Review Article

พยาธิกำเนิดของโรคไขมันเกาะตับและโมเดลสัตว์ทดลองขนาดเล็ก

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บทคัดย่อ

พยาธิกำเนิดของโรคไขมันเกาะตับและโมเดลสัตว์ทดลองขนาดเล็ก

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โรคไขมันเกาะตับ (fatty liver disease, FLD) ทั้งโรคไขมันเกาะตับที่ไม่ได้เกิดจากแอลกอฮอล์ (non-alcoholic fatty liver disease, NAFLD) และโรคไขมันเกาะตับที่เกิดจากแอลกอฮอล์ (alcoholic fatty liver disaese, AFLD) เป็นปัญหาสุขภาพรุนแรงทั่วโลก กลไกของการเกิดโรค NAFLD และ AFLD มีความคล้ายคลึงกันโดยเริ่มจากการมีไขมันสะสมในตับจนถึงการเกิดภาวะตับอักเสบจากการมีไขมันพอกที่ตับ ภาวะตับแข็ง และมะเร็งตับ เป็นที่น่าสนใจที่ทั้ง NAFLD และ AFLD เป็นโรคที่มักเกิดร่วมกับโรคอื่น ได้แก่ โรคหลอดเลือดหัวใจและมะเร็ง จากอุบัติการณ์ที่เพิ่มมากขึ้นของ NAFLD ในปัจจุบันจึงมีความพยายามพัฒนาทั้งการป้องกันและการรักษา ถึงแม้ว่าจะมีการศึกษาอย่างมากแต่ความรู้เกี่ยวกับกลไกการเกิด NAFLD ยังคงไม่สมบูรณ์ ทฤษฎี “การโจมตีสองครั้ง” โดยการโจมตีครั้งแรกเกิดจากรูปแบบการใช้ชีวิตทั่วไปที่นิยมบริโภคอาหารไขมันสูง ภาวะอ้วนและโรคเบาหวาน ตามด้วยการโจมตีครั้งที่สองจากการที่ตับมีความไวต่อภาวะอักเสบมากขึ้นนั้นได้กลายเป็นทฤษฎีที่ล้าสมัยแล้ว ปัจจุบันจึงเป็นทฤษฎี “การโจมตีหลายครั้ง” ซึ่งเกิดจากหลายปัจจัย รวมถึงปัจจัยต่างๆ ที่เหนี่ยวนำ NAFLD ได้แก่ ภาวะเบาหวาน ฮอร์โมนที่หลั่งจากเนื้อเยื่อไขมัน ปัจจัยโภชนาการ แบคทีเรียในลำไส้ และปัจจัยทางพันธุกรรมและเหนือพันธุกรรม ในขณะที่ AFLD นั้นมีสาเหตุหลักจากการดื่มแอลกอฮอล์ในปริมาณมาก ก่อให้เกิดการทำลายตับโดยตรงจากสารเฮปาโตท็อกซิน 1 ร่วมกันกับปัจจัยก่อโรคตับอื่นๆ รวมถึงโรคไวรัสตับอักเสบและ NAFLD โมเดลสัตว์ทดลองของ NAFLD อาจแบ่งออกเป็นสองประเภท ได้แก่ ประเภทที่เกิดจากการกลายพันธุ์ และประเภทที่เกิดจากการเหนี่ยวนำด้วยอาหารหรือยา โมเดลหนูถีบจักรที่ขาดเลปติน (*ob/ob*) หรือไม่ตอบสนองต่อเลปติน (*db/db*) และโมเดลหนูที่ขาดเมไธโอนีน/โคลีนในอาหาร เป็นโมเดลที่นิยมใช้ในงานวิจัยจำนวนมาก ส่วนการทำลายยีนเป้าหมายและการใช้อาหารที่มีสารอาหารสูงในการเหนี่ยวนำ NAFLD เป็นโมเดลที่กำลังได้รับความสนใจมากขึ้น เนื่องจากนักวิจัยต้องการลดช่องว่างความแตกต่างระหว่างโมเดลสัตว์ทดลองและกลไกการเกิดโรคในมนุษย์ ส่วนโมเดลที่ใช้ในการศึกษา AFLD มีในสัตว์หลายประเภท ตั้งแต่ลิง หมูแคระ และสัตว์ฟันแทะ อย่างไรก็ตาม โมเดลสัตว์ฟันแทะในการศึกษาโรค AFLD อาทิ หนูถีบจักรที่ถูกตัดยีนออก (knock-out mouse) ช่วยเพิ่มความจำเพาะในการทดสอบสมมุติฐานกลไกการเกิดโรค จากโมเดลเหล่านี้ทำให้มีรายงานทฤษฎีใหม่ๆ ที่น่าจะเป็นไปได้ในการอธิบายกลไกการเกิดโรค NAFLD และ AFLD อย่างไรก็ตาม แม้ว่าจะมีความก้าวหน้าอย่างมากในความรู้เกี่ยวกับกลไกการเกิดโรคจากการศึกษาด้วยโมเดลเหล่านี้ ก็ยังคงจำเป็นต้องศึกษาเพิ่มเติมต่อไปเพื่อให้สามารถอธิบายกลไกการเกิดโรคได้ชัดเจนและจำเพาะยิ่งขึ้น เพื่อการพัฒนาวิธีการรักษาโรค NAFLD และ AFLD ต่อไป

คำสำคัญ: โรคไขมันเกาะตับ, โรคไขมันเกาะตับที่ไม่ได้เกิดจากแอลกอฮอล์, โรคไขมันเกาะตับที่เกิดจากแอลกอฮอล์, ไขมันเกาะตับ, โมเดลสัตว์ทดลองขนาดเล็ก

