible mechanism is thought to be via the ROS-mediated increased expression of matrix metalloproteinase-2 in HSC (Galli et al., 2005). (Perez de Obanos, Lopez-Zabalza et al. 2007).

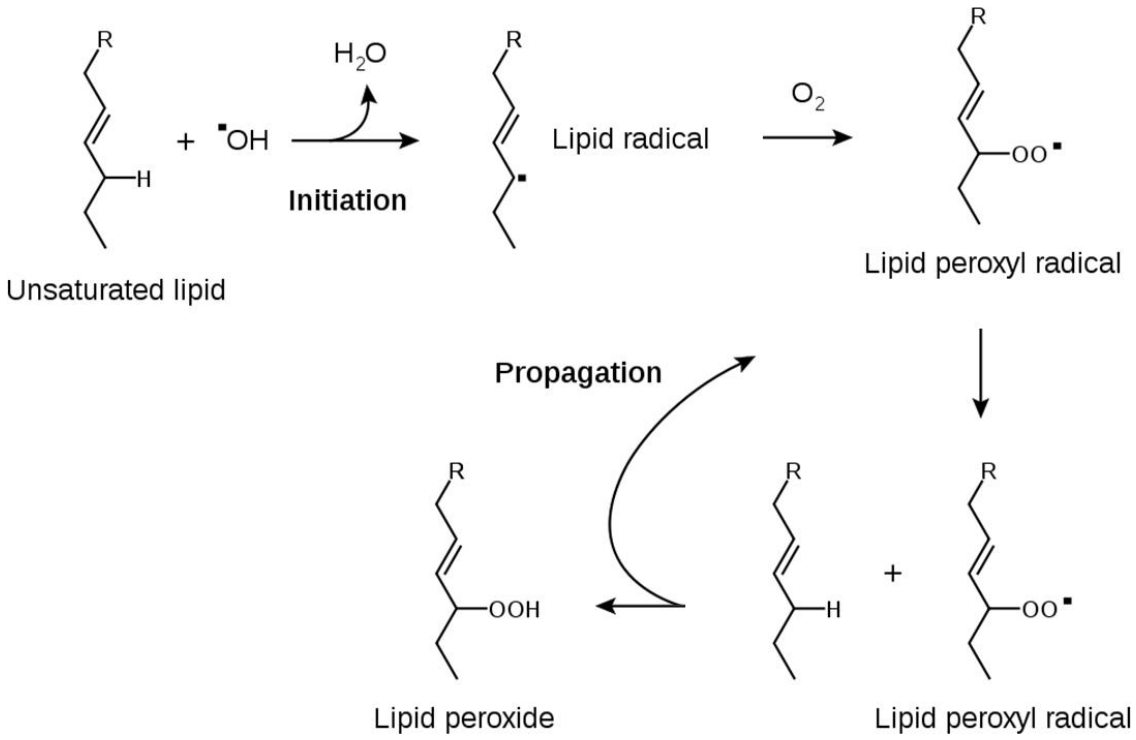
3.2. Lipid peroxidation

ROS are relatively short-lived molecules that exert local effects. They can attack polyunsaturated fatty acids and initiate lipid peroxidation, with formation of aldehyde by-

products such as 4-hydroxy-2nonenal (HNE) and malondialdehyde (MDA) (Poli and Schaur, 2000). These molecules have a longer half-life and can diffuse from their site of origin to reach intracellular and extracellular targets, further amplifying the effects of oxidative stress. As a major feature of NAFLD (Madan et al., 2006), increased lipid peroxidation products are thought to be involved in the progression of NAFLD to fibrosis (Albano, Mottaran et al. 2005).

The overall process of lipid peroxidation consists of three steps: initiation, propagation, and termination (Figure 5). The initiation is evoked by the attack on a fatty acid or fatty acyl side chain of any chemical species that has sufficient reactivity to abstract a hydrogen atom from a methylene carbon in the side chain (Mead, 1976). The greater the number of double bonds in a fatty acid side chain, the easier is the removal of a hydrogen atom, which is why polyunsaturated fatty acids are particularly susceptible to peroxidation. The hydrogen atom has a single electron and its removal leaves behind an unpaired electron on the carbon atom to which it was originally attached. The resulting carbon-centred lipid radical can have several fates, but the most likely one in the cells is to undergo molecular rearrangement, followed by reaction with O_2 to give a peroxyl radical. Peroxyl radicals can combine with each other if they meet, or they can attack membrane proteins, but they are also capable of abstracting hydrogen from adjacent fatty acid side chains and so propagating the chain reaction of lipid peroxidation. Termination of the chain reaction can only occur when there are sufficient antioxidants present to neutralize or inactivate the free radicals or an equal amount of free radicals having a high probability to react with each other. If not quickly and appropriately terminated, a single initiation event can result in conversion of hundreds of fatty acid side chains into lipid

Figure 5. Lipid Peroxidation.



Lipid peroxidation is the process proceeded by a free radical chain reaction mechanism.

hydroperoxides (Halliwell and Chirico, 1993).

4. Antioxidant Defense

To counteract excessive reactive species, the body is endowed with antioxidants. In a healthy body, free radicals and antioxidants maintain a ratio. A shift in this ratio towards free radicals gives rise to oxidative stress. In a state of oxidative stress antioxidants are depleted. To detoxify the hazardous compounds, the liver contains enzymes (eg., superoxide dismutase, glutathione peroxidase, catalase, etc.) and high levels of low molecular weight antioxidants (eg., glutathione, vitamin C, etc.) that inactivate reactive oxygen species. Common non-enzymatic and enzymatic antioxidants are listed in Table 3.

Superoxide dismutases (SODs), catalase, glutathione peroxidase and glutathione are the major components comprising hepatic antioxidant defense system. SODs catalyze removal of superoxide radicals. A copper-zinc SOD is present in the cytosol and in the intermembrane space of the mitochondria; a manganese-containing SOD is present in the mitochondrial matrix. Both of these enzymes are critical for prevention of ROS-induced toxicity (Battaini et al., 2003). Catalase and the glutathione peroxidase system both help to remove hydrogen peroxide. Catalase is found primarily in peroxisomes, and catalyzes a reaction between two hydrogen peroxide molecules, resulting in the formation of water and O_2. The glutathione peroxidase system consists of several components, including the enzymes glutathione peroxidase and glutathione reductase and the cofactors such as glutathione (GSH) and nicotinamide adenine dinucleotide phosphate (NADPH). GSH is an essential component of this system and serves as a cofactor for glutathione transferase, which helps remove certain drugs and chemicals as well as other reactive molecules from cells. GSH is probably the most important non-enzymatic antioxidant present in cells. As

Table 3. Antioxidants and Scavenging Enzymes

Antioxidants	**Description**
Glutathione	major hydrophilic antioxidant in extracellular compartment
Vitamin C	hydrophilic antioxidant in extracellular fluid
Vitamin E	scavenges in hydrophobic compartment
SOD	superoxide dismutase, present in all mammalian cells
Catalase	enzyme mainly in peroxisome
Glutathione Peroxidase	mainly present in cytosol

a naturally occurring major thiol and synthesized in cytosol in its reduced form, GSH is translocated from cytosol to mitochondria and nucleus, and serves as an antioxidant and for detoxifying electrophilic compounds. Intracellular GSH is maintained in its thiol form by glutathione disulfide (GSSG) reductase, which requires NADPH. Some drugs can be detoxified by conjugation with the thiol group of GSH (e.g., acetaminophen). Depletion of mitochondrial GSH by chronic oxidative stress occurs usually in pericentral hepatocytes, where most of the liver injury originates (Grimanelli et al., 2003). Occupying 70-80% of the liver mass, hepatocytes are the main cell type responsible for hepatic lipid metabolism and detoxification of exogenous and endogenous substrates, making them the major targets for the reactive species related oxidative damages.

In summary, a compromised antioxidant defense system observed in liver diseases is usually associated with the generation of a large amount of free radicals either through drug or chemical metabolism and a high level of consumption of antioxidants in the liver cells. Proper antioxidant therapy may, therefore, help reduce free radicals and potentiate cell survival.

B. Hepatocellular Fatty Acid Uptake and Intracellular Transport

1. Introduction

Long-chain fatty acids (LCFA), in addition to providing the cell with energy, are substrates for membrane biogenesis and act as signalling molecules. These fatty acids and their derivatives directly or indirectly regulate cellular processes such as differentiation, development and gene expression as well as the activities of enzymes, membrane receptors and ion channels. Dietary fats have profound effects on gene expression and

fatty acid-activated transcription factors (nuclear receptors) may have a fundamental role in regulating energy balance through their sensing of fatty acid flux in metabolically active tissues. Because of the functional roles of these fatty acids and of their role in NAFLD together with their structural features and physicochemical properties, it is important to understand the mechanisms for uptake and retention of these molecules.

2. Membrane Associated Transport Proteins in Hepatocellular Fatty Acid Uptake

Historically the mechanism by which fatty acids are translocated across the plasma membrane of various cell types, including endothelial and parenchymal cells, was a matter of debate for many years. Because of their lipophilic nature, fatty acids were thought to move freely within lipid membranes and, therefore, the transmembrane transport of fatty acids will occur by simple diffusion, with the fatty acid flux being determined by the net gradient across the membrane (De Grella and Light, 1980; Doody et al., 1980; Kamp and Hamilton, 1993). The latter is governed by the physicochemical partition of fatty acids between extracellular albumin, the membrane lipid phase, intracellular fatty acid binding protein and the respective aqueous phases (Rose et al., 1989). In the early 1980s, it was reported from different groups that the uptake of fatty acids into various cell types showed saturation kinetics. This process could be inhibited by non-metabolizable fatty acid analogues and the pretreatment of cells with proteases, suggesting the possible involvement of membrane-associated proteins in their uptake (Abumrad et al., 1981; Abumrad et al., 1984). By using a variety of techniques a number of such membrane proteins have been identified thereafter (Table 4).

Table 4. Membrane-associated Fatty Acid Binding Proteins

Protein	*M* (kDa)	Main Occurrence
Membrane FABP	22	Adipose Tissue[1]
$FABP_{pm}$	40-43	Adipose Tissue, heart, intestine, liver, placenta[2]
FA receptor (FAR)	56-60	Heart, kidney[3]
FA transport protein (FATP)	63	Adipose tissue, heart, skeletal muscle (brain, kidney, lung, liver)[4]
FA translocase (FAT/CD36)	88	Adipose tissue, heart, skeletal muscle, intestine, spleen, (testis)[5]

Proteins were identified in the rat, except for FATP (mouse). FA, fatty acid. Data from [1] (Trigatti et al., 1991), [2] (Sorrentino et al., 1988; Stremmel, 1988; Berk et al., 1990; Campbell et al., 1995), [3] (Fujii et al., 1987; Fujii et al., 1987), [4] (Schaffer and Lodish, 1994), [5] (Harmon et al., 1991; Abumrad et al., 1993).

In 1985, Stremel and colleagues (Stremel et al., 1985) reported the partial purification by affinity chromatography of a 40-43 kDa fatty acid binding protein, designated $FABP_{pm}$ (Sorrentino et al., 1991). $FABP_{pm}$ is an abundant component of the liver plasma membrane as it is estimated to constitute 2% of plasmalemmal proteins (Zhou et al., 1990). Monoclonal antibodies directed against liver $FABP_{pm}$ were found to inhibit fatty acid uptake by hepatocytes (Sorrentino et al., 1988; Stremmel, 1988), suggesting that the majority of overall fatty acid uptake is mediated by $FABP_{pm}$.

Studies done by Abumrad (Abumrad et al., 1984) on the inhibitory action of stilbene compounds and sulfo-N-succinimidyl derivatives of fatty acids (Harmon et al., 1991) on fatty acid permeation into rat adipocytes gave evidence for the involvement of a 80-90 kDa membrane protein in cellular fatty acid binding and/or transport across the plasma membrane. This protein was cloned and called membrane fatty acid translocase (FAT) (Abumrad et al., 1993). The 472 amino acid protein (53 kDa) is a substantially glycosylated integral membrane protein of 88 kDa.

Using an expression cloning technique, Schaffer and Lodish (Schaffer and Lodish, 1994) identified a 63 kDa membrane protein in mouse adipocyte, later it was designated as fatty acid transport protein (FATP). It was shown to be integrally associated with the plasma membrane (Schaffer and Lodish, 1994; Stump et al., 1995). Transfected fibroblast cell lines expressing FATP exhibit a several-fold increase in [^{14}C]oleate uptake compared to control cells (Schaffer and Lodish, 1994). Evidence suggested that FATPs are membrane anchored proteins specific for LCFA since uptake of fatty acids shorter than 10 carbon chain length are unaffected by modulating FATP expression (Schaffer and Lodish, 1994). A hypothetical model of FATP mediated long chain fatty acid (LCFA)

uptake was proposed by Stahl (Stahl et al., 2001). Extracellular LCFAs may directly bind to FATP complexes and be transported into cells. Alternatively, LCFAs could bind firstly to CD36, which hands the LCFAs to FATP dimers. After entering the cells, intracellular LCFAs are coupled to coenzyme A by long-chain fatty acyl-CoA synthetase, preventing their efflux, while fatty acid binding proteins (FABPs) act as a cytoplasmic buffer for incorporated LCFAs and then deliver them to specific subcellular compartments undergoing different biological reactions.

3. Intracellular Fatty Acid Transport via Fatty Acid Binding Proteins (FABPs)

With the isolation of the first "fatty acid binding protein" (FABP) nearly 40 years ago (Ockner et al., 1972), a large variety of 14–16-kDa FABPs (127–137 residues) have been reported since then. They were found abundantly present in the cytoplasm of tissues and involved in cellular long chain fatty acid uptake, translocation, and metabolism (Table 5). These cytosolic proteins bind various amphiphilic molecules, such as long-chain fatty acids, bile acids and retinoids (Veerkamp and Maatman, 1995). As a result of their interaction with these essential lipids, the FABPs potentially have multiple tasks inside the cell. Aside from cellular uptake and transport of lipids, the FABPs also likely play a role in the regulation of lipid metabolism (Kaikaus et al., 1990; Wolfrum et al., 1999). The entire spectrum of possible functions of the different FABPs types is currently still being investigated by various biochemical, biophysical, and biological approaches (Noy, 2000; Storch and Thumser, 2000).

Despite the high degree of divergence of their amino acid sequences and binding properties, the overall protein fold has been conserved in all iLBP types. A representative

Table 5. Members of FABP Family

FABP type	Abbreviation	Tissue Occurrence	Liagnds
Liver FABP	L-FABP	Liver, intestine, kidney, lung, pancreas	FA, acyl-CoAs, heme, bilirubin, eicosandoids
Intestinal FABP	I-FABP	Intestine, stomach	FA
Heart FABP	H-FABP	Heart, kidney, skeletal muscle, aorta, adrenals, brain, testes, lung, stomach	FA
Adipocyte FABP	A-FABP	Adipose tissue	FA, retinoic acid
Epidermal FABP	E-FABP	Skin, brain, lens, capillary, endothelium, retina	FA, eicosanoids
Ileal FABP	IL-FABP	Intestine, ovary, adrenals, stomach	FA, bile acids
Brain FABP	B-FABP	Brain	FA
Myelin FABP	M-FABP	Peripheral nervous system	FA, retinoids
Testicular FABP	T-FABP	Testis	FA

This table is adapted from Wang, Guqi. 2004. Role of fatty acid binding protein in liver regeneration and cellular protection in Winnipeg, Manitoba. Ph.D. Thesis, University of Manitoba, pp2. FA, fatty acid.