Pathogenesis of fatty liver diseases (FLD) and their small animal models

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Abstract

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Fatty liver disease (FLD), including non-alcoholic (NAFLD) and alcoholic fatty liver disease (AFLD), are serious health problems worldwide. Both NAFLD and AFLD have similar pathological spectra, ranging from simple hepatic steatosis to steatohepatitis, liver cirrhosis, and hepatocellular carcinoma. Notably, NAFLD and AFLD are frequently associated with extrahepatic complications, e.g. cardiovascular disease and malignancy. NAFLD is increasingly prevalent and represents a growing challenge in terms of prevention and treatment, despite advances in this field, knowledge on the pathogenesis of NAFLD is still limited. The classical 'two-hit' hypothesis, having hepatic accumulation of lipids secondary to sedentary lifestyle, high fat diet, obesity and insulin resistance as the first hit, and sensitization of the liver to inflammation acting as the 'second hit', is now outdated. The "multiple hit" hypothesis considers multiple insults acting together on genetically predisposed subjects to induce NAFLD and provides a more accurate explanation of NAFLD pathogenesis. Such "hits" include insulin resistance, hormones secreted from the adipose tissue, nutritional factors, gut microbiota, and genetic/epigenetic factors. Excessive alcohol intake can lead to AFLD through its direct action as a hepatotoxin1 as well as potentiation of other liver diseases including chronic viral hepatitis and NAFLD. Animal models of NAFLD may be divided into two broad categories; those caused by genetic mutation and those with an acquired phenotype produced by dietary or pharmacological manipulation. The genetic leptin-deficient (ob/ob) or leptin-resistant (db/db) mouse and the dietary methionine/choline-deficient model have been employed in the majority of published research. More recently, targeted gene disruption and the use of supra-nutritional diets to induce NAFLD have gained greater prominence as researchers have attempted to bridge the phenotype gap between the available models and the human disease. On the other hand, several models for experimental AFLD exist, including non-human primates, micropigs and rodents. Most researchers employ rodent models of AFLD. Knock-out mice have increased the specificity of the hypotheses that can be directly tested. Based on these models systems, several plausible hypotheses have been applied to explain the pathogenesis of NAFLD and AFLD. However, despite significant advances in our understanding of the mechanisms by development of these models, further studies are still needed in order to translate these to more understandable and specific mechanisms of these diseases to develop new treatments for either NAFLD or AFLD.

Keywords: fatty liver disease, non-alcoholic fatty liver disease, alcoholic fatty liver disease, steatosis, small animal model

Introduction

Fatty liver disease or hepatic steatosis is a pathological condition in which fat or simple triglyceride is accumulated in hepatocytes, defined by accumulated triglyceride concentration exceeding 5.5% or 55 mg/g liver (Basaranoglu *et al*., 2015). The prevalence of steatosis tends to be increasing in industrialized countries especially in Asian and Western region (Liu, 2012). High prevalence of steatosis is relevant to sex, ethnic, age, and concurrent disease, which is more frequent among people with obesity, diabetes, and alcoholics (Adams *et al*., 2005). Fatty liver disease is considered as the first step in the spectrum of liver diseases caused and not caused by alcohol consumption (Browning and Horton, 2004). The progression of non-alcoholic fatty liver disease (NAFLD) and alcoholic fatty liver disease (AFLD) is a spectrum of liver diseases ranged from simple hepatic steatosis, to steatosis with inflammation (steatohepatitis) and necrosis, to fibrosis, cirrhosis, and potentially to hepatocellular carcinoma (HCC) (Browning and Horton, 2004; Arteel, 2010). Hepatic steatosis can be diagnosed via several methods including the elevation of serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels as biomarkers, imaging techniques, for instance, computerized tomography (CT) and magnetic resonance imaging (MRI) (Paschos and Paletas, 2009). Nonetheless, Nalbantoglu and Brunt (2014) suggest that liver biopsy remains as the gold standard via identifying specific pathological lesions of the disease, both range and state. Fat accumulates in the liver of patients with hepatic steatosis mainly in the form of triglycerides (Jacome-Sosa and Parks, 2014; Buzzetti *et al*., 2015). Triglycerides are derived from the esterification of glycerol and free fatty acids (FFAs). They then enter storage or secretory pools. FFAs are obtained either from diet or adipose tissue via lipolysis and/or from hepatic *de novo* lipogenesis (DNL). Once in the hepatocytes, FFAs form fatty acyl-CoAs via acyl-CoA synthases activity and enter either esterification or β-oxidation pathways (Ferramosca and Zara, 2014).

Non-alcoholic fatty liver disease (NAFLD).

NAFLD represents a spectrum of liver disease encompassing steatosis, non-alcoholic steatohepatitis (NASH), and cirrhosis in the absence of alcohol abuse (Anstee and Goldin, 2006; Buzzetti *et al*., 2015). NASH has been reported worldwide and is increasingly recognized as the leading cause for liver dysfunction and cirrhosis in the non-alcoholic, viral hepatitis negative population in Europe and North America (Anstee and Goldin, 2006). Prevalance of NAFLD in Asia including Thailand have been noted lower than in western countries (Ashtari *et al*., 2015). It was reported that NAFLD is strongly associated with obesity, metabolic syndromes, dyslipidemia, insulin resistance (IR), and type II (non-insulin dependent) diabetes mellitus, and documented as risk factors of NAFLD (Powell *et al*., 1990; Anstee and Goldin, 2006; Buzzetti *et al*., 2015). NAFLD is now considered to be a hepatic manifestation of metabolic syndromes (Marchesini *et al*. 2003; Anstee and Goldin, 2006).

Pathogenesis of NAFLD.

The underlying mechanism for the development and progression of NAFLD is complex and multifactorial. Different theories have been formulated. The classical “two-hit hypothesis” was initially used to describe the pathogenesis of NAFLD. According to this theory, the first hit is the hepatic accumulation of fat secondary to sedentary lifestyle, high fat diet, obesity, and IR. The second hit is the sensitization of liver to activation of inflammatory cascades and fibrogenesis (Leamy *et al*., 2013; Peverill *et al*., 2014). However, later on, it was found that this view is too simplistic to explain the pathogenesis of NAFLD. As a result, a “multiple hits hypothesis” is now used to explain the pathogenesis and progression of NAFLD (Buzzetti *et al*., 2015). Those multiple hits include as follows.

Insulin Resistance.

In patients with NAFLD, fat is accumulated in the liver as triglycerides formed by esterification of glycerol and FFAs (Jacome-Sosa and Parks, 2014; Buzzetti *et al*., 2015). FFAs in the blood stream come from 1) gut absorption from diet, 2) lipolysis of adipose tissue, and 3) hepatic DNL (Musso *et al*., 2013; Buzzetti *et al*., 2015). Once FFAs enter

hepatocytes, they form fatty acyl-CoAs through acyl-CoA synthases activity. Fatty acyl-CoAs may enter either esterification or β-oxidation pathways (Buzzetti *et al*., 2015). Hepatic DNL can be increased by activation of transcription factors such as sterol regulatory element-binding protein-1 (SREBP-1), carbohydrate response element-binding protein (ChREBP), and peroxisome proliferator-activated receptor (PPAR) (George and Liddle, 2008). SREBP-1c regulates the activation of DNL and is stimulated by insulin. SREBP-2 involves in cellular cholesterol homeostasis in which its abnormal function results in hepatic fat accumulation. ChREBP is activated by glucose and increases DNL but also provides more substrate for triglyceride and FFAs synthesis (Buzzetti *et al*., 2015). An increase in DNL via activation of these transcription factors can results in an increase in hepatic fat accumulation. Generally, insulin receptor substrate 2 (IRS-2) functions by inhibiting SREBP-1c (Schreuder *et al*., 2008). In patients with IR, IRS-2 is down-regulated. Therefore SREBP-1c is un-inhibited and over-expressed, resulting in an increase in DNL and hepatic fat accumulation. Moreover, β-oxidation of FFAs is inhibited in IR patients, promoting hepatic fat accumulation (Buzzetti *et al*., 2015). FFAs in hepatocytes activate serine-kinase which induces the defects of insulin signaling pathways, resulting in IR. Insulin suppresses adipose tissue lipolysis while IR lowers this suppression, resulting in the increase in efflux of FFAs to the liver (Lewis *et al*., 2002).

Mitochondrial Dysfunction.

Structural and functional alterations in mitochondria contribute to the pathogenesis of NAFLD (Buzzetti *et al*., 2015). Structural alterations include depletion of mitochondrial DNA, morphological, and ultrastructural changes. Functional alterations include respiratory chain and mitochondrial β-oxidation (Buzzetti *et al*., 2015). Alteration of mitochondrial or peroxisomal function may impair ability to handle the increased lipid flux, resulting in impairment of fat homeostasis, generation of lipid derived toxic metabolites, and overproduction of reactive oxygen species (ROS), contributing to hepatocytes necro-inflammation and worsening of mitochondrial damage (Cusi, 2009; Buzzetti *et al*., 2015).

Endoplasmic Reticulum Stress.

An increased protein synthetic input, a primary dysfunction of endoplasmic reticulum (ER) or lack of adenosine triphosphate (ATP) can lead to unfolded proteins accumulation within the ER, activating unfolded protein response (UPR), which is an adaptive response aiming to resolve ER stress (Wang and Kaufman, 2014). Hyperglycemia, ATP depletion due to mitochondrial injury, hypercholesterolemia, phosphatidylcholine depletion, and oxidative stress, are factors that induce UPR in NAFLD (Seki *et al*., 2005). UPR leads to activation of c-Jun N-terminal kinase (JNK), an activator of inflammation and apoptosis which leads to steatosis and steatohepatitis. UPR also activates SREBP-1c pathways, resulting in an increase in hepatic DNL (Buzzetti *et al*., 2015).

Adipose Tissue Dysfunction.

Adipose tissue generally secretes hormones (adipokines) such as leptin and adiponectin. Obesity-related adipocyte hypertrophy and/or IR result in an imbalance of adipokines (Gregor and Hotamisligil, 2007). Adipose tissue contributes to the maintenance of low grade inflammatory states by producing pro-inflammatory cytokines; serum levels of interleukin-6 (IL-6) and adipocytes expression of tissue necrosis factor alpha (TNF-α) are increased in obese patients, and subsequently declined following weight loss. The increases in pro-inflammatory cytokines accelerate the progression of NAFLD from simple steatosis to NASH and fibrosis (Plessis *et al*., 2015). Leptin is a 16-kDa anorexigenic hormone with pro-inflammatory actions that prevents lipid accumulation in non-adipose sites (Kakuma *et al*., 2000). In the liver, this is achieved by lowering the expression of SREBP-1. However, obese subjects are leptin resistant, increasing leptin level, resulting in its pro-fibrogenic action. It was suggested that leptin stimulates Kupffer cells to produce transforming growth factor beta 1 (TGF-β1) and subsequently activate hepatic stellate cells (Kakuma *et al*., 2000; Buzzetti *et al*., 2015). Adiponectin improves hepatic and peripheral IR, and exhibits anti-inflammatory and hepatoprotective activities by enhancing

the deacylation of the sphingolipid ceramide independently of AMP-activated protein kinase (AMPK) by blocking the activation of nuclear factor-kappa B (NF-ΚB), secreting anti-inflammatory cytokines, and inhibiting the release of pro-inflammatory cytokines such as TNF-α and IL-6. In obese patients, reduced adiponectin and increased leptin levels may result in hepatic steatosis and activation of inflammation and fibrogenesis (Buzzetti *et al*., 2015).