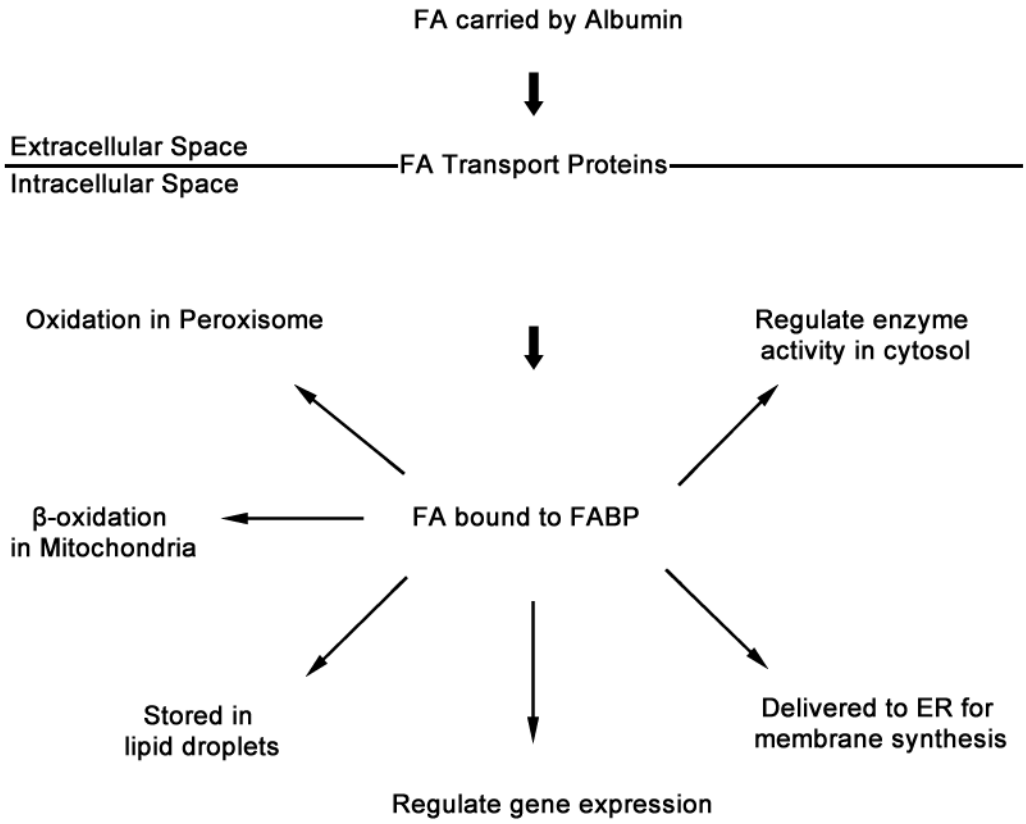
example of the typical FABP fold (i.e. H-FABP) is shown in Figure 6. The FABPs consist of 10 antiparallel β-strands to form a β-sheet structure that wraps around to create a β-barrel fold (Banaszak et al., 1994). Because of the clamshell appearance, this fold has also been dubbed "β-clam" (Sacchettini, 1988). The bottom of the clam is sealed off by a number of hydrophobic side-chains clustered around the N-terminus, whereas the mouth of the clam is almost completely covered by a helix-turn-helix domain consisting of two short α-helices. There is a so-called "gap" in the β-sheet between β-strands D and E, where no hydrogen-bonding network between the backbone atoms exists (Cowan et al., 1993; Sacchettini, 1988; Xu et al., 1993). This gap produces an opening in the protein surface, but one that is not large enough to serve as a ligand entrance. Instead, a region located between the turns βC-βD and βE-βF as well as a helix II has been postulated as the so-called "entry portal" for various ligand molecules (Sacchettini and Gordon, 1989).

All FABPs bind long chain fatty acids with differences in ligand selectivity, binding affinity and binding mechanism (Chmurzynska, 2006) as a result of small structural differences between isoforms. As lipid chaperones, FABPs may actively facilitate the transport of lipids to specific compartments in the cell, such as to the lipid droplet for storage; to the endoplasmic reticulum for signalling, trafficking and membrane synthesis; to mitochondria or peroxisome for oxidation; to cytosolic or other enzymes to regulate their activity; to the nucleus for lipid-mediated transcriptional regulation; or even outside the cell to signal in an autocrine or paracrine manner (Furuhashi and Hotamisligil, 2008) (Figure 7).

Figure 6. The common iLBP fold.

Ten antiparallel β-strands (from A to J) form a β-barrel cavity. Two α-helices (αI and αII) form a "cap" closing this structure. The internal cavity is the fatty acid binding site. Data is obtained from Protein Data Bank (ID: 1LFO) and made by PyMOL Molecule Graphics System.

Figure 7. Putative functions of FABP in the cell.



Fatty acid (FA) trafficking accompanied by the fatty acid-binding proteins (FABPs) in the cell is shown. As lipid chaperones, FABPs have been proposed to play a role in the transport of lipids to specific compartments in the cell. ER, endoplasmic reticulum.

C. Liver Fatty Acid Binding Protein

1. Introduction

Liver type FABP was first discovered in 1969 when Levi and his co-workers studied the binding of bilirubin and bromosulfophthalein to rat liver cytosol (Levi et al., 1969). Two fractions involved in binding to these ligands were found and one of them had a molecular mass of 10-20 kDa, termed as Z protein, which is the original name of FABP. During the 1970s, several experiments were performed in different laboratories to investigate the distribution of Z protein in various tissues (Mishkin et al., 1972; Ockner et al., 1972; Ketterer et al., 1976). Z protein, which binds long chain fatty acids, and with a molecular weight of approximately 15 kDa was identified in the cytosol of intestinal, liver, kidney and other tissues, was eventually termed liver fatty acid binding protein or L-FABP for short.

2. Gene, Expression, and Three Dimensional Structure of L-FABP

Analysis of the amino acid sequences of human L-FABP showed that it was comprised of 127 amino acid residues. Further statistical analysis of the amino acid located at the centromeric p12-q11 region of chromosome 2 (Chen et al., 1986). The sequences of human and rat L-FABPs revealed significant sequence homology (Chan et al., 1985). One year later, chromosomal localization of human L-FABP was peroxisome proliferator response element (PPRE) is important for the regulation of L-FABP (Veerkamp and Maatman, 1995; Storch and Corsico, 2008). This PPRE contains an imperfect direct repeat sequence that binds and is activated by peroxisome proliferator-activated receptor-α (PPAR-α) (Schachtrup et al., 2004). Intervention through

hypercholesterolaemic and hyperlipidaemic drugs like statins and fibrates can up-regulate its expression in the liver (Landrier et al., 2004; Rakhshandehroo et al., 2010).

Cellular intestine and liver concentrations of L-FABP are roughly 2-5% of the cytoplasmic protein. In addition to liver and intestine, L-FABP may also be found, to a lesser extent, in kidney and colon. All are tissues with highly active fatty acid metabolism. L-FABP is found abundantly in the cytoplasm and nucleus of hepatocytes (Bordewick et al., 1989; Fahimi et al., 1990). Hepatocyte concentration of L-FABP can reach up to 0.4mM (Burnett et al., 1979).

The full length cDNA of human L-FABP was isolated and determined in 1985 by Lowe et al. (Lowe et al., 1985), which was deposited in GeneBank (code: M10050.1; Table 6). Lowe's study was confirmed by Thompson et al. (Thompson et al., 1999). They compared the sequences of L-FABPs from various species and determined L-FABPs' tertiary structure. It is composed by two α-helices in helix-turn-helix of about 17 residues, forming a "lid" for the binding cavity. The rest of ten anti-parallel β-strands form a barrel-like binding pocket. The volume of the L-FABP ligand binding pocket is the largest of any of the intracellular FABP family (Thompson et al., 1997). L-FABP is unique among the FABP family in its ability to bind more than one LCFA and in the size of ligand that can accommodate. It contains a high affinity site and a low affinity site (Richieri et al., 1994; Norris and Spector, 2002). In the higher affinity site, the LCFA is oriented with carboxyl buried deep in the interior, while in the weaker affinity site an LCFA is oriented with carboxyl facing the surface of the protein binding pocket opening (He et al., 2007). Occupancy of the lower affinity LCFA binding site may depend upon prior binding of an LCFA to the higher affinity site. L-FABP's binding cavity can bind

Table 6. Human L-FABP mRNA

0-30	**M**SFSGKYQLQ SQENFEAF**M**K AIGLPEELIQ
31-60	KGKDIKGVSE IVQNGKHFKF TITAGSKVIQ
61-90	NEFTVGEE**C**E LET**M**TGEKVK TVVQLEGDNK
91-120	LVTAFKNIKS VTELNGDIIT NT**M**TLGDIVF
121-127	KRISKRI

Human L-FABP mRNA is composed of 127 amino acids, with one cysteine residue at position 69, and four methionine residues at position 1, 19, 74, and 113, respectively. All methionine and cysteine residues are bolded in this table. Taken from GeneBank (M10050.1).

up to two LCFAs (Frolov et al., 1997) as seen in Figure 8.

3. L-FABP Biological Functions

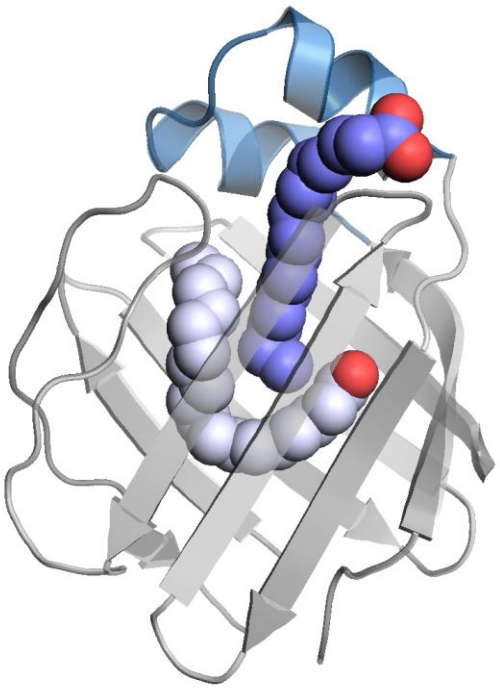
3.1. L-FABP – Intracellular Transporter

A well studied aspect of L-FABP's biological function is it's function as an intracellular transporter, carrying LFCA and its oxidation products (Kansau et al., 1996; Christian et al., 2004), bile acids (Barton et al., 1996), eicosanoids (Christian et al., 2004), and hypolipidemic drugs (Li et al., 2005), due to their high binding affinity. The primary function of L-FABP is thought to be the intracellular translocation of long chain fatty acids and their metabolites, making L-FABP able to control the cellular handling of amphiphilic compounds carefully, to allow their restricted use in the cell, while keeping them from damaging intracellular organelles, at least under normal circumstances. The abundance of L-FABP would thus provide a large buffering capacity, preventing large fluctuations in non-protein-bound fatty acid concentrations.

3.2. L-FABP – Novel Endogenous Hepatic Antioxidant

Several studies have shown that, L-FABP is an effective endogenous hepatic antioxidant (Wang et al., 2005; Rajaraman et al., 2007; Yan et al., 2009). After constructing a mammalian expression plasmid containing the human L-FABP gene sequence, these plasmids were transfected into Chang cells, although derived from human liver tissue but devoid of L-FABP expression (Wang et al., 2005). Following transfection, *in vivo* oxidative stress was induced by both hydrogen peroxide and hypoxia/reoxygenation. This procedure increased intracellular ROS content as well as inducing cellular apoptosis. L-FABP reduced caspase-1 activity in these cells, suggesting

Figure 8. Rat L-FABP bound to two oleates.



Two α-helices of rat L-FABP are shown in blue while the other ten anti-parallel β-strands are in grey. Rat L-FABP can bind up to two FA in its binding cavity. The first one shown as white is completely buried within the confines of the cavity while the blue is the second FA. Both of the FA carboxyl groups are shown in red. Data is obtained from Protein Data Bank (ID: 1LFO) and made by PyMOL Molecule Graphics System.

that L-FABP may provide a protective function against ROS induced apoptosis (Wang et al., 2005). Also, L-FABP cDNA transfected cells were associated with reduced levels of ROS products. This important study showed some promising preliminary results of L-FABP acting as a cytoprotectant in an oxidative stress cell model. To link the study to a pharmacological mechanism, a rat hepatoma cell model was utilized (Rajaraman et al., 2007). After treatment of cells with dexamethasone and clofibrate, *in vitro* oxidative stress was induced by hydrogen peroxide. The intracellular L-FABP content was found to be increased almost 3 fold in the clofibrate treated group with lowered levels of free radicals compared to controls, while the dexamethasone treated group was observed to express a much lower amount of L-FABP. Dexamethasone treatment also resulted in a significant increase in oxidative products with higher lactate dehydrogenase (LDH) release. Along with the other unchanged *in vitro* antioxidative molecular levels and enzyme activities such as glutathione peroxidase, superoxide dismutase and catalase, this study revealed one of the clofibrate hepatoprotective actions could be through increased cytosolic L-FABP.

The exact mechanism of L-FABP's cytoprotective function was later investigated. In rat L-FABP amino acid sequence, there are seven methionine (Met) groups located at the positions 1, 19, 22, 74, 85, 91 and 113 (Figure 9). A hypothesis that L-FABP could directly participate in antioxidative function through its cysteine and methionine groups was proposed because methionine and cysteine residues are prone to oxidation in proteins (Stadtman et al., 2003). As proposed, L-FABP's *in vitro* antioxidative function was shown to be associated with its Met residues (Yan et al., 2009). After *in vitro* oxidative stress induction using AAPH and AMVN (Figure 10), a hydrophilic and hydrophobic

free radical generator, respectively, Yan and colleagues found that a mass shift of about 80Da in the AAPH treated L-FABP while AMVN treatment gave a mass shift of 16Da (Figure 11) using MALDI-TOF mass spectrometry analysis. This indicated that five of the seven methionine residues were oxidized when free radicals were generated from a hydrophilic environment while at least one of them was oxidized when the hydrophobic free radicals formed. Further data showed that Met^{1}, Met^{22}, Met^{113} were oxidized by the lipophilic free radicals at different degree. In L-FABP, Met^{1} and Met^{113} were the most susceptible residues to free radicals, contributing to its scavenging property to inactivate hydrophilic and hydrophobic free radicals (Figure 12). This *in vitro* study gave an insight upon L-FABP's cellular antioxidative mechanism. As the oxidation of methionine residues in proteins can be repaired by methionine sulfoxide reductases (Msrs) *in vivo* (Moskovitz et al., 2000), the cyclic redox processes of L-FABP's Met residues during the free radical inactivation process could contribute to L-FABP's antioxidant activity.