Genetic Determinants.

Genetic variants, especially in the form of single nucleotide polymorphisms (SNPs), influence hepatic FFAs flux, oxidative stress, response to endotoxins and cytokine production and activity, influencing NAFLD development and progression (Anstee *et al*., 2011). Patatin-like phospholipase 3 (PNPLA3) SNP contributes to the development and progression of NAFLD, in particular the I148 M (rs738409 C/G) variant. PNPLA3 gene encodes for a protein called adiponutrin which has significant homologies to enzymes implicated in lipid metabolism processes and could exert a lipolytic activity on triglycerides (Anstee and Day, 2013). The PNPLA3 148M allele is associated with lower DNL and expression of the lipogenic transcription factor SREBP-1c, despite a substantially increased hepatic fat content, while hepatic β-oxidation is apparently not influenced by the PNPLA3 genetic variant. Furthermore, hepatic lipid accumulation in PNPLA3 148M carrier is associated with a decrease in secretion of triglyceride-rich lipoproteins from the liver (Mancina *et al*., 2015). A variant of transmembrane 6 superfamily member 2 (TM6SF2) gene is also possibly associated in NAFLD pathogenesis. TM6SF2 protein promotes very low density lipoprotein (VLDL) secretion, while the rs58542926 variant, through a loss of function, is associated with hepatic steatosis, lower plasma levels of VLDL and higher ALT levels (Buzzetti *et al*., 2015).

Dietary Factors.

Dietary factors, both in terms of quantity and caloric intake but also of specific nutrients, contribute to the development of NAFLD and NASH (Buzzetti *et al*., 2015). Fructose is a lipogenic, pro-inflammatory dietary factor that results in oxidative stress and upregulation of TNF-α (Buzzetti *et al*., 2015). It is metabolized by specific hepatic kinases, independently of insulin action, into fructose-1-phosphate before being converted into triose phosphate, which enters the glycolytic pathway generating substrates for DNL. In a mouse model, fructose-induced NAFLD is associated with bacterial overgrowth and increased intestinal permeability. Fructose can also induce copper deficiency in mice and subsequent NAFLD (Moore *et al*., 2014). Conversely, coffee, mono-unsaturated fats, and moderate alcohol consumption were found to prevent NAFLD while combination of high fat diet and intermittent alcohol consumption was associated with an increase in steatosis, inflammation, and fibrosis (Molloy *et al*., 2012; Kanuri *et al*., 2016).

In Vitro Models for NAFLD.

Primary Cell Cultures and Immortalized Cell Lines.

In general, primary cell cultures and immortalized cell lines are widely used to develop *in vitro* models. Primary human hepatocytes (Gomez-Lechon *et al*., 2004), Kupffer cells, stellate cells or sinusoidal endothelial cells are physiologically relevant models for clinical conditions (Dambach *et al*., 2005). However, ethical issues and limited number of human liver samples make it extremely complicated to employ primary human cell cultures. The other option would be primary rodent cells, which depending on the model used may more or less mimic the situations found in human. However, methods used for isolation need to be well established in order to achieve reproducible outcomes and sometimes cells loose tissue-specific functions when cultured for an extended time. An alternative to primary cell cultures are immortalized cell lines, which have an extended replicative capacity, a stable phenotype, and enable for the use of the same consistent cells throughout a study (Kanuri and Bergheim, 2013).

Co-Culture Model.

In the complex architecture of *in vivo* hepatic tissue, the interaction of two or more cells is a common

phenomenon. Accordingly, reproducing these settings in *in vitro* experiments can be a reliable model to investigate the cell to cell interaction in the progression of disease. RAW 264.7 macrophages and AML-12 cells being models of Kupffer cells and murine hepatocytes, respectively, were concomitantly exposed to fructose and/or lipopolysaccharide (LPS) in the presence of inhibitors (Spruss *et al*., 2011). The results obtained from the co-culture model suggest that an activation of Kupffer cells by endotoxin may trigger the induction of inflammatory cytokines in other cell types in the liver (e.g., hepatocytes), which play a vital role in the progression of fructose-induced non-alcoholic fatty liver disease (Spruss *et al*., 2011).

Three-Dimensional Cell Culture Model.

Until now, most of *in vitro* studies have utilized two-dimensional monolayer cell cultures to elucidate the molecular mechanisms involved in the progression of NAFLD. However, recent studies have shown that three-dimensional liver cell cultures may better mimic the physiological settings of the liver rather than two-dimensional monolayer liver cell cultures (Kanuri and Bergheim, 2013). Janokar *et al*. 2011 developed a model of 3D spheroids by using elastin-like peptide polyethyleneimine (ELP-PEI) and showed that ELP-PEI coated surfaces influence the morphology of H35 rat hepatoma cells to create 3D spheroids. This model was predominantly used to investigate the transcriptional regulation of genes involved in hepatic steatosis. Furthermore, the results for the first time have shown the transcriptional dynamics (e.g., activation, suppression) of NF-ΚB in response to TNF-α stimulation (Janokar *et al*., 2011).

In Vivo Models for NAFLD.

Research models of NAFLD may be divided into two broad categories, those caused by either spontaneous or induced genetic mutation and those with an acquired NAFLD phenotype. The latter group may be produced by either dietary or pharmacological manipulation (Anstee and Goldin, 2006).

A Model of Genetically Determined Leptin Deficiency (The ob/ob Mouse).