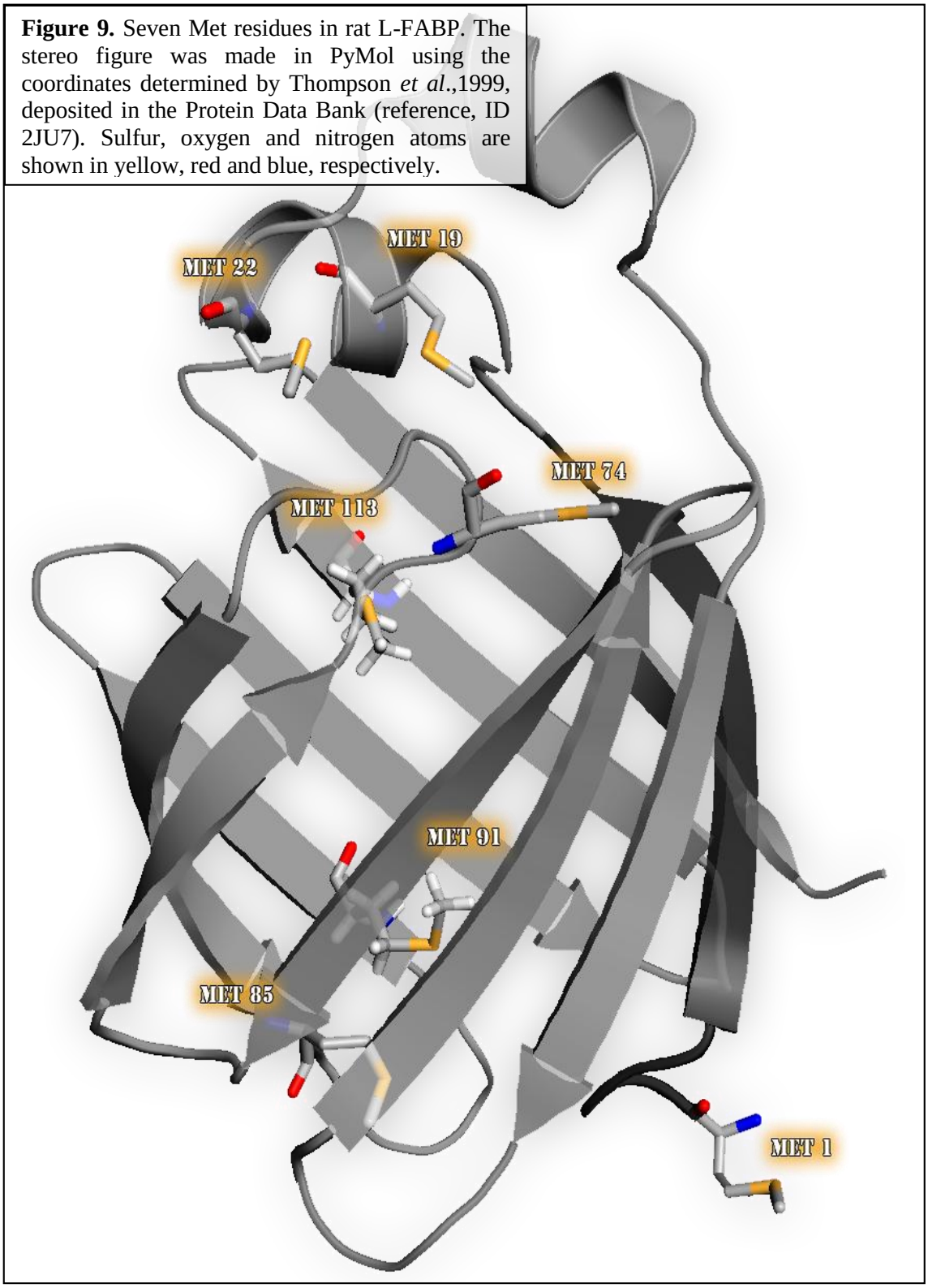
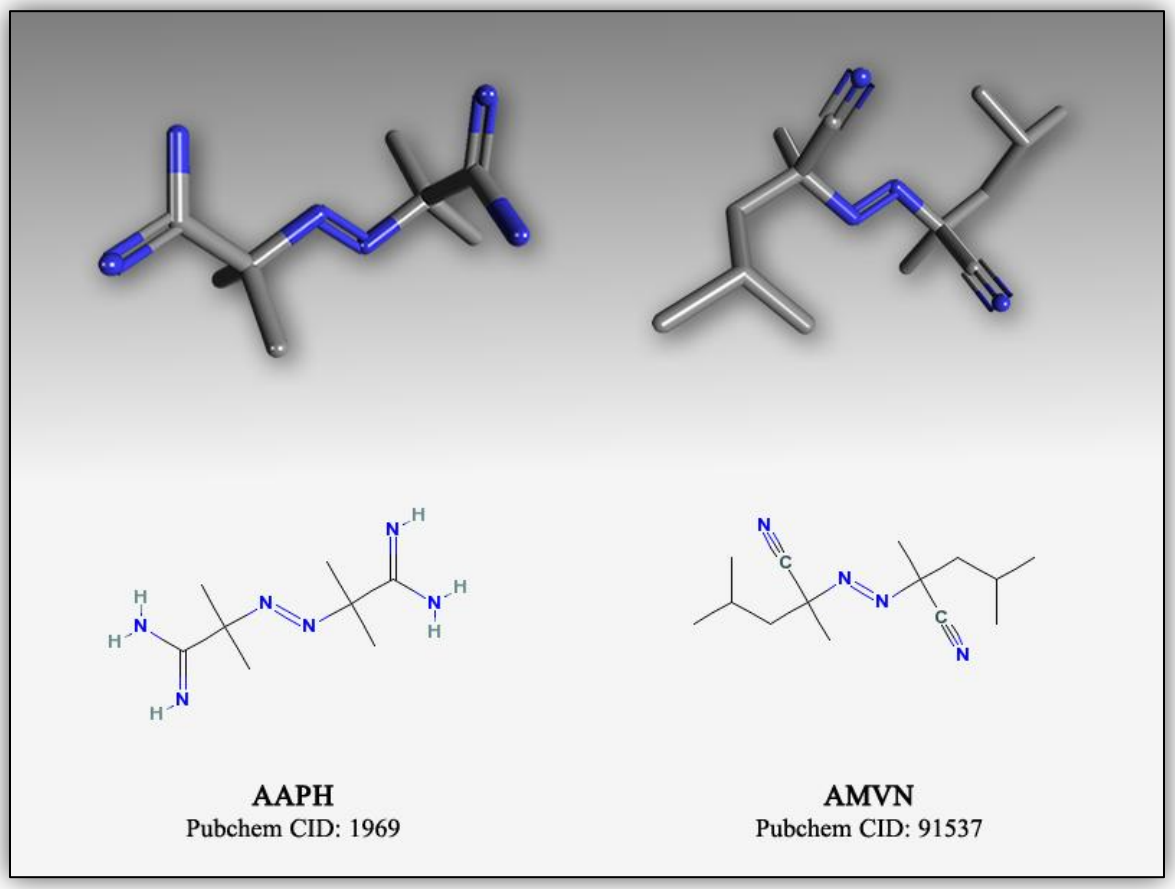


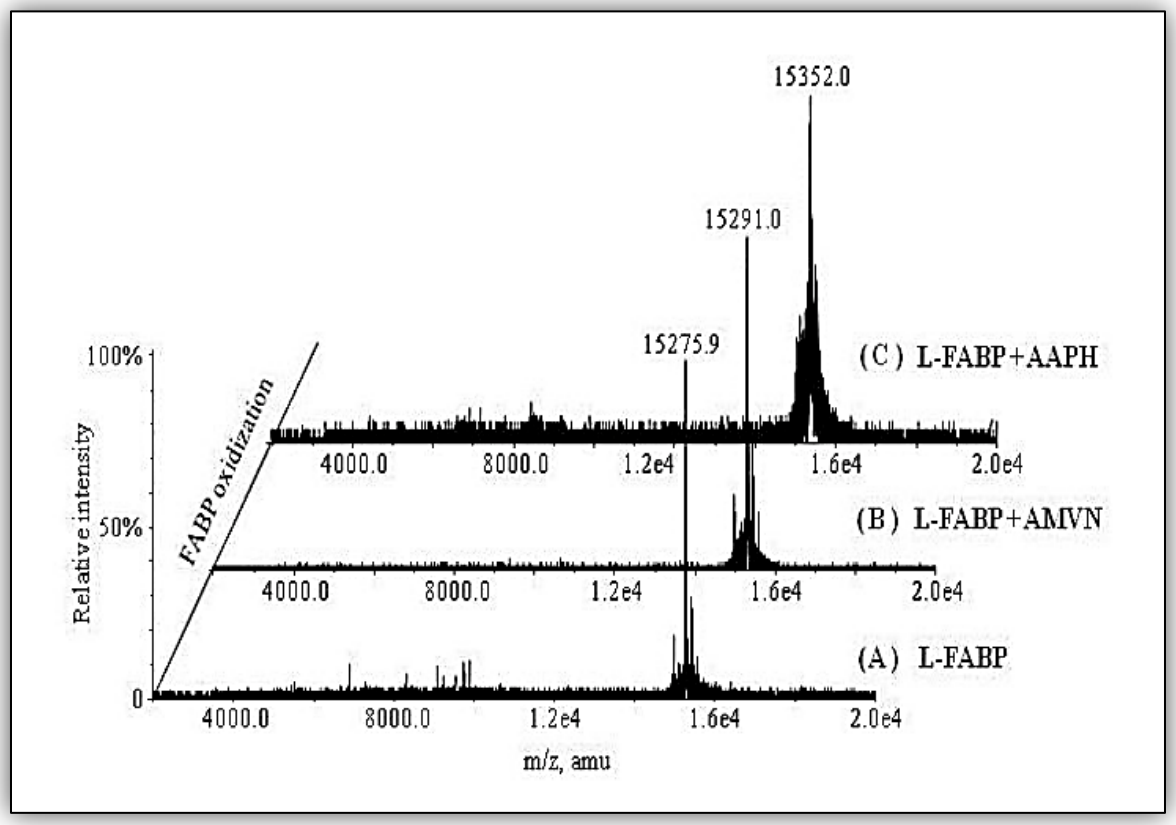
Figure 9. Seven Met residues in rat L-FABP. The stereo figure was made in PyMol using the coordinates determined by Thompson *et al*.,1999, deposited in the Protein Data Bank (reference, ID 2JU7). Sulfur, oxygen and nitrogen atoms are shown in yellow, red and blue, respectively.

Figure 10. Structures of AAPH and AMVN.



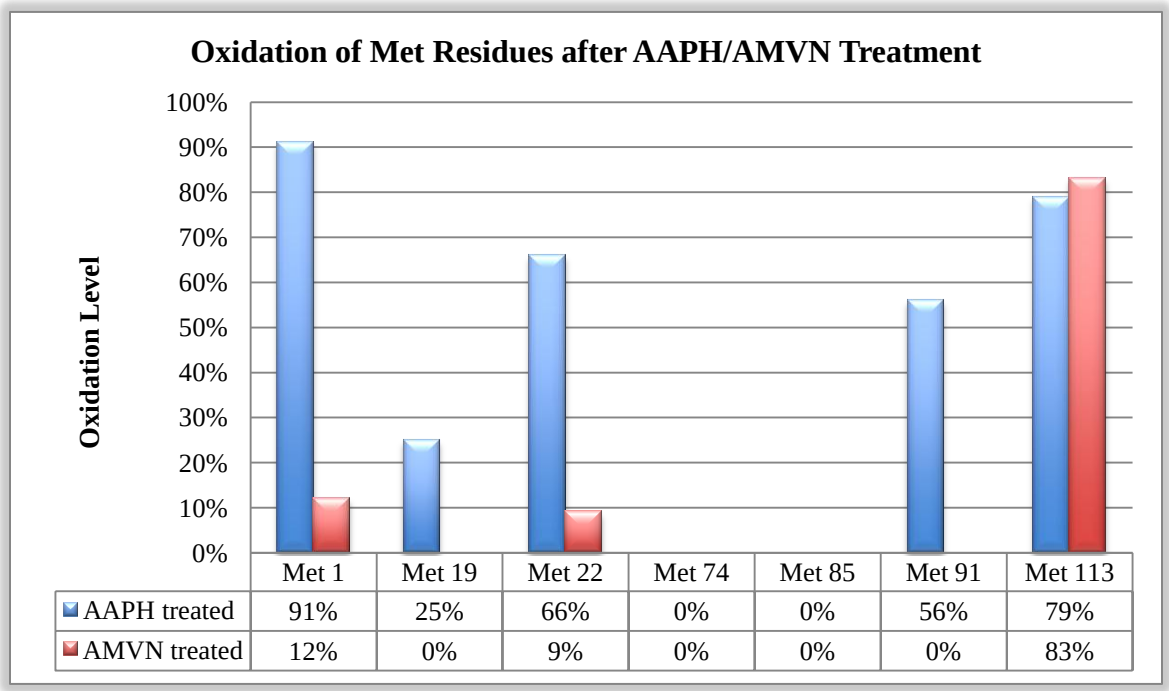
AAPH is a water-soluble azo compound, which is used extensively as a free radical generator, often in the study of lipid peroxidation and the characterization of antioxidants. AMVN is a synthetic azo compound that dissociates spontaneously to form carbon-centered free radicals in lipid environments. AMVN is commonly used as a free radical initiator in studies of tocopherol trapping, antioxidant status, and lipid peroxidation. Structures obtained from PubChem.

Figure 11. MALDI-TOF MS spectrum of recombinant L-FABP and L-FABP incubated with AAPH or AMVN.



L-FABP was purified by column chromatography and subjected to MALDI-TOF MS analysis. (A) the high intensity peak at m/z 15,275.9 corresponded to the molecular mass of purified recombinant L-FABP, non-oxidized state; (B) the high intensity peak at m/z 15,291.0 corresponded to the molecular mass of the recombinant L-FABP incubated with AMVN, a mass shift of 16Da comparing to (A); (C) the high intensity peak at m/z 15,352.0 corresponded to the molecular mass of the recombinant L-FABP incubated with AAPH, a mass shift of about 80Da comparing to (A). This figure was originally published in Journal of Lipid Research. Yan et al. Molecular mechanism of recombinant liver fatty acid binding protein's antioxidant activity. *Journal of Lipid Research*. 2009; Vol 50(12):2445-2454. © the American Society for Biochemistry and Molecular Biology.

Figure 12. Oxidation of Met Residues after AAPH/AMVN Treatment.



After AAPH/AMVN treatment, the oxidized L-FABP was subjected into MALDI-TOF Mass Spectrometry to identify the oxidation of Met residues. In both groups, Met1 and Met113 are the most susceptible residues to free radicals, while Met74 and Met85 could not react with free radicals generated both from hydrophilic and lipophilic generator. Met113 is the most effective endogenous free radical scavenging residues compared to other Met residues. Furthermore, this study showed that L-FABP has a better free radical scavenging property in the hydrophilic environment. This figure is reproduced from the research originally published work in Journal of Lipid Research by Yan et al. Molecular mechanism of recombinant liver fatty acid binding protein's antioxidant activity. *Journal of Lipid Research*. 2009; Vol 50(12): 2445-2454. © the American Society for Biochemistry and Molecular Biology.

II. Hypothesis and Objectives

Hypothesis

Reports indicating that modulating L-FABP expression influences the cellular antioxidant defence system *in vivo* (Wang, Gong et al. 2005; Rajaraman, Wang et al. 2007; Wang, Shen et al. 2007) and the possible mechanism for this function is the cyclic redox reaction with intracellular reactive species via its methionine groups. Moreover, the intracellular L-FABP level can be easily increased using clofibrate in 1548 rat hepatoma cell line (Rajaraman et al., 2007), it is logical to investigate whether L-FABP can be utilized as an endogenous antioxidant in NAFLD. Thus, the hypothesis to be tested in this study is that L-FABP can protect hepatocytes against the free radicals generated in a NAFLD cell culture model.

Objective

The principal objectives of this work were to: 1) establish and evaluate an *in vitro* NAFLD cell culture model; and 2) evaluate the cytoprotective effects of pharmacological modulation of L-FABP level via clofibrate on hepatocellular antioxidant status in this model.

III. Materials and Methods

A. Materials

Unless specified all chemicals were purchased from Sigma-Aldrich and were of analytical grade.