The *ob/ob* mouse carries a spontaneous mutation first observed whilst intercrossing inbred mouse strains, resulting in leptin deficiency. This autosomal recessive trait renders animal hyperphagic, inactive, obese, and severely diabetic with marked hyperglycemia (Mayer *et al*., 1951; Anstee and Goldin, 2006). Histological features include enlarged islets of Langerhans (consistent with compensatory hyperplasia due to IR) and hepatic fat deposition (Anstee and Goldin, 2006). Early parabiotic experiments demonstrated that the *ob/ob* mice were unable to produce a satiety factor but could respond to such a factor from a donor animal (Coleman, 1978). The *ob/ob* mouse is a complex model of obesity-related steatosis. Lipid and carbohydrate metabolism is deranged in several ways; the expanded white adipose tissue mass in the *ob/ob* mice and the increased expression of TNF-α promote adipose tissue lipolysis releasing long chain fatty acids (LCFAs). The increased circulating LCFAs are delivered to the liver. In addition, SREBP-1c is activated and accumulated in the *ob/ob* hepatocyte nuclei, promoting fatty acid synthase activity and de novo synthesis (Shimomura *et al*., 1999). The increased synthesis and storage of lipid in the liver coincident with expanded adipose tissue stores contribute to hepatic steatosis and obesity (Anstee and Goldin, 2006).

A Model of Genetically Determined Leptin Resistance (The db/db Mouse).

Mutations in the diabetes (*db*) gene resulted in autosomal recessive diabetic, obese phenotype similar to the *ob/ob* mouse (Anstee and Goldin, 2006). The *db/db* mice have normal or elevated level of leptin but are resistant to its effects. Studies have shown that the *db* gene encodes the leptin receptor (Tartaglia *et al*. 1995). The mutated receptor isoform lacks its signaling activity, resulting in loss of function and consequently leptin resistance (Chen *et al*. 1996).

Models of Genetically Determined Increased Circulating LCFAs.

The transmembrane protein CD36 (fatty acid translocase) is an important fatty acid transporter expressed in peripheral tissues including muscle and adipose tissue. CD36 null mouse exhibits elevated circulating LCFAs and triglyceride levels and hepatic IR due to impaired fatty acid storage. The affected mice develop steatosis and fail to suppress hepatic gluconeogenesis (Coburn *et al*., 2000; Goudriaan *et al*., 2003).

Models of Genetically Determined Hepatic Lipogenesis: SREBP-1 promotes hepatic lipogenesis. Two non-obese transgenic mouse models (PEPCK-nSREBP-1a mice and aP2-nSREBP-1c mice) with severe hepatic steatosis have been described (Shimano *et al*. 1996). PEPCK-nSREBP-1a mice overexpress SREBP-1a in the liver under the control of a phosphoenolpyruvate carboxykinase promoter (Shimano *et al*. 1996). These animals exhibit histological steatosis but not steatohepatitis or dyslipidaemia, although ALT levels are elevated. aP2-nSREBP-1c mice overexpress SREBP-1c in adipose tissue. These animals have a lipoatrophic phenotype associated with steatosis (Shimomura *et al*., 1998). The study has shown that SREBP-1-mediated hepatic lipogenesis proceeds even in the presence of IR (Shimomura *et al*. 1999).

Models of Genetically Determined Reduced -β-Oxidation.

Several induced and spontaneous genetic mutations that are associated with steatosis due to defective β-oxidation have been described. The mutations all affect key regulatory transcription factors, the import of LCFAs into mitochondria, and enzymatic activity within the oxidative cascade. Systemic carnitine deficiency is induced by a mutation in the carnitine transporter gene or organic cation transporter 2 (*Octn2*) and leads to failure of fatty acid transport into mitochondria for β-oxidation (Anstee and Goldin, 2006). This model develops extreme steatosis within days of birth. Similarly, acyl-CoA oxidase ($AOX^{-/-}$) mutant mice carry a deletion in a key enzyme in peroxisomal β-oxidation. Initially, animals are phenotypically normal but over an 8-week period, mice develop severe steatosis, but this then spontaneously resolves as steatotic hepatocytes are replaced. Mice carrying a homozygote PPAR-α knockout do not accumulate fat under normal fed conditions but fail to upregulate fatty acid oxidation, and so develop severe steatosis when fatty acid delivery to the liver is increased by fasting (Kersten *et al*., 1999; Koteish and Diehl, 2001).

Models of Acquired Reduced β-Oxidation (Methionine/ Choline Deficiency).

Impaired β-oxidation may be induced by drugs including an estrogen antagonist, tamoxifen, or the carnitine palmitoyltransferase I (CPT1) inhibitor, etomoxir (Koteish and Diehl, 2001), however, the main research model in which an acquired defect in mitochondrial β-oxidation is used to induce steatosis is based on feeding a diet deficient in methionine and choline. Choline is an essential nutrient with roles in cell membrane integrity, transmembrane signaling, phosphatidylcholine synthesis, neurotransmission, and methyl metabolism. The role of dietary choline deficiency in promoting hepatic steatosis was thought to be due to impair synthesis of phosphatidylcholine resulting in diminished VLDL assembly and secretion, and consequently reduced TG clearance (Yao and Vance, 1990). Mice fed a diet that is deficient in both choline and the essential amino acid methionine (MCD) develop inflammation and hepatic fibrosis in addition to simple steatosis (Weltman *et al*., 1996). The magnitude of the effect of this diet varies according to species, strain and gender of animals (Kirsch *et al*., 2003).

Fructose-Rich Diets.

A shift in dietary patterns towards a sugar rich diet may be a risk factor for development of NAFLD in human (Spruss and Bergheim, 2009). Carbohydrate-rich diets (e.g., high-sucrose or high-fructose diets) have been used to induce the development of NAFLD in mouse models. C57BL/6 mice *ad libitum* access to different mono- and disaccharides in drinking water revealed that fructose had

the most damaging effect on the liver despite having the least impact on body weight gain (Bergheim *et al*., 2008). Furthermore, fructose in chow (e.g., up to 60% of total calories derived from fructose) or drinking solutions (e.g., 30% fructose solution) not only leads to the development of NAFLD but also IR (Bergheim *et al*., 2008). Similar to what was reported from high fat diet (HFD) models, diets rich in fructose lead to oxidative stress, expression of pro-inflammatory cytokines, and SREBP-1c in the liver, and elevated endotoxin levels in portal blood as well as alterations of adipocytokine expression in visceral adipose tissue in a dose- and time-related manner (Bergheim *et al*., 2008). However, similar to the results found for feeding HFDs, high fructose diets did not cause liver injury as severe as that found in the MCD diet model, despite more closely resembling not only the pathological and molecular alterations but also the dietary patterns found in humans with NAFLD (Kanuri *et al*., 2013).

Western-Style or Fast Food Diet: Combination of Fat and Sugar.

A combination of both fat and fructose with slightly elevated cholesterol content referred to as Western style or fast food diet has repeatedly been used as a dietary model to induce NAFLD in rodents. Charlton *et al*. 2011 showed that after feeding C57BL/6 mice for 6 months with a fast food diet consisting of a high-fat chow (40% of energy from fat with 2% cholesterol) and drinking water enriched with high fructose corn syrup (HFCS), 42 g/L final concentration, the mice not only became overweight and IR but also developed NASH with an increase in fibrosis signature-gene expression.

Alcoholic Fatty Liver Disease (AFLD).

Alcohol consumption is a leading cause of global morbidity and mortality, with much of the burden resulting from initiation of AFLD. Excessive alcohol intake leads to liver damage through its direct action as a hepatotoxin1 as well as potentiation of other liver diseases, including chronic viral hepatitis and NAFLD. Patients with continued excessive alcohol consumption are at risk for development of fibrosis and cirrhosis. Twenty to 40% of patients with steatosis progressed to fibrosis, of which 8-20% developed cirrhosis (Altamirano and Bataller, 2011). As in other liver diseases, patients with cirrhosis are at risk for hepatic decompensation (ascites, variceal bleeding, and encephalopathy) and HCC (Orman *et al*., 2013). Although the most important risk factor for AFLD is the absolute amount of alcohol intake, multiple other factors play a role in host susceptibility. Women are at greater risk of AFLD, as are Mexican, black non-Hispanic Americans, and Thailand. Obesity may potentiate the hepatotoxic effects of alcohol, presumably through mechanisms similar to those result in NAFLD. Smoking and the pattern of alcohol intake are associated with the increased risk of AFLD (Orman *et al*., 2013; Summart *et al*., 2017).

Pathogenesis of AFLD.

Steatosis is the first response of the liver to alcohol abuse. It is defined histologically as the deposition of fat in hepatocytes (Salaspuro *et al*., 1981; Gao and Bataller, 2011; Orman *et al*., 2013). Early studies indicate that alcohol consumption increases the ratio of reduced nicotinamide adenine dinucleotide to oxidized nicotinamide adenine dinucleotide ($NADH/NAD^+$) in hepatocytes, which disrupts mitochondrial β-oxidation of fatty acids and resulted in steatosis. Alcohol intake has also been shown to augment the supply of lipids to the liver from the small intestine, increasing mobilization of fatty acids from adipose tissue and uptake of fatty acids by the liver (Gao and Bataller, 2011; Orman *et al*., 2013). Alcohol exposure, directly or indirectly, regulates lipid metabolism-associated transcription factors. This stimulates lipogenesis and inhibits fatty acid oxidation. Ethanol increases fatty acid synthesis in hepatocytes via upregulation of SREBP-1c, promoting fatty acid synthesis (Orman *et al*., 2013). Alcohol consumption could directly increase transcription of SREBP-1c gene via its metabolite acetaldehyde or indirectly up-regulate SREBP-1c expression by activating processes and factors

that stimulate SREBP-1c expression, such as endoplasmic reticulum response to cell stress, adenosine, endocannabinoids, LPS signaling via Toll-like receptor (TLR), and its downstream proteins. Alcohol also down-regulates factors that reduce SREBP-1c expression, such as AMPK, Sirtuin1, adiponectin, and signal transducer and activator of transcription 3 (STAT3) (Gao and Bataller, 2011). Alcohol consumption inhibits fatty acid oxidation in hepatocytes mainly via inactivation of the PPAR-α, a nuclear hormone receptor that controls transcription of a range of genes involved in FFA-transport and oxidation. Acetaldehyde, an ethanol metabolite, directly inhibits the transcriptional activation activity and DNA-binding ability of PPAR-α in hepatocytes (Orman *et al*., 2013). In addition, ethanol can indirectly inhibit PPAR-α via up-regulation of cytochrome P450 2E1-derived oxidative stress and adenosine, both of which inhibit PPAR-α, or via down-regulation of adiponectin and zinc, which each activates PPAR-α (Gao and Bataller, 2011). In addition to regulation of fat metabolism-associated transcription factors, ethanol can affect the activities of enzymes involved in fat metabolism by inhibiting AMPK, which reduces fat metabolism and fatty liver (Orman *et al*., 2013). AMPK is a serine-threonine kinase that phosphorylates and subsequently inactivates acetyl-CoA carboxylase (ACC), a rate-limiting enzyme for fatty acid synthesis. Inactivation of ACC reduces level of malonyl-CoA, a precursor in fatty acid synthesis and an inhibitor of carnitine palmitoyltransferase 1, a rate-limiting enzyme for fatty acid oxidation. In addition, AMPK directly phosphorylates and inhibits SREBP activity in hepatocytes, promoting steatosis. In this manner, AMPK inhibits fatty acid synthesis but promotes fatty acid oxidation via inactivation of ACC enzyme activity. Alcohol inhibits AMPK activity in the liver, leading to a decrease in phosphorylation with an increase in ACC activity, followed by a decrease in carnitine palmitoyltransferase 1 activity (Gao and Bataller, 2011; Orman *et al*., 2013). Finally, autophagy has an important role in removing lipid droplets in hepatocytes. Long-term alcohol consumption inhibits autophagy. However, a recent study showed that short-term ethanol exposure activates autophagy by generating reactive oxygen species and inhibiting the mammalian target of rapamycin, indicating that acute ethanol activation of autophagy could have a compensatory role to prevent the development of steatosis during the early stages of alcoholic liver injury (Gao and Bataller, 2011).