1. 1548 rat hepatoma cell line

L-FABP expressing CRL-1548 hepatoma cell line (derived from epithelial liver hepatoma of *rattus norvegicus*) was purchased from the American Type Culture Collection (ATCC, Rockville, Maryland, USA).

2. Chemicals

HyClone Classical liquid media Minimum Essential Medium with Earle's Balanced Salts (MEM/EBSS) was purchased from Thermo Fisher Scientific (Nepean, Ontario, Canada). Fetal bovine serum, oleate, palmitate, fatty-acid-free bovine serum albumin, sodium pyruvate, penicillin, streptomycin, Nile Red, 2,7-dichlorofluorescein diacetate (H_2DCFDA), 4',6-Diamidino-2-phenylindole dihydrochloride (DAPI), poly-L-lysine hydrochloride, trypsin-EDTA, L-glutamine and monoclonal anti-β-actin antibody (CAT#: A2228, produced in mouse) were purchased from Sigma-Aldrich (Oakville, Ontario, Canada). Cell proliferation reagent WST-1 was purchased from Roche (Roche Diagonstics GmbH, Mannheim, Germany). Anti-mouse lgG, horseradish peroxidase linked whole antibody (CAT#: NA931, produced in sheep) and anti-rabbit lgG horseradish peroxidise linked whole antibody (CAT#: RPN4301, produced in goat) was purchased from GE Healthcare (GE Healthcare, Pittsburgh, USA). Rainbow marker was purchased from Invitrogen (Invitrogen Canada Inc, Burlington, Canada). L-FABP

polyclonal antibody was generated in our laboratory by Dr. Guqi Wang (University of Manitoba, Canada).

B. Methods

1. Cell Culture and FA/Clofibrate Treatment

Rat hepatoma cell line 1548 were grown in MEM/EBSS supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin, 10% fetal bovine serum, 4 mM L-glutamine and 0.011% sodium pyruvate in a humidified, 37ºC incubator in an atmosphere of 95% air and 5% CO2. Cells were maintained in 75 cm^2 culture flask (Corning, Cat#: 430725).

Cells were seeded at a density of $2.5x10^4$ cells/well for WST-1, Nile Red and DCF assay, 96-well plates, and 1×10^5 cells/well for Nile Red Stating, 12-well plates in MEM/EBSS medium and incubated overnight for adherence. The following day, cell culture media was replaced with freshly prepared medium containing oleate and palmitate mixture (2:1) in presence of 3% fatty-acid-free bovine serum albumin. Cells were treated with 0, 0.5, 1, 2, and 3 mM of fatty acid (FA) mixture for 1 and 2 days at 37°C in a humidified incubator in an atmosphere of 95% air and 5% CO_2. Treatment also included clofibrate to increase L-FABP levels (Wang et al., 2007). Clofibrate (500 µM) was dissolved in dimethyl sulfoxide (DMSO) and later added to the medium (DMSO < 0.1% v/v in final volume). Control cells were incubated with DMSO alone. For drug treatment groups, cells were either treated with various concentrations of FA for 2 days followed by a 2-day treatment of clofibrate without FA, or treated with FA for 2 days but on the second day clofibrate intervention was introduced and the third day cells were treated only with clofibrate without FA (Table 7). Previous studies indicated clofibrate

Table 7. FA/Clofibrate Treatments

	Day 1	Day 2	Day 3	Day 4
Treatment #1	FA	FA		
Treatment #2	FA	FA		
Treatment #3	FA	FA	CLO	CLO
Treatment #4	FA	FA+CLO	CLO	

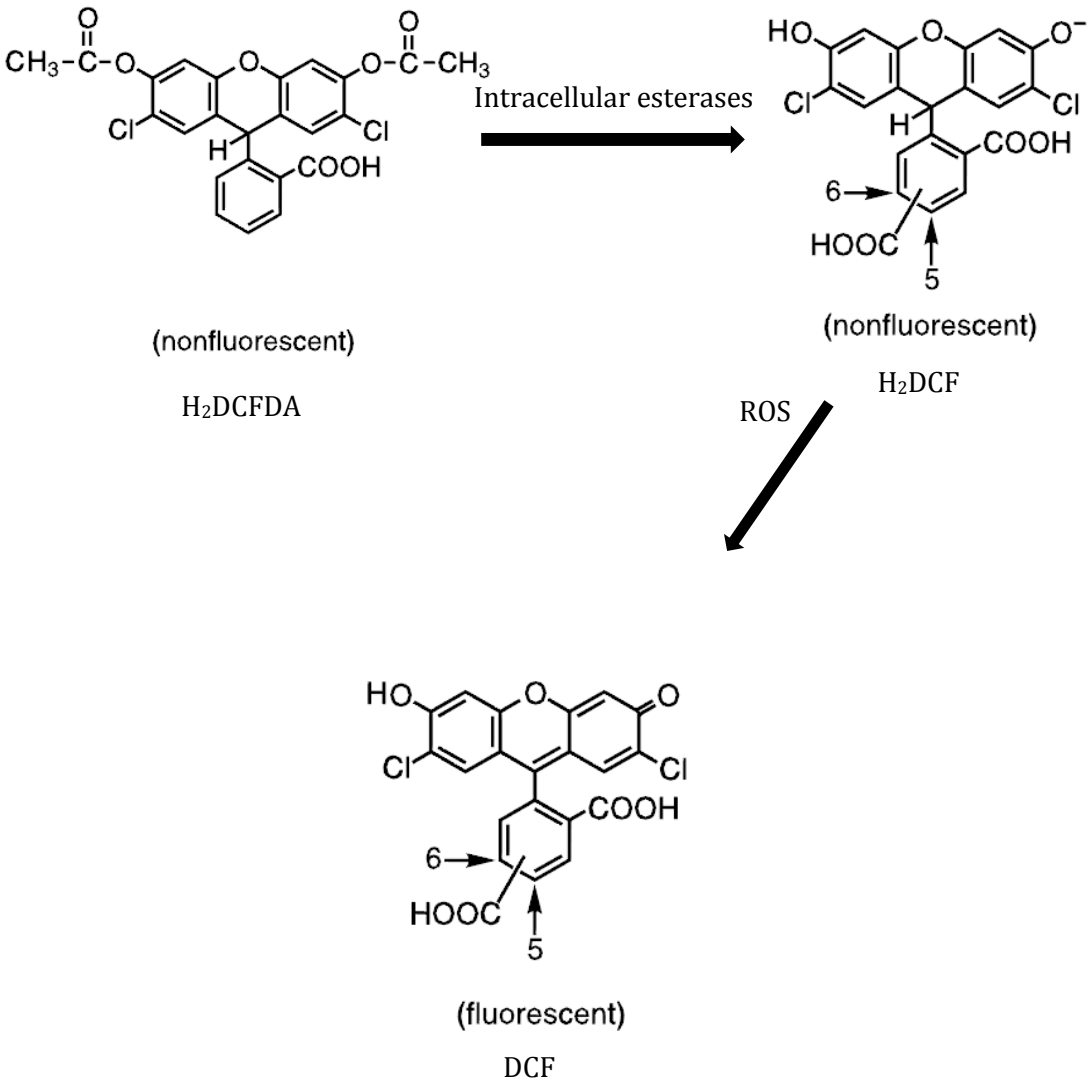
Cells were treated with FA (0, 0.5, 1, 2 mM of palmitate and oleate mixture) for 1 or 2 days. 2-day clofibrate intervention was introduced either on day 2 or day 3.

was able to induce significantly increased L-FABP expression after two-day treatment via stabilization of L-FABP mRNA (Yan et al., 2010). L-FABP is also known to be an efficient antioxidant to scavenge intracellular free radicals (Wang et al., 2005; Rajaraman et al., 2007). In current study, clofibrate treatment was employed to induce intracellular L-FABP level after the induction of ROS production through cellular steatosis. At the end of the incubation period, cells were prepared for the various assays described below.

2. DCF Fuorescence Assay

This assay (Figure 13) is based on the principle that H_2DCFDA, a non-polar compound, readily diffuses into cells where it is hydrolyzed by intracellular esterases to the non-fluorescent derivative 2,7-dihydrodichlorofluorescin (H2DCF), which is polar and trapped within the cells. In the presence of intracellular reactive oxygen species, this compound is oxidized to the highly fluorescent compound 2,7-dichlorofluorescein (DCF). Fluorescence intensity of DCF from reactive oxygen species (ROS) formed in cell cultures was assessed using a Wallac 1420 multilable counter (Perkin Elmer). A 10 mM (4.87 mg/ml) stock solution of 2,7-dichlorofluorescein diacetate (H_2DCFDA) was prepared freshly in ethanol and stored at – 20°C until required and finally diluted to 100 μM with the medium without FBS prior to each study. Following incubation with fatty acids, cells were washed twice with warm FBS-free medium to remove the extracellular albumin and incubated with 100 μM 2,7-dichlorofluorescin diacetate ($DCFH_2$-DA) at 37°C in the dark for 30 min. After washing with warm PBS once, the DCF fluorescent intensity was measured in fluorescence plate reader with the excitation at 485 nm and the emission at 535 nm. Total protein in each well was measured according to the Bradford

Figure 13. The molecular structures of 2,7-dihydrodichlorofluorescin-diacetate (H_2DCFDA) and its subsequent reaction products involved in the fluorescence assay.



assay (Bradford, 1976). DCF intensity in each well was normalized by its total protein content (μg).

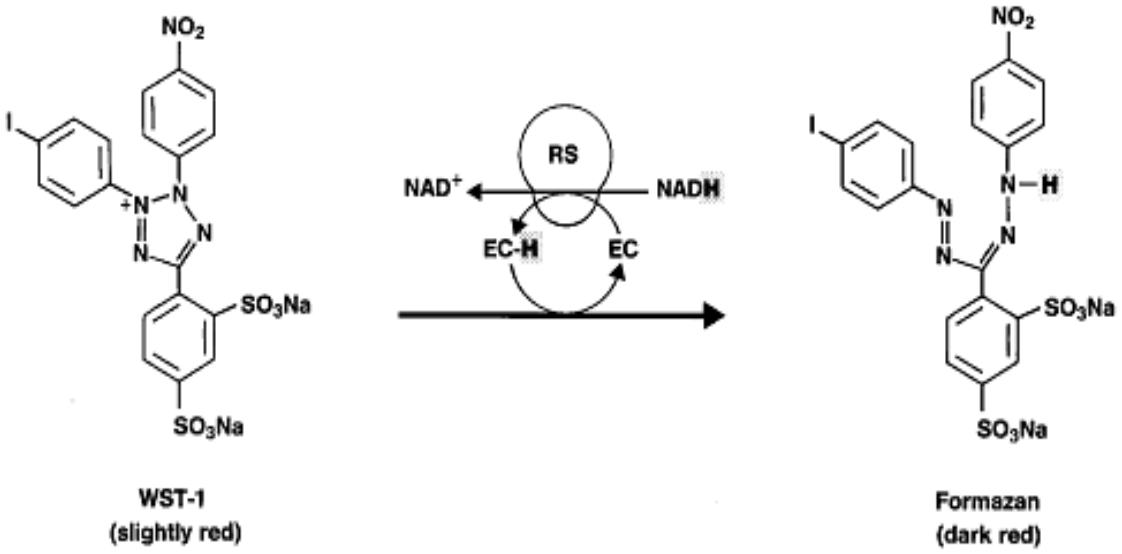
3. WST-1 Cell Proliferation Assay

The cell proliferation reagent WST-1 is based on the metabolic activity of viable cells (Figure 14). Metabolically active viable cells reduce tetrazolium salts to formazan compounds as shown in Figure 14, while non-viable cells do not. WST-1 (10 μl/well) was added to cells that had already been treated with the fatty acid mixture (0, 0.5, 1, and 2 mM) for 5 days, followed by 1 or 2 hr incubation with WST-1 reagent according to manufacturer's suggestion to acquire the highest sensitivity. Cell viability was measured using the ELx 808 Ultra Microplate Reader (BIO-TEK Instrument. INC.) at 450 nm.

4. Intracellular Lipid Droplets Observation

Nile Red is a fluorescent dye that stains lipid droplets by dissolution into the droplets (Greenspan et al., 1985); while DAPI is a commonly used dye for cell nucleus stain. After 2-day fatty acid treatment, cells were visualized for intracellular lipid droplets under optical microscopy (Nikon). Nile Red and DAPI fluorescent staining was introduced to further assess the intracellular accumulation of lipid droplets. One cover slip (Fisher, Cat# 12-545-81) was put into each well of the 12-well plate followed by immersion in 70% alcohol for 10 min. After vacuum and evaporation of the alcohol, sterile 0.2 mg/ml Poly-L-Lysine was used to coat the cover slips for cells to attach. Then cells were seeded at a density of 1×10^5 cells/well and incubated at 37°C in a humidified incubator overnight. The next day cell cultures were replaced with the medium containing the fatty acids. After treatment for 48 hr, cells were twice rinsed with warm

Figure 14. Cleavage of the tetrazolium salt WST-1 (4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) to formazan.



This figure is adapted from Roche Diagonstics GmbH, Mannheim, Germany. (www.roche-applied-science.com/pack-insert/1644807a.pdf; Accessed: July 19, 2011). EC: electron coupling reagent; RS: mitochondrial succinate-tetrazolium-reductanse system.

PBS and fixed by 4% formaldehyde in PBS at room temperature for 30 min. After rinsing cells were stained with 1 µg/ml Nile Red at room temperature for 15 min in the dark. After a further rinse with warm PBS twice, 5 µg/ml DAPI was added into each well and further incubated for 5 min. After the final rinse, cover slips were mounted onto glass slides with 20 µl self-made anti-fade mounting medium (0.1% P-phenylenediamine and 90% glycerol in PBS, adjusted to pH 8.0-9.0 with 0.2 M sodium bicarbonate buffer, pH 9.2) and observed under a fluorescent microscope.

5. Intracellular Lipid Droplets Assessment

Although it was reported that nile red can fluoresces in the presence of phospholipids, cholesterol, cholesteryl esters, and triacylglycerols, the fluorescence of nile red interacting with lipid droplets comprising neutral lipids (mainly triacylglycerols and sterol esters) is selectively detected when examination of the fluorescence of nile red stained cells at wavelengths below 570 nm (Greenspan et al., 1985). Following fatty acid treatment, cells were rinsed with 200 µl warm PBS in each well in the 96-well plate. Two micrograms per millilitre Nile Red and 1 µg/ml DAPI were prepared in PBS to co-stain the cells. Cells in each well were stained with 100 µl co-stain solution at 37°C for 10 min, protected from light. After the single wash with warm PBS once, an additional 200 µl PBS was loaded onto each well. Fluorescent analysis was then performed using a Wallac 1420 multilable counter (Perkin Elmer) at excitation 485 nm and emission 535 nm for Nile Red while excitation at 355 nm and emission at 460 nm for DAPI. Nile Red intensity in each well was normalized by its DAPI signal.

6. BCA Protein Assay

The BCA Protein Assay is a detergent-compatible formulation based on bicinchonimic acid (BCA) for the colorimetric detection and quantization of total protein. The procedure relies on the well-known reduction of Cu^{+2} to Cu^{+1} by proteins in an alkaline medium (the Biuret reaction). The highly sensitive and selective colorimetric detection of the cuprous caption (Cu^{+1}) uses a unique reagent containing bicinchoninic acid. The blue-colored reaction product of this assay is formed by the chelation of two BCA molecules with one cuprous ion. The water-soluble complex exhibits a strong absorbance at 562 nm that is linear with increasing protein concentrations over a 20-2,000 µg/ml range. The BCA method is not a true end-point method; that is, the final color continues to develop. However, following incubation the rate of continued color development is sufficiently slow to allow large numbers of samples to be assayed together.

BCA Protein Assay stock solutions include: Reagent A (containing sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate in 0.1 M sodium hydroxide), Reagent B (containing 4% cupric sulfate), albumin standard ampoules, 2mg/ml (containing bovine serum albumin (BSA) at 2.0 mg/ml in 0.9% saline and 0.05% sodium azide). BCA Protein Assay solution was made by mixing stock solution A and B in a ratio of 50:1 on a daily basis. Dilutions were made of the fractions and a series of dilutions of a BSA solution. Each dilution was assayed with the BCA reagents. Each standard and sample (10 µl) was pipetted into the appropriate microwell plate, which contained 200 µl/well of BCA working reagent (A: B= 50:1). Plates were mixed on a shaker for 30 seconds before incubating the covered plate at 37°C for 30 min. After incubation, absorbance of each well was measured on an ultramicroplate reader (ELx 808,

Bio-TEK instruments, INC, Winooski, USA) at 595 nm. Finally, from the BSA standard dilutions, a standard curve relating absorbance to protein concentration was prepared. All determinations were done in triplicate.

7. Western Blot Analyses

Control and treated cells were grown to confluence in 75 cm^2 culture flasks following which the cells were washed twice by ice-cold PBS, scrapped, harvested, and centrifuged. The resulting cell pellet was then suspended in 500 µl PBS containing 1mM PMSF, 5 µg/ml aprotinin, 10 µg/ml leupeptin, pH 7.4, 4ºC in 1.5 ml Fisher microcentrifuge tubes and lysed using a Fisher Sonic Dismembrator (Model 300, FisherScientific, USA). After sonication, samples were centrifuged at 13,000 x g (µSpeedFuge, SFR 13K, Savant) for 20 min at 4ºC. Protein concentration of the supernatant was determined by the BCA protein assay.

7.1. Sample Preparation

A 60 µg equivalent volume of cellular proteins (≤ 30 µL) was added to 10 µL sample loading buffer (0.0625 M Tris-HCl, 10% glycerol, 2% SDS, 0.1% bromophenol blue, 5% β-mercaptoethanol, pH 6.8) and boiled in 1.5 ml Fisher microcentrifuge tubes for exactly 5 minutes at 100^{o}C. Samples were then allowed to cool to room temperature.

7.2. Preparation of Gel

The separating gel (12%) and the stacking gel (5%) solutions were prepared by mixing the ingredients listed below in Table 8 (for two gels using 1 mm spacer). The electrophoretic apparatus was assembled using short glass plates and 1.5 mm spacers (Bio-Rad, CA, USA). The separating gel was allowed to set for 45 min using distilled

Table 8. Formula for Electrophoretic Gel Preparation

Ingredients	**12 % Separating Gel**	**5 % Stacking Gel**
Distilled H_2O	3.3 mL	3.4 mL
Tris HCl (1.5 M or 1 M)	2.5 mL (1.5 M)	0.63 mL (1 M)
10 % w/v SDS	100 μL	50 μL
Acrylamide mixture (30%)	4 mL	0.83 mL
10 % $(NH_4)S_2O_8$	100 μL	50 μL
TEMED	4 μL	5 μL

H_2O as a sealant following which it was discarded by inverting the apparatus. The stacking gel solution was then poured over the separating gel and a plastic comb was inserted. After 30 min, the comb was pulled out and the gel was immersed in the Electrode Running Buffer.

7.3. Sample Loading and Electrophoresis

Samples were vortexed to ensure homogeneity and centrifuged for 30 seconds. Using a 100 μL Eppendorf pipette, 20-30 μl (adjusted for protein content) of the samples were loaded into the wells created by the comb. Electrophoresis was carried out at a constant voltage 90 Volts for 90 minutes using a Bio-Rad Miniprotean II cell powered by Bio-Rad Powerpac 1000 (Bio-Rad, CA, USA). At the end of the procedure, the clearly demarcated stacking gel component was cut off using a sharp lancet and the separating gel was equilibrated in the transfer buffer for 30 min.

7.4. Membrane Transfer and Blocking

The Millipore Imunogoblin-P PVDF membranes were activated in pure methanol for 30 seconds, followed by 2 minutes wash with Millipore pure water. Membranes, sponges and filter papers were then soaked in the Transfer Buffer for 1 hr prior to transfer blotting. Transfer sandwich was assembled in the following order: Sponge – Filter paper – Gel – Membrane – Filter paper – Sponge. Transfer was performed at 100 Volts for 1 hr using a Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad, CA, USA). Thereafter, membrane was blocked with 5% powdered skimmed milk in TBS for 1 hr at room temperature.

7.5. Antibody Detection

The membrane was incubated with either the polyclonal antisera raised against L-FABP (used at 1:200 in 5% powdered skimmed milk in TBS) (Wang et al., 2004) or a monoclonal mouse anti-β-actin antibody (used at 1:2000 in 5% powedered skimmed milk in TBS) at 4^{o}C overnight. The membrane was subsequently incubated with either a peroxidase-conjugated anti-rabbit antibody for L-FABP (used at 1:1000 in 2% powdered skimmed milk in TBST) or a horseradish peroxidase linked anti-mouse lgG antibody for β-actin (used at 1:1000 in 2% powdered skimmed milk in TBST) for 1 hr at room temperature. After the secondary antibody incubation, membranes were placed in a high salt buffer (TBS buffer supplemented with 0.5M NaCl and 0.2% SDS) for decreasing the background according to Millipore's suggestion and incubated for another 30 minutes with gentle shaking. Finally, after rinsing with Mili-Q water, the antigen-antibody complex was detected by enhanced chemiluminescence (ECL Plus system, GE Healthcare, USA). The densitometry of the bands was performed using ImageJ (Frederick, USA).

8. Statistical Analyses

Data are presented as mean ± SEM. The n value refers to number of replicates performed for each study. Statistical analyses were carried out by *t* test (unpaired) where 2 groups were compared while a one-way ANOVA was used for multiple comparisons followed by a Bonferroni post-test using GraphPad Prism 5.0.

IV. RESULTS

1. Cell Morphology

Intracellular lipid accumulation was detected by both microscopy (Figure 15) and Nile Red staining (Figure 16) after 2-day incubation with media containing the FA mixture. As FA concentration increased, intracellular lipid droplets increased in a dose-dependent manner. Both density and diameter of the cytoplasmic lipid droplets increased when cells were exposed to the high fat environment.

2. Intracellular Neutral Lipid Accumulation Assessment

Similar to the cell morphology observation, the intracellular neutral lipid content in different concentrations of FA-treated groups showed dose-dependent lipid accumulation (Figure 17). FA-treated groups showed a 226%, 387%, and 808% of lipid accumulation comparing to the control group when incubated with 0.5, 1, and 2 mM FA ($p < 0.001$).

3. Cell Viability

Lipotoxicity induced by FA in the rat hepatoma cells using the cell proliferation reagent WST-1 is shown in Figure 18. FA induced a dose-dependent lipotoxicity in 1548 cells. At 3 mM fatty acid mixture, the lipid load was sufficient to damage hepatocytes resulting in cells detaching from the culture plates (data not shown). On the first treatment day cell viability in the 2 mM FA group was greatly reduced. Almost half

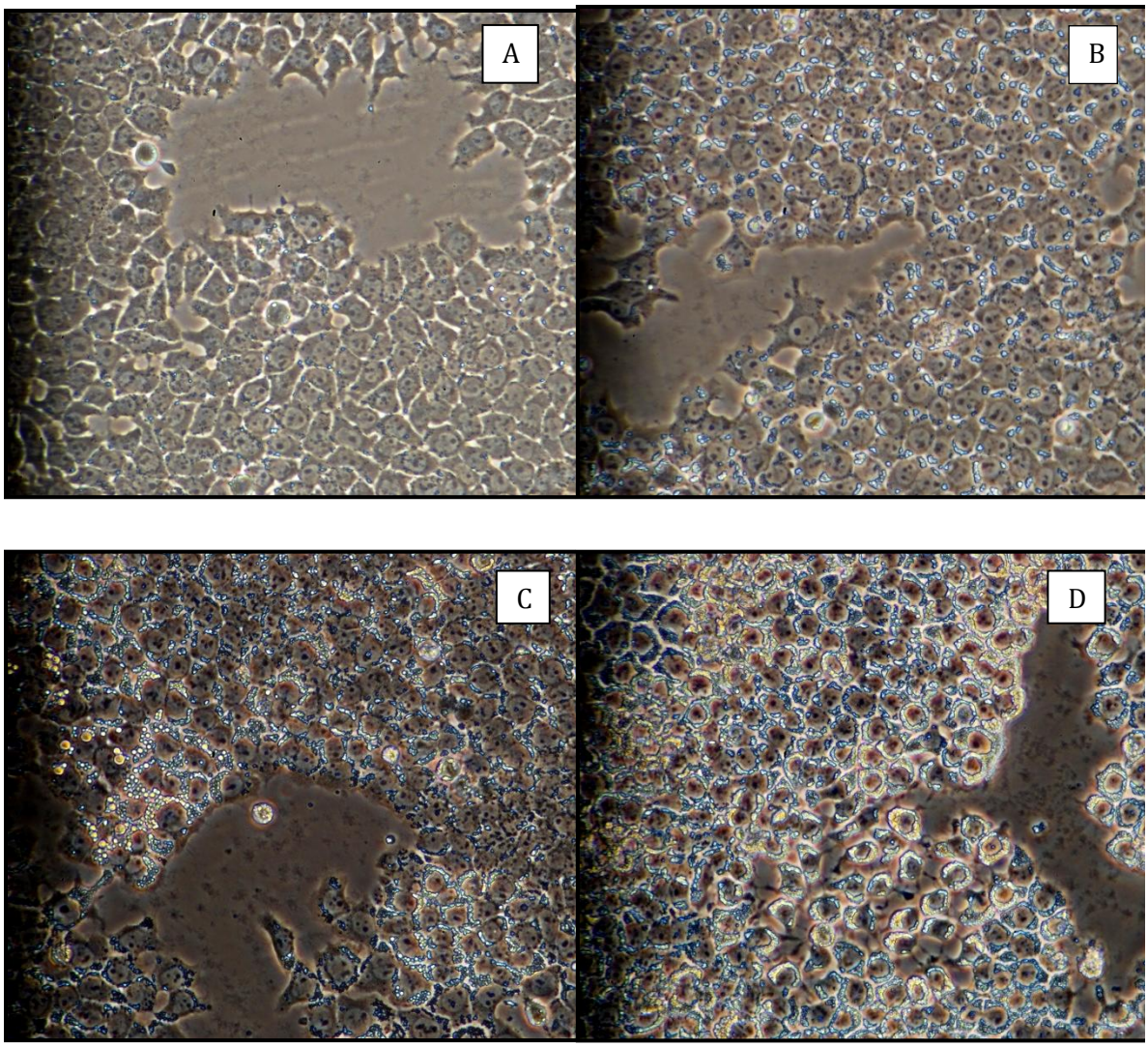


Figure 15. Optical microscopy observations of 1548 cells treated with various concentrations of FA. Cells were grown in MEM/EBSS medium containing oleate and palmitate (2:1) mixture in presence of 3% BSA either untreated **(A)**, or cells treated with 0.5 mM FA **(B)**, or 1 mM FA **(C)**, or 2 mM FA **(D)**. Cell morphology greatly changed when treated with different concentrations of FA (20X magnification).

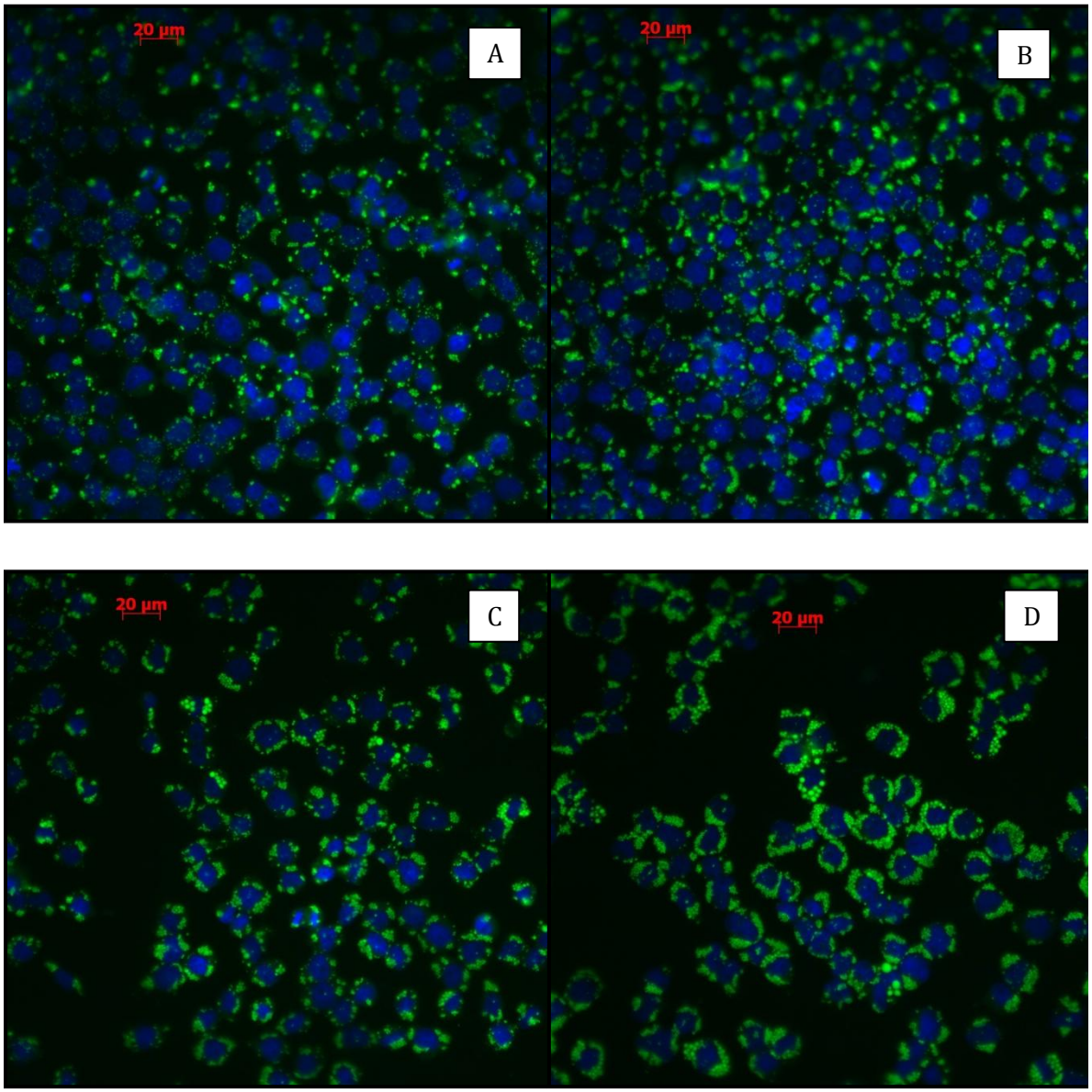


Figure 16. Fluorescent microscopy observations of 1548 cells treated with various concentrations of FA, stained with Nile Red and DAPI. Cells were grown in MEM/EBSS medium containing oleate and palmitate (2:1) mixture in presence of 3% BSA either untreated **(A)**, or cells treated with 0.5 mM FA **(B)**, or 1 mM FA **(C)**, or 2 mM FA **(D)**. Cytoplasmic lipid droplets were stained in green, surrounding the nucleus of the cells, which was stained in blue by DAPI. The green signal in control cells (A) was thought to be endogenous background (20X magnification).