In Vitro Models for AFLD.

In Vitro Acute Alcohol Abuse Model.

The *in vitro* AFLD model is based on supplementation of culture media with pure alcohol, usually 200 mmol/L of ethanol. One of the major concerns on the *in vitro* alcohol treatment using concentrations above 100 mmol/L is the direct cytotoxic effect (Dolganiuc and Szabo, 2009). At lower concentrations (< 100 mmol/L), alcohol changes the redox status of the cells and alters intercellular junctions, increases the cell membrane fluidity, and affects the composition of lipid rafts, all of which may contribute to alcohol-mediated increase in transcellular and paracellular permeability, and thus effect on the cell function. Alcohol also affects the expression of adhesion molecules, which may be a concern when using adherent cell types due to possible cell detachment. Additional concerns arise from the possibility of modified *ex vivo* function of some primary cells, including hepatocytes, stellate cells, and their precursors, due to limited *ex vivo* environment compared to *in vivo* condition (Dolganiuc and Szabo, 2009). One other main characteristic of the *in vitro* acute alcohol abuse (AAA) model is its simplicity, often considered as an advantage or disadvantage depending on the research goal. Most of the *in vitro* study involves culture of a single cell type or co-culture of several cell types (Dolganiuc and Szabo, 2009). While such an approach brings forward the differential effect of alcohol on pure cell populations, and/or their intercellular interaction, it still lacks the systemic alcohol metabolism and inter-cellular interactions (Dolganiuc and Szabo, 2009).

Human-Induced Pluripotent Stem Cells Model.

In recent years, human-induced pluripotent stem cells (iPSCs) have been generated from diverse human somatic cells, which can then be differentiated into a spectrum of mature human cell types including functional hepatocytes, enabling unlimited supply of hepatocytes that retain the same genetic information (Tian *et al*., 2014). Tian *et al*. (2014) exposed different stages of iPSC-induced hepatic cells to ethanol at a pathophysiological concentration (100 mmol/L). Ethanol at the pathophysiological dosage significantly reduces the expression of α-fetoprotein, an early hepatic cell marker, and induces cell apoptosis, during differentiation of iPSC-derived endoderm into hepatic progenitor cells. Proliferative activity of more mature stage hepatic cells is significantly lowered. Amount of lipid droplets are increasingly detected in ethanol-treated iPSC-derived hepatocytes compared to controls (Tian *et al*., 2014).

Precision-Cut Liver Slices from Rats.

Klassen *et al*. (2008) have developed the use of precision-cut liver slices (PCLS) as an *in vitro* culture model to investigate how ethanol causes alcohol-induced liver injury. The PCLS retained excellent viability as determined by lactate dehydrogenase and ATP levels over a 96-h period of incubation. More importantly, the major enzymes of ethanol detoxification, including alcohol dehydrogenase, aldehyde dehydrogenase, and cytochrome P450/2E1, remained active, and PCLS readily metabolized ethanol and produced acetaldehyde. Normal lobular hepatic architecture is maintained, and the cell-cell and cell-matrix interactions mimic the *in vivo* situation. PCLS also contains hepatocytes and the other non-parenchymal cells of the liver, namely Kupffer cells, endothelial cells, and stellate cells, in which these other cell types likely contribute to the deleterious effects of ethanol (Klassen *et al*., 2008).

In Vivo Models for AFLD.

It is well known that AFLD results from the dose- and time-dependent alcohol consumption. Numerous species have been used to study liver pathology caused by alcohol, including rodents, micropigs, and non-human primates (Arteel, 2010). Of these species, the pathology in non-human primates (e.g. baboon) is arguably the most similar to that in human. However, high maintenance cost limits the utility to the field. Cost issues are a similar concern with the micropig model of AFLD. As a result, the majority of alcohol research performed in rodents (i.e., rats and mice) (Arteel, 2010).

Acute Animal Models of Alcohol Exposure.

Acute administration over the course of hours or a few days is the simplest model of alcohol exposure. An acute model of alcohol exposure can clearly be used to a model that damage due to an acute binge drinking episode. Acute exposure can in principle be used to mimic very early changes caused by ethanol (Wheeler *et al*., 2001). However, the major effects of ethanol on liver are generally biochemical (e.g., alterations in lipid/glucose metabolism) in acute models (Arteel, 2010). Pathologic changes caused by acute ethanol exposure are generally minor under these conditions (e.g., lipid accumulation) (Arteel, 2010). Although acute ethanol causes limited pathological changes in the liver, it is useful for studying the modified responses of liver to other xenobiotics. Specifically, acute ethanol exposures at doses relatively non-toxic enhance the toxicity of other compounds. In addition to liver pathology, acute ethanol exposure has been utilized to study the effects of *in vivo* ethanol exposure on subsequent responses to stimuli in culture. For example, Kupffer cells exposed to acute ethanol *in vivo* are more easily stimulated and produce more inflammatory cytokines than naïve cells (Enomoto *et al*., 1998). Additionally, hepatocytes are sensitized to cytotoxic cell killing by ethanol administration (Arteel, 2010).