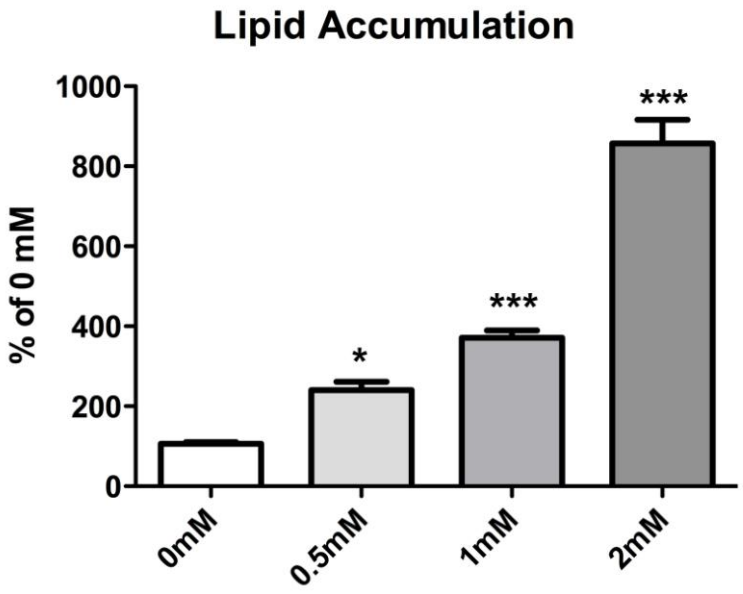


Figure 17. Lipid accumulation in 1548 cells following 2-day treatment with differing concentrations of FA. Cells were cultured in black 96-well plates. Nile Red fluorescence (485 nm excitation/535 nm emission) was normalized by DAPI signal (355 nm excitation/460 nm emission) in each well. All treated groups had statistically increased intracellular lipid content. Values are mean ± SEM, n=8, * $p < 0.05$, *** $p < 0.001$ comparing to control group. Results are expressed as % of control.

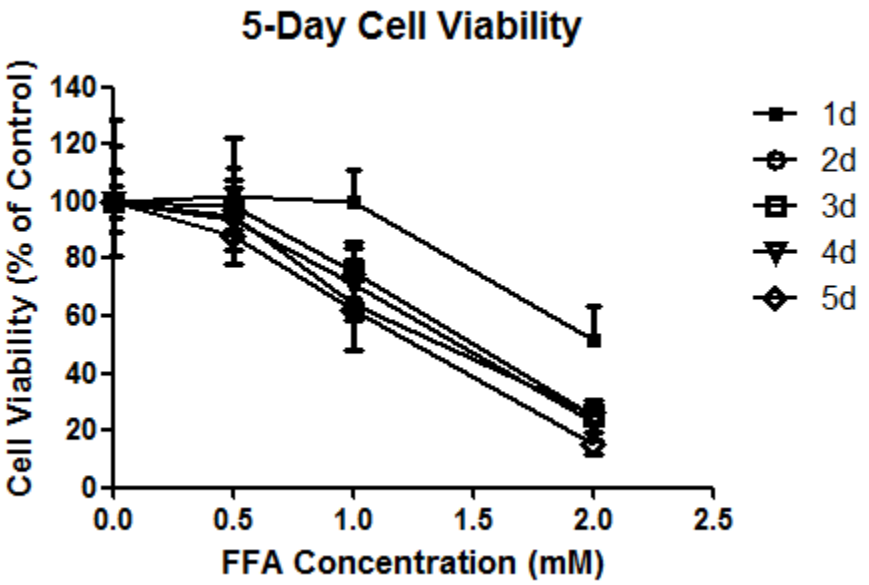


Figure 18. Cell viability of 1548 cells during a 5-day FA treatment. Significant loss of cell viability was observed on the first day of FA treatment in the 2 mM group compared to controls ($p < 0.001$). Loss of cell viability in the 1 mM was found on the second day of treatment, which was statistical different comparing to controls ($p < 0.01$). There were no significant differences between the 0.5 mM and control groups in the 2-day treatment. All FA treated groups showed no further significant changes in cellular viability from day 2 to day 5. Values are mean ± SEM, n=10. Results are expressed as % of control.

of the cells were nonviable as a result of the high fat overload, while there were no significant viability losses observed in the 0.5 and 1 mM FA treated groups. On the second day of FA treatment, the 1548 cells showed a 9.8%, 35.9%, and 75.1% decrease in viability at concentrations of 0.5 mM, 1 mM, and 2 mM of FA, respectively. The 1 mM, and 2 mM FA groups showed highly significant differences ($p < 0.01$) in cell viability compared to the control group. There was no statistical difference between the 0.5 mM FA treated and control groups. Moreover, there were no statistical differences amongst all groups from day 2 though day 5 treatments, suggesting that 2-day incubation of FA is sufficient to induce lipid-overload cytotoxicity in 1548 cells.

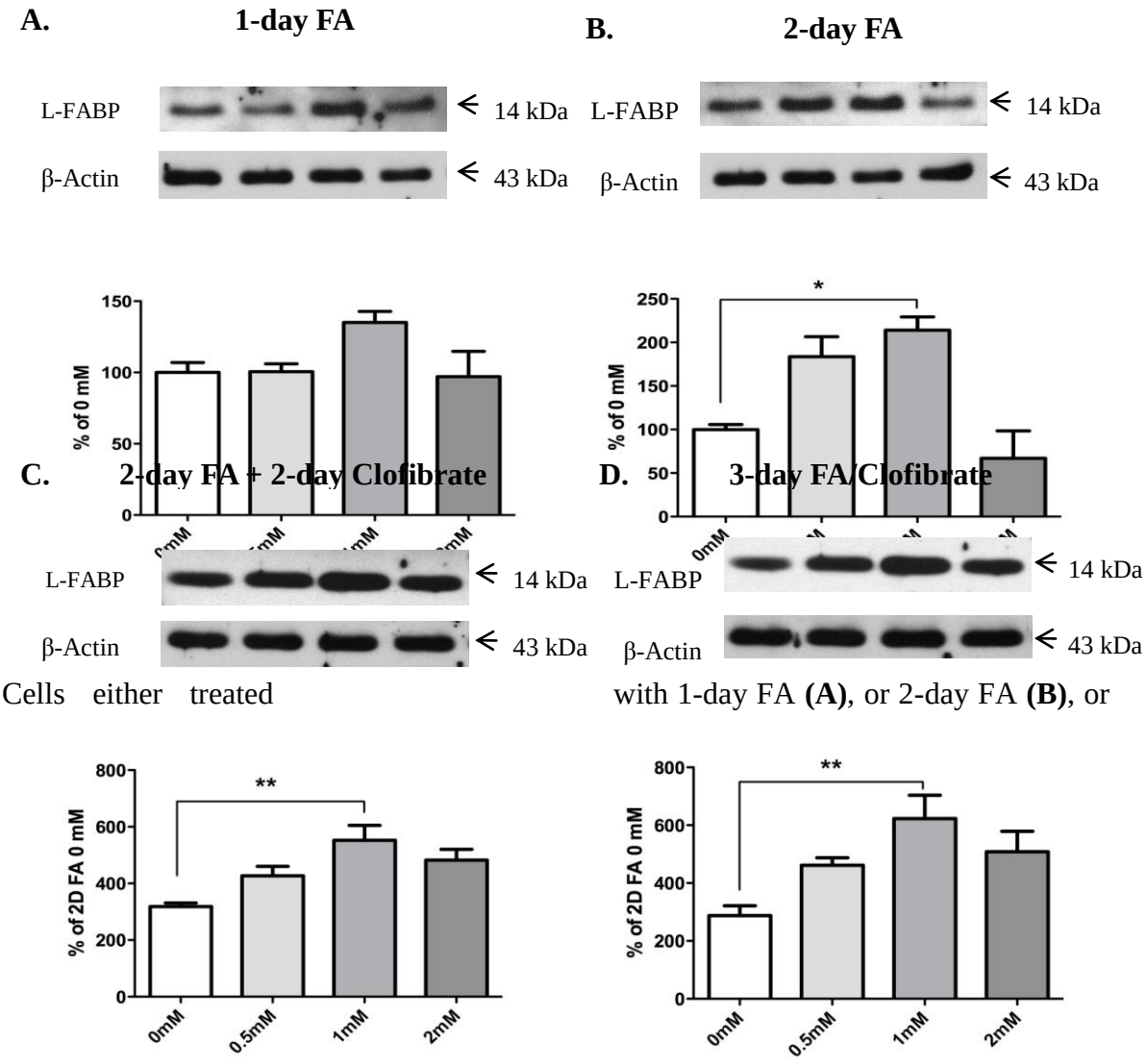
4. L-FABP Levels

Western blot analysis was used to detect the expression of L-FABP in control and FA treated cells (Figure 19). Significantly increased expression of L-FABP occurred only in the 1 mM FA treated group after 2 days ($p<0.05$). Following 1-day FA incubation did not induce significant L-FABP expression in either experimental group (A in Figure 19). The L-FABP expression in the 2 mM FA treated group decreased dramatically following the 2-day FA treatment compared to 0.5 mM and 1 mM groups (B in Figure 19). After clofibrate administration, L-FABP expressions in all FA-treated cells increased, of which 1 mM showed statistical increases in L-FABP expression in two FA-clofibrate combination treatment groups ($p < 0.01$).

5. Free Radical Levels

The diacetate ester of DCF (H_2DCFDA) was employed as it easily diffuses into cells and is hydrolyzed by intracellular esterases to the fluorescent product DCF. Figure

Figure 19. L-FABP Levels in FA and FA/Clofibrate Combined Treatment.



Cells either treated with 1-day FA **(A)**, or 2-day FA **(B)**, or 2-day FA followed by 2-day clofibrate treatment **(C)**, or 1-day FA followed by co-treatment of FA and clofibrate on the second day followed by clofibrate-only treatment on the third day **(D)**. Bottom - Histogram representing integrated density values for L-FABP from four different experiments. Band densities were quantified and expressed as mean ± SEM. **p < 0.05, **p < 0.01*.

20 shows the FA induced oxidative stress, as measured by DCF fluorescence, was significantly enhanced as FA concentration increased in all treated groups. One-day treatment of FA resulted in 151 ± 5%, 266 ± 21%, and 332 ± 19% increase in ROS levels

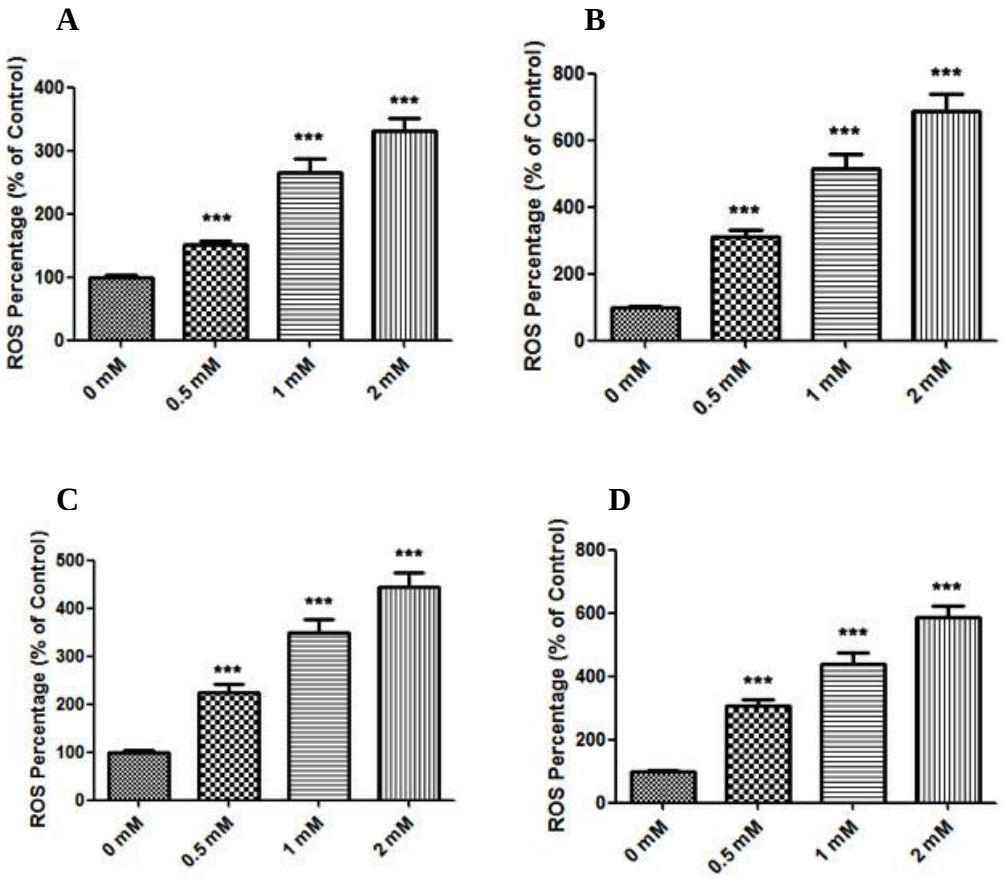
for the 0.5 mM, 1 mM, and 2 mM FA treated groups, respectively (Figure 20, A; $p < 0.001$). ROS production was further increased after 2-day FA treatment. Amount of free radicals generated were 310 ± 21%, 514 ± 43%, and 688 ± 48% in 0.5, 1, and 2 mM groups, respectively (Figure 20, B; $p < 0.001$). After introduction of clofibrate treatment, the total free radical levels were significantly reduced, especially when clofibrate intervention was introduced on the second day with FA treatment. ROS levels were lowered to 224 ± 17%, 349 ± 28%, and 445 ± 30% in 0.5, 1, and 2 mM groups, respectively (Figure 20, C; $p < 0.001$). When clofibrate was introduced as a late intervention after 2-day FA treatment for 2 days, ROS formations was still reduced in cells although levels were not reduced as much as when the clofibrate treatment was initiated on the second day. ROS generations were reduced to 307 ± 20%, 439 ± 34%, and 585 ± 37% in 0.5, 1, and 2 mM groups, respectively (Figure 20, D; $p < 0.001$).

Further comparisons of intracellular ROS generations between 1-day and 2-day FA treatment; FA treatment on day 1 followed by FA treatment on day 2 together with clofibrate treatment on day 2 followed by clofibrate only treatment on day 3; 2-day FA treatment and 2-day FA followed by 2-day clofibrate treatment as well as the two FA/clofibrate co-treatments, were shown in Figure 21. Without clofibrate treatment, the intracellular ROS level was elevated on the second day of FA treatment and all treated groups showed statistically increased DCF signals compared to the first day of FA treatment. After introduction of clofibrate, treatment of 1-day FA followed by co-

treatment of FA and clofibrate on the second day followed by clofibrate-only treatment on the third day was found more efficient in removing intracellular free radicals compared to the other combined treatment regimen, especially in the high FA treated

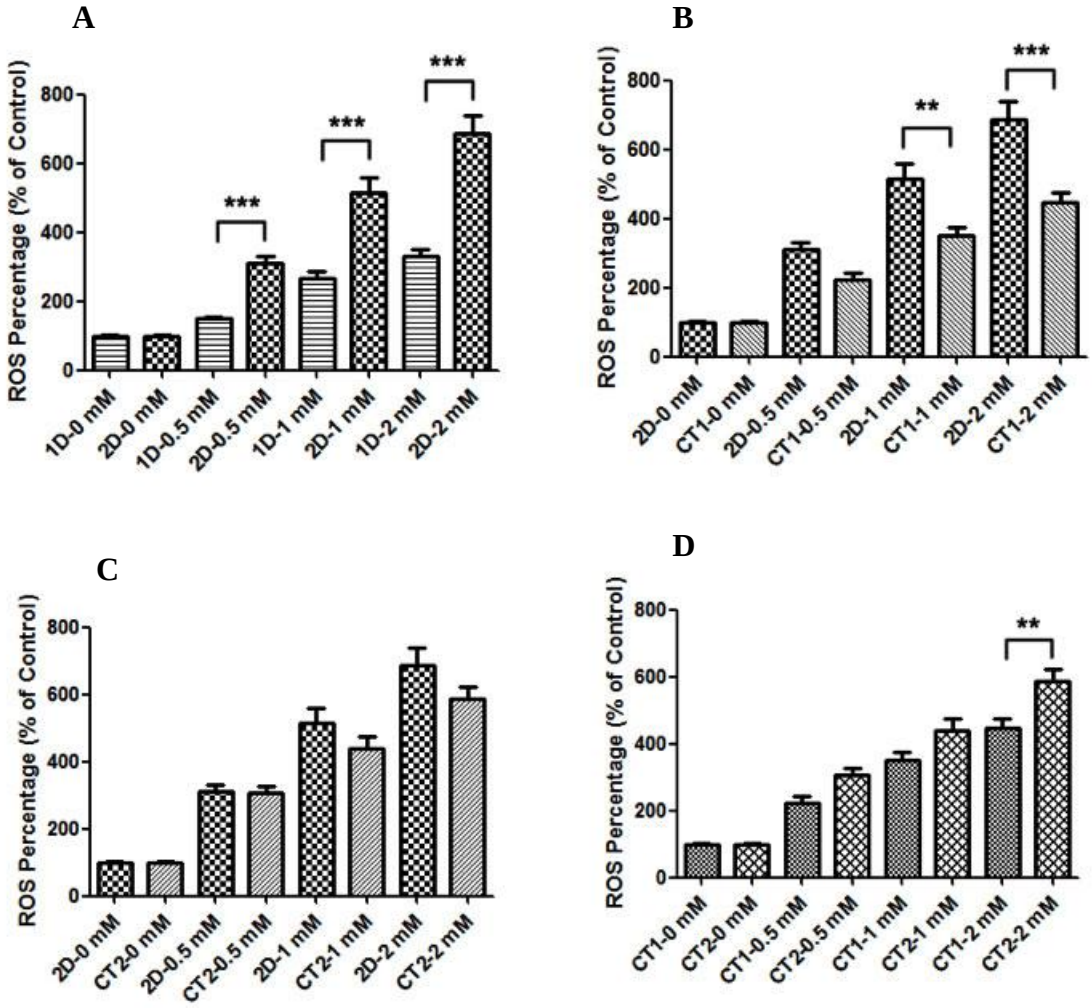
groups (Figure 21, B and C; $p < 0.05$). Although the DCF signal of all groups in the 2-day FA treatment followed by 2-day clofibrate treatment were reduced, the data showed no statistical difference from the 2-day FA treatment in each group (Figure 21, C), which indicated that an early rather than the late intervention of clofibrate may help cells scavenge free radicals even if cells were exposed to a high fat environment. Comparison of the two co-treatments showed that only the level of ROS products in 2 mM treated group in 2-day FA followed by 2-day clofibrate treatment was significantly higher than the other co-treatment group (Figure 21, D; $p < 0.05$).

Figure 20. ROS levels in hepatoma cells.



Cells were either treated with 1-day FA **(A)**, or 2-day FA **(B)**, or 2-day FA followed by 2-day clofibrate treatment **(C)**, or 1-day FA followed by co-treatment of FA and clofibrate on the second day followed by clofibrate-only treatment on the third day **(D)**. Cells were cultured in black 96-well plates. DCF fluorescence was recorded by fluorescence plate reader with excitation 485 nm and the emission 535 nm. Results are expressed as % of control and normalized by the total protein (μg) in each well. All treated groups had significant increased intracellular ROS products compared to control group in each experiment (n = 4, *** $p < 0.001$).

Figure 21. Comparisons of ROS Production between FA-only and FA-clofibrate combined treatments.



Comparison of 1-day with 2-day FA treatment **(A)**, or comparison between 2-day FA treatment and the treatment of 1-day FA followed by co-treatment of FA/clofibrate on the second day followed by clofibrate-only treatment on the third day **(B)**, or comparison between 2-day FA treatment and the treatment of 2-day FA followed by 2-day clofibrate treatment **(C)**, or comparison between the two FA/clofibrate combined treatments **(D)**. Results are expressed as % of control and normalized by the total protein (μg) in each well. All treated groups had significant increased intracellular ROS products compared to control in each experiment (n = 4, $^{**}p < 0.05$, $^{***}\ p < 0.01$). 1D, 1-day FA treatment; 2D, 2-day FA treatment; CT1, 1-day FA followed by co-treatment of FA/clofibrate on the second day followed by clofibrate-only treatment on the third day; CT2, 2-day FA followed by 2-day clofibrate treatment.

V. Discussion

1. Establishment of the Cell Culture Model for Studying NAFLD

Several animal models are available for studying the cellular outcomes of NAFLD (Sundaram et al., 2005; Anstee and Goldin, 2006; Wortham et al., 2008; Wouters et al., 2008). However, these models are associated with various drawbacks. For example: 1) genetic or dietary models may be relatively time-consuming to either successfully select the correct strain of mice or develop the symptoms of NAFLD for establishing the model, which may vary from several weeks to months to develop steatohepatitis and fibrosis, e.g., foz/foz mice may need ~300 days (Arsov et al., 2006); 2) developing and maintaining an animal model is very costly; 3) the effects of gender, strain and species of the animal makes it hard to establish a clean control especially for a complicated disease like NAFLD which is associated with many metabolic dysfunctions; 4) some models do not or only partially reflect the true picture of human NAFLD in terms of pathogenesis and disease mechanisms (Nanji, 2004; London and George, 2007; Larter and Yeh, 2008). The models either only mimic the histopathologic features or the pathogenic features associated with human NAFLD. The methionine and choline deficient diet model does not develop peripheral insulin resistance but instead has lower fasting plasma glucose and seems to be insulin-sensitive after 4 weeks of treatment (Rinella et al., 2008). These changes are not observed in human NAFLD patients.

In fact, appropriate cell culture models were developed to overcome some of these drawbacks. Besides the advantages of readily available, unlimited life span, stable phenotype and standardized culture conditions in using cultured liver cells, it has been reported that the *in vitro* hepatocellular models designed for the investigation of NAFLD

demonstrated similar disease features found in *in vivo* observations in terms of lipid accumulation pattern and hepatocyte lipoapoptosis (Feldstein et al., 2003). One of the examples is the primary culture of hepatocytes isolated from steatosis patients provided the closest pathogenesis that observed in human liver tissue. In addition, NAFLD *in vitro* models also enabled researchers to study the effect of lipid overload upon the hepatocyte within a short period of time by directly exposing the cells to high concentration of fatty acids. For instance, when FaO cells were exposed to 0.1% triglycerides, it was reported intracellular ROS level rapidly peaked after 6 hr, suggesting ROS overproduction was an early event triggering hepatocyte apoptosis (Shimabukuro et al., 1998). Thus, well-defined *in vitro* hepatocellular models may be useful to experimentally investigate the biochemical effects of elevated fatty acids levels in hepatocytes (Gomez-Lechon et al., 2007).

However, it is important to note that there are limitations when using *in vitro* models to study NAFLD. For example, the primary culture of liver cells may be limited by the liver tissue sample quantities and quality. Their relatively short *in vitro* life after isolation also makes it difficult to maintain them for further long-term studies. It is also important to realize that most of the *in vitro* studies investigated the acute injury to the liver cells comparing to the chronic exposure of lipid overload observed in the liver tissue of human NAFLD patients.

In the present study, we utilized 1548 rat hepatoma cells, exposed to a high fat medium to establish an NAFLD cell culture model, which was defined as dramatically increased intracellular lipid content either observable in cell morphology (Figure 15 and 16) or detectable through Nile Red fluorescent assay (Figure 17) and enhanced cellular

oxidative stress status (A and B in Figure 20). All lipid accumulation and ROS production showed the characteristic time- and dose-dependent patterns. These features of NAFLD are present in hepatoma cells fed a mixture of oleate:palmitate (2:1) within two days. These data are consistent with other studies using either *in vitro* cultured human hepatocytes (Donato et al., 2006) or human hepatoma cell lines such as HepG2 (Jin et al., 2009), whose *in vitro* steatosis was also induced by mixtures of these fatty acids. Compared to animal models, this cell culture model also enabled us to control for the FA or pharmacological administration. Overall, our NAFLD cell culture model demonstrated to be less-expensive and easy to control drug levels, and most importantly, mimics the major characteristics of human fatty liver disease.

2. Evaluation of the in vitro NAFLD Cell Culture Model

2.1. Hepatocellular Steatosis Induction

Ideally, lipids for inducing steatosis in experimental studies should be similar to those responsible for inducing NAFLD in patients. The choice of lipids to induce steatosis lies in that oleic and palmitic acids are the most abundant FA in liver triglycerides in both normal subjects and patients with NAFLD (Araya et al., 2004). Saturated fatty acids such as palmitate have been shown to be responsible for the promotion of acute harmful effect of lipid accumulation in the liver (Gomez-Lechon et al., 2007) and induction of hepatocyte lipoapoptosis in NAFLD (Malhi et al., 2006; Ibrahim et al., 2011), while unsaturated fatty acids such as oleate are thought to render hepatocytes vulnerable to oxidative stress (Garcia et al., 2011). Thus, this provides the

rational for using oleate and palmitate to develop an *in vitro* NAFLD model with a high fat accumulation profile.

Several studies established the *in vitro* NAFLD model in either isolated primary human hepatocytes or hepatocyte cell lines by exposing cells to mixtures of oleate and palmitate (Donato et al., 2006; Gomez-Lechon et al., 2007; Jin et al., 2009; Garcia et al., 2011). The extent of lipid accumulation in the present cell culture model was comparable to those observed either in other *in vitro* primary hepatocyte culture models from NAFLD patients or NAFLD animal models. It was found that there was almost 3.8-fold increase in lipid load in 24-hr cultured hepatocytes obtained from liver tissue with steatosis compared to the hepatocytes isolated from human tissuse without steatosis (Gomez-Lechon et al., 2007). In NAFLD studies utilizing animal models, the accumulated hepatic triglyceride levels varied from almost two-fold (Sundaram et al., 2005; Baumgardner et al., 2008) to nearly nine-fold increase (Brix et al., 2002). As observed in another NAFLD *in vitro* cell culture model administrating 1 mM mixture of the same proportional (2:1) palmitate and oleate to HepG2 cells for 48 hr (Garcia et al., 2011), a 4-fold of increased intracellular neutral lipid content was observed.

In the current study, administering 1 mM FA showed nearly 4-fold elevated intracellular FA content in the 1548 cells while 0.5 and 2 mM groups showed 226% and 808% of increased lipid loads, respectively, after two-day FA treatment. Thus, the pattern of lipid accumulation in the present *in vitro* NAFLD cell culture was comparable to those observed either in hepatocytes from liver tissue of patients with steatosis or widely used NAFLD animal and cell models.

2.2. Induced Lipotoxicity Comparable to Other in vivo and in vitro Models

Lipoapoptosis, a characteristic feature of NASH (Alkhouri et al., 2009), is specifically induced by saturated fatty acids (Eitel et al., 2002; Listenberger et al., 2003). Gomez-Lechon et al. found that the higher amount of palmitate in the mixture (oleate/palmitate, 1:2 and 0:3 ratios) the more caspase 9 dependent apoptotic cells were identified in both isolated human hepatocytes and HepG2 cells (Gomez-Lechon et al., 2007). These data indicated that the lipotoxicity produced by FA are involved in the caspase-dependent cell death pathway. Various reports depict that cytosolic accumulation of saturated FA especially palmitate induce apoptosis involving a mitochondrial basis for its pathogenesis. In the HepG2 cells palmitate triggers the c-JunNH_2-terminal kinase (JNK)-dependent mitochondrial apoptotic pathway by Bax activation. JNK induces apoptosis in a transcription-independent process by activating the proapoptotic Bcl-2 protein Bim. This BH3 domain only protein is able to activate Bax, thus, triggering mitochondrial dysfunction and eventually hepatocyte death (Malhi et al., 2006).

Although unsaturated fatty acids such as oleic acid do not directly induce hepatocyte lipoapoptosis, they sensitize hepatocytes to death ligands such as tumour necrosis factor related apoptosis inducing ligand (TRAIL) (Malhi et al., 2007). TRAIL as well as tumour necrosis factor alpha (TNF-α) were found to be the major death receptor ligands mediating hepatocyte apoptosis when they are challenged with unsaturated fatty acids (Malhi et al., 2006).

Additionally, fatty acid-induced endoplasmic reticulum (ER) stress was associated with palmitate-mediated cell death through the JNK pathway activation. Such evidence was identified in isolated primary hepatocytes from methionine-choline

deficient NAFLD mice model and several ER-stress markers were found in H4IIE rat hepatoma cell line fed with high concentrations of palmitate acid (Pfaffenbach et al., 2010). ER is a central organelle of eukaryotic cells for lipid synthesis, protein folding and eventual maturation. Also, ER is the major signal transducing organelle that senses and responds to changes of the homeostasis. ER stress is referred to as any disturbance of the ER homeostasis affecting the folding of proteins in the ER lumen and accumulation of improperly folded proteins. It initiates a critical physiological response termed Unfolded Protein Response (UPR) leading to an increased ER resident chaperones and a decreased overall protein synthesis (Trauner et al., 2010). Failure of the UPR to combat ER stress leads to hepatocyte apoptosis (Pfaffenbach et al., 2010). Several UPR mediators can be activated in ER stress (Trauner et al., 2010). One of them is protein kinase RNA-like ER kinase (PERK), which can be activated by oxidative stress, leading to inhibition of the eukaryotic translation initiator factor-2alpha (eIF-2α) and thus resulting in a global decreased protein translation (Wu and Kaufman, 2006). UPR activation via eIF-2α phosphorylation has been observed in the NAFLD patients (Puri et al., 2008). Excessive ER stress resulting in increased hepatic triglycerides via inhibition of apolipoprotein B100 secretion potentially worsens steatosis (Ota et al., 2008). Studies by Wei et al demonstrated that, in H4IIE rat liver cells, palmitate induced ER stress via extrusion of ER calcium is a possible factor causing calcium-mediated mitochondrial cytochrome c release and eventually resulting in the activation of the intrinsic apoptotic pathway in H4IIE hepatome cells (Wei et al., 2007; Wei et al., 2009). This may explain the dose-dependent pattern of the decreased cell viability when 1548 cells, derived from H4IIE,

were challenged by high concentrations of fatty acid mixtures containing palmitate in the present study (Figure 18).