Chronic Models of Alcohol Exposure: Ad libitum Feeding.

A major advantage of *ad libitum* ethanol feeding is that it is not technically demanding, generally requiring only daily changes of the ethanol-containing liquid. Oral alcohol administration by drinking is clearly the most relevant to

clinical AFLD (Arteel, 2010). Therefore, initial animal model of ethanol exposure attempted to mimic human condition and utilized *ad libitum* access to ethanol-containing water. While this model was quite simple, there are major concerns as rodents have a strong aversion to the taste or smell of ethanol, and therefore do not consume large enough quantities of ethanol to produce significant liver pathology (Rodd-Henricks *et al*., 2002; Arteel, 2010). In an attempt to overcome the aversion of ethanol, alternative techniques of *ad libitum* ethanol delivery have been developed, such as forced choice model of alcohol exposure, in which ethanol is incorporated into a liquid diet that serves as the only source of calories. The ethanol intake for rats on *ad libitum* diet is sufficient to produce liver injury, especially fat accumulation (Lieber and DeCarli 1989).

Chronic Models of Alcohol Exposure: Enteral Feeding.

One mechanism of bypassing aversion to ethanol in experimental animals is by eliminating *ad libitum* control of ethanol exposure. Ethanol can be enterally delivered directly to the stomach via a surgically implanted intragastric tube. Ethanol is infused at a constant rate over the course of the day instead of given *ad libitum* (Wheeler *et al*., 2000).The major advantage of the enteral feeding model is that the pathology obtained in this model, in as little as 4 weeks of enteral diet, mimics AFLD in human, including micro- and macro-vesicular steatosis, apoptosis, mixed inflammatory cell infiltrate, and focal necrosis (Arteel, 2010). Furthermore, fibrosis and cirrhosis can be produced in rats using this model by including carbonyl iron in the diet or prolonging the time course of enteral feeding. Fibrotic changes associated with later stages of alcohol-induced pancreatic damage also occurred as early as after 8 weeks of enteral feeding (Kono *et al*., 2001). Another advantage of the enteral model is the high level of control on diet delivery. This effect avoids control-paired caloric concerns associated with *ad libitum* feeding (Arteel, 2010). Although the pathology caused by enteral alcohol feeding is more severe and therefore more relevant to the situation in human than *ad libitum* feeding, there are limitations associated with use of this model. First, the enteral model is a technically demanding method of ethanol delivery. Although the surgery is not complex, it does require a dedicated rodent surgery facility to perform this model. Furthermore, due to the higher levels of alcohol achieved in this model, the relative amount of husbandry required during enteral feeding compared to *ad libitum* protocol is greater (Arteel, 2010).

Alternative Models of Experimental Alcoholic Liver Disease.

Another reliable approach for induction of fibrosis and cirrhosis in rats is feeding of a diet deficient in choline (Hironaka *et al*., 2000). Choline deficiency causes significant steatosis presumably via decreases in lipotropes as early as 1-2 weeks following initiation of diet. Long-term feeding of choline-deficient diet causes hepatitis, fibrosis, and cirrhosis, and hepatocellular carcinoma after prolonged feeding (Arteel, 2010). The advantages of this model are technical ease of feeding diet and induction of advanced liver pathology associated with chronic AFLD (Arteel, 2010).

Conclusion.

The prevalence of fatty liver disease tends to be increasing in industrialized countries. It can be classified according to the underlying causes as NAFLD and AFLD. Major advances have been made in our understanding of the mechanisms for the development of fatty liver diseases. The pathogenesis of NAFLD is strongly associated with obesity, dyslipidemia, and IR. The multiple-hit hypothesis is now currently used to explain the pathogenesis and progression of NAFLD instead of the classical two-hit hypothesis. Those multiple hits include insulin resistance, mitochondrial dysfunction, endoplasmic reticulum stress, adipose tissue dysfunction, genetic determinants, and dietary factors. There may be additional factors contributing to the occurrence and progression of NAFLD, adding to the list of “hits”. Because of the advancements in our understandings about the pathogenesis of NAFLD, several models, both *in vitro* and *in vivo*, have been developed, targeting the pathways that contribute to NAFLD. *In vitro*

models include cultures that try to best mimic the liver environment, including the co-culture model and three-dimensional liver cell cultures. *In vivo* models include genetically modified animal models that result in fat accumulation in the liver and acquired NAFLD phenotype animals where they were fed modified diets to induce NAFLD. AFLD is the accumulation of fat in the liver induced by alcohol consumption. The contribution of alcohol to AFLD is via both its direct hepatotoxicity and through pathways that dysregulates hepatic lipid metabolism. Experimental models of AFLD are mainly performed by ethanol exposure. *In vitro* models are produced by supplementing the culture media with ethanol. The cultures that represent the liver in this case include hepatocyte cell lines, hepatocytes differentiated from human-induced pluripotent stem cells, and precision-cut liver slices from rats. *In vivo* animal models are produced by alcohol administration, either acute or chronic. Acute alcohol exposure models are produced by feeding alcohol over the course of hours or a few days. Chronic alcohol exposure models can be produced by adding alcohol to drinking water or delivering directly to the stomach via intragastric tube. Other alternative models for AFLD that produce the same pathological conditions without administering alcohol are animals fed with choline deficient diets. Both *in vitro* and *in vivo* model have many advantages that their genome is similar to human genome, which high stability and high producibility (Vandamme, 2014) These models can be employed for further study and fill the knowledge gap of fatty liver diseases, either to create a better understanding of the pathogenesis and progression of the disease, or to develop new therapeutic options for patients with fatty liver disease, which is still growing in number and having limited treatment of choice.

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