According to the study by Gomez-Lechon et al, a concentration range of 0-2 mM of an oleate to palmitate ratio of 2:1 was associated with similar time- and dose-dependent cytotoxicity in both isolated human hepatocytes and HepG2 cells (Gomez-Lechon et al., 2007). The lowest cell viability reduction was observed during the first 36-hr treatment. This study provided the rational for choosing a similar dose range and ratio of FAs to induce steatosis in 1548 cells. In the Gomez-Lechon et al study, the 2 mM treated HepG2 cells showed almost 50% nonviable cells while no statistical significant differences in cell viability between other treated (0.5 and 1 mM) and the control groups were noted following 24 hr. However, in the present study, we did found statistical difference in cell viability after exposure to 0.5 and 1 mM fatty acid after 24 hr (Figure 17). In addition, our finding that 3 mM of FA mixture resulted in the 1548 cells detaching from the culture vessels (data not shown) after 24 hr treatment was also consistent with what was reported in their HepG2 NAFLD cell model (Gomez-Lechon et al., 2007). Compared to the data from the study of Gomez-Lechon et al showing less cell death in 2 mM FA treated group after 24 hr using isolated human hepatocytes than the HepG2 cells after lipid overloading (Gomez-Lechon et al., 2007), our data demonstrated a similar cell viability decreasing pattern as what they observed in HepG2 model (loss of 50% cell viability after 24 hr, Figure 18). These data suggest that hepatoma cell lines were more sensitive to FA and less tolerant of lipid overloading induced lipoapoptosis.

2.3. Time- and Dose-dependent intracellular ROS Generation

Although the incorporation of FA into triglycerides in the form of lipid droplets has been suggested as a protection strategy for hepatocytes to fight against FA overload (Choi and Diehl, 2008; Trauner et al., 2010), the prolonged accumulation of excessive hepatic lipid content, which hepatocytes are unable to handle, results in severe lipid peroxidation and makes the liver highly vulnerable to the subsequent oxidative stress and injury (Madan et al., 2006). ROS has been found to be the main contributor to hepatic lipid peroxidation. In the present study elevated cytochrome P450 enzyme 2E1 (CYP2E1) activity (Kohjima et al., 2007) and palmitate-induced mitochondrial ROS over-production (Nakamura et al., 2009b) may contribute to the time- and dose-dependent augment of intracellular ROS (Figure 20, A and B).

CYP2E1 was thought to be a major source for ROS generation in hepatocytes when challenged with a lipid overload. Higher expression of CYP2E1 in NAFLD are known to lead to oxidative stress (Aubert et al., 2011). This may be due to the poor coupling property of CYP2E1 with NADPH-cytochrome P450 reductase, resulting in generation of significant amounts of superoxide radical, hydrogen peroxide and consequent lipid peroxidation response (Lieber, 1997). Elevated hepatic CYP2E1 due to abnormal lipid accumulation was identified in NAFLD patients (Chalasani et al., 2003) and NAFLD animal models (Gyamfi et al., 2008). Palmitic and oleic acids have been reported to induce an increased CYP2E1 expression in primary human hepatocyte culture (Raucy et al., 2004). Although there was no direct evidence indicating the inducible expression of CYP2E1 in the 1548 rat hepatoma cell line, however, this cell line is derived from the hepatoma H-35 strain (Pitot et al., 1964) and is capable of hydroxylating

bile acids (Reuber, 1961) through the cytochrome P450 dependent monooxygenases (Bjorkhem and Danielsson, 1974; Gurtoo et al., 1978). Moreover, derivative cell lines from H-35 such as FaO cells were reported to constitutively express inducible CYP2E1 (De Waziers et al., 1995; Zhukov and Ingelman-Sundberg, 1999). Thus, in the current NAFLD model induced CYP2E1 activity may be one of the possible contributors for the elevated ROS production.

Elevated ROS production may be closely correlated to the accumulation of FA induced mitochondrial dysfunction. Li et al found that incubating isolated mouse hepatocytes and HepG2 with 0.2 mM palmitate could induce significant mitochondrial ROS generation within 24 hr (Li et al., 2008). Utilizing another cell line derived from the 1548 cell line, H4IIE3C, Nakamura et al found a 58% increase in intracellular ROS production induced by palmitate at 0.25 mM within 8 hr treatment and this induction was dose-dependent (Nakamura et al., 2009a). Further investigation of ROS generation pathways elucidated that the source of palmitate-induced ROS was from impaired flux of electrons into the mitochondrial respiratory chain resulting in abnormal reduction of oxygen. The potential mechanism was that palmitate induced significantly increased expression of carnitine palmitoyltransferase-1a (CPT-1a), the rate-limiting enzyme in mitochondrial fatty acid β-oxidation, which accelerated β-oxidation. FA metabolism through normal β-oxidation supplies mitochondrial respiratory chain with electrons. This acceleration gave rise to large amounts of electrons entering the respiratory chain, finally leading to ROS production (Nakamura et al., 2009b). In the present study, the H4IIE rat hepatoma cells were challenged with a higher concentration of palmitate, implying that a

similar mechanism was responsible for the observed time- and dose-dependent ROS over-production.

2.4. Expression of L-FABP in NAFLD Cell Culture Model

The role of L-FABP in NAFLD may involve binding and solubilizing the LCFAs and their peroxidation products to provide an intracellular buffer system preventing lipid accumulation to induce cytotoxicity. Representing as much as 2-5% of cytosolic protein and concentration of 200-1,000 μM (Paulussen and Veerkamp, 1990; McArthur et al., 1999), L-FABP has high capacity to bind LCFAs (Ek et al., 1997) and their peroxidation products (Raza et al., 1989). L-FABP enhances cellular LCFA and LCFA-CoA uptake and increasing their solubility in aqueous cytosolic environment (Jolly et al., 1998; Schroeder et al., 1998), thus is protective against the detergent effects of excess fatty acids. Accounting for 90% of the cytosolic LCFA binding capacity in liver (Martin et al., 2003), L-FABP is able to maintain intracellular LCFA pool size by delivering the LCFAs to mitochondria for enhancing LCFA oxidation (Glatz et al., 1984; Atshaves et al., 2004) and esterification (Murphy and Schroeder, 1997).

Previous findings in our lab suggested L-FABP might be another potent endogenous antioxidant (Wang et al., 2005; Rajaraman et al., 2007; Wang et al., 2007). This provided the rational for investigating the potential cytoprotective role of L-FABP in NAFLD, which is associated with oxidative stress. The antioxidative mechanism thought to be responsible for this action may be through scavenging free radicals via redox cycling of several L-FABP's methionine groups (Yan et al., 2009). Using 1548 rat hepatoma cells to establish NAFLD in cell cultures makes it convenient to investigate the potential role of L-FABP in this model as the L-FABP expression can be easily induced

via clofibrate pharmacological intervention (Rajaraman et al., 2007) and significantly increased intracellular L-FABP content can be achieved within two days of the clofibrate treatment (Yan et al., 2010).

Induction of L-FABP expression comes from activation of the peroxisome proliferator-activated receptor-α (PPARα). L-FABP expression can be up-regulated by peroxisome proliferators (Brandes et al., 1990), peroxisome proliferator drugs (Wolfrum et al., 2001), and other ligands able to activate PPARα (Schroeder et al., 2008). LCFAs as well as their intracellular activated forms, LCFA-CoA are such ligands capable of activating PPARα, thus inducing L-FABP expression. Palmitic acid and oleic acid were reported to activate PPARα (Wolfrum et al., 2001). Kaikaus et al, however, found that neither palmitic acid nor oleic acid at 0.8 mM induced L-FABP expression in rat primary hepatocytes (Kaikaus et al., 1993a). This finding indicated that majority of these lipids at this concentration are predominantly be metabolized via mitochondrial β-oxidation rather than enter the nucleus to interact with the PPARα receptor (Kaikaus et al., 1993c). This may provide for an explanation as to why the hepatoma cells in the present study treated with the FA mixture showed no significant increase in L-FABP expression compared to the control group within the two-day FA treatment (A and B in Figure 19). A recent study found decreased L-FABP expression in humans with severe stages of NAFLD, but patients with simple steatosis over-expressed L-FABP in liver (Charlton et al., 2009). The reason for this dysregulation of L-FABP was possibly explained as an adaptive reaction of the body when challenged with high fat content at an early stage like simple fatty liver, whereas during the progressive stage of NAFLD the fatty acid disequilibrium and impaired lipid metabolism results in dramatically increased level of free radicals

randomly damaging the intracellular compartments and affecting the stability of other macromolecules produced in the cell including L-FABP and its mRNA. Similarly, in the present study the increased L-FABP expression was observed with the 1 mM FA treated group on the second day of FA treatment, implying a possible similar *in vitro* hepatocellular adaptation to the lipid overload, which minimized the elevated lipotoxicity by either solubilization these molecules or targeting them for oxidation or esterification.

3. Pharmacological Modulation of L-FABP and L-FABP Antioxidative Contribution

The various amino acid groups differ in their susceptibility to oxidation by ROS (Stadtman, 1993). Among them, the sulphur containing residues cysteine and methionine are the most easily oxidized amino acids by ROS (Vougier et al., 2003). The widespread presence of methionine sulfoxide reductases reduces either free or protein-bound methionine sulfoxide formed by the oxidation of methionine (Levine et al., 1999). In cells, methionine participates in oxidation processes through redox cycling reactions, the so-called "methionine regeneration", and thus implementing its repair over oxidative modified proteins (Stadtman et al., 2003). Rat L-FABP contains one cysteine and seven methionine groups (Gordon et al., 1983) in its amino acid sequence. This makes L-FABP an attractive endogenous intracellular antioxidant against ROS along with its high intracellular level.

Studies done by our lab indicate that L-FABP is able to scavenge intracellular ROS content either in a human hepatoma cell line over-expressing this protein (Wang et al., 2005) or in rat hepatoma cells when induced with clofibrate (Rajaraman et al., 2007). Clofibrate treatment induced a dose- and time-dependent L-FABP expression pattern in rat 1548 hepatoma cells within four days (Yan et al., 2010). L-FABP's antioxidative

function was further confirmed by showing no significant changes of superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) activities during clofibrate treatment (Yan et al., 2010). However, it should be noted that, as an important PPARα activator, clofibrate was found not appear to be of clinical benefit in the treatment of NASH in one pilot human trial (Laurin et al., 1996).

In the current study, two different combined treatments of FA and clofibrate in 1548 cells were compared in terms of L-FABP expression induction and cytosolic ROS level alteration. The 2-day FA followed by 2-day clofibrate treatment simulated the situation that upon NAFLD diagnosis patients stop a high fat diet intake and received clofibrate intervention to increased the L-FABP expression; while in the other combined treatment, the patients may still have a high fat diet during the initial intervention of clofibrate however the intervention was introduced earlier. Through clofibrate treatment, increased L-FABP expressions were observed in both combined treatments (Figure 20, C and D). This is likely achieved through enhanced transcription of the L-FABP gene via activation of PPARα and stabilization of L-FABP mRNA by increasing its half-life up to 25 hr in 1548 cells (Yan et al., 2010).

Comparing the 3-day combined treatment to the 2-day FA treatment groups, attenuation of the ROS signal was observed in the 1 and 2 mM groups (Figure 21, B). When clofibrate intervention was introduced on the second day, these groups showed approximately 2.1-fold and 1.8-fold induced L-FABP expression by clofibrate, respectively (Figure 19, B). Although L-FABP expression inductions were also identified in another combined treatment (2 day FA followed by 2 day clofibrate), lower but no significant decrease in intracellular ROS products were observed (Figure 21, C). This

implied that an early induction of L-FABP might protect the cells from lipid induced free radical damage. The possible mechanisms behind this protection may be via binding excessive lipids and their oxidation products to target them to mitochondria and peroxisome to stimulate their oxidation (Corton et al., 2004). Another plausible explanation as previously reported by our group was that L-FABP might act as an antioxidant, trapping the ROS thus inactivating them with the help of the methionine residues in L-FABP, thus breaking the lipid peroxidation chain reaction. This was consistent with the recent finding that the less lipid peroxidation products were observed after clofibrate-induced PPARα induction in a methionine and choline deficient mouse NASH model (Donthamsetty et al., 2008).

In current study, the free radical levels in 1 and 2 mM FA treated groups were statistically reduced after introducing early intervention of clofibrate to induce L-FABP expression (FA one day, followed by one day FA/clofibrate, followed by clofibrate only treatment, Figure 21, B). However, when late clofibrate intervention was introduced after 2-day FA exposure, free radical levels were not statistically reduced (2-day FA followed by 2-day clofibrate, Figure 21, C). This may be attributed to the less L-FABP expression observed in the late clofibrate intervention compared to the early intervention (Figure 19, C and D). This leads to the speculation that antioxidant therapy alone may not be sufficient to treat NAFLD.

Chang et al reported that, combination treatment such as that utilizing both antioxidants (breaking the chain reaction of lipid peroxidation) and cytoprotectants (for instance, by stabilizing cellular and organelle phospholipid membranes or inactivating mitochondria-dependent apoptotic pathways or inhibiting pro-inflammatory molecules

production, etc.), targeting different components of the NAFLD pathogenesis would be more effective in treating this disease (Chang et al., 2006). Several candidates of cytoprotectant have been proposed and tested in some small-sized and short-term pre-clinical trials. Of them, ursodeoxycholic acid (UDCA), a hydrophilic dihydroxy bile acid found in human, was the found to be able to improve transaminases and steatosis in initial pilot studies (Laurin et al., 1996; Kiyici et al., 2003). Treatment of NASH using UDCA in preliminary human studies showed its protective property including stabilization of hepatocyte plasma membranes (Heuman et al., 1996) and inhibition of apoptosis via inhibiting bile salt induced mitochondrial dysfunction (Rodrigues et al., 1998). However, the results were not confirmed in a large randomized placebo-controlled two-year trial conducted by Lindor et al (Lindor et al., 2004). In the trial no significant difference of histological improvement was observed between the placebo group and patient received UDCA intervention. Thus, the potential benefits of other cytoprotective agents such as taurine (Obinata et al., 1996), lecithin (Buchman et al., 1992) and silymarin (Crocenzi and Roma, 2006) are underway. Taurine is able to conjugate bile acids and further inhibit the activity of CYP2E1 helping to suppress ROS-induced cell apoptosis, thus it should be beneficial in NASH (Chang et al., 2006). Whether these exogenous substrates are able to improve necrosis and inflammation caused by a number of pro-inflammatory molecules involving TNF-α is not clear (Tilg and Diehl, 2000). Pentoxifylline, a xanthine derivative shown to inhibit TNF-α production (Duman et al., 2007), is such a promising candidate. The significant improvements in transaminases (Adams et al., 2004; Satapathy et al., 2004), steatosis and lobular inflammation (Satapathy et al., 2007), have been observed in several small pre-clinical trials. Thus, studies looking at the combined use of different

cytoprotectants and antioxidants such as L-FABP may prove beneficial in treating late stage indices of NAFLD.

VI. CONCLUSION

In the present study an *in vitro* hepatocellular steatosis NAFLD model was established with lipid accumulation through overloading rat hepatoma cells with various concentrations of long chain fatty acid mixtures at the set proportion (2:1) of saturated (palmitic acid) to unsaturated (oleic acid) fatty acid. This *in vitro* NAFLD cell culture model demonstrated typical patterns including elevated lipid accumulation, increased lipotoxicity and augmented free radical generating properties, which are all comparable to those observed in published studies using human steatosis samples and other *in vitro* NAFLD animal or cell models. The convenience of inducing steatosis within two days makes it a useful model to study the pathogenesis of NAFLD *in vitro*.

In the present study, experimental evidence suggested that early pharmacological intervention using clofibrate improved hepatocellular steatosis through lowering intracellular ROS content. This may be correlated to the induced L-FABP expression and antioxidant function of this protein. Thus, it leads to the conclusion that, L-FABP may act as an effective antioxidant for the early stage of NAFLD to prevent the hepatocellular damage induced by oxidative stress.

The finding that late intervention of clofibrate to induce L-FABP did not improve the outcome of free radical scavenging to the same extent comparing to the early intervention, suggests the significance of using combination treatment of antioxidants and cytoprotectants as an effective therapeutic modality to fight against NAFLD.

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