Pathogenesis of and major animal models used for nonalcoholic fatty liver disease

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Abstract
Nonalcoholic fatty liver disease (NAFLD) and its pathologically more severe form, nonalcoholic steatohepatitis (NASH), have become prevalent worldwide and carry an increased risk of developing hepatocellular carcinoma and other metabolic diseases. Diverse animal models have been proposed to replicate particular characteristics of NAFLD and NASH and have provided significant clues to the critical molecular targets of NASH treatment. In this review, we summarize the histopathology, pathogenesis, and molecular basis of NAFLD progression and discuss the benchmark animal models of NAFLD/NASH.

Keywords
Nonalcoholic fatty liver disease, nonalcoholic steatohepatitis, histopathology, pathogenesis, dietary model, genetic model

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Introduction
Nonalcoholic fatty liver disease (NAFLD) represents a progressive liver disorder ranging from simple liver steatosis to nonalcoholic steatohepatitis (NASH), fibrosis, cirrhosis, and ultimately hepatocellular carcinoma, in the absence of excessive alcohol intake.\textsuperscript{1,2} NAFLD is becoming one of the most alarming chronic liver diseases because it is one of the fastest growing indicators for adult liver transplantation and a major cause of hepatocellular carcinoma.\textsuperscript{3–6} Development of NAFLD has a strong association with metabolic abnormalities such as obesity, insulin resistance (IR), and type 2 diabetes, and NAFLD itself is a risk factor for cardiovascular disease. Patients with NAFLD are at high risk of dying from cardiovascular disease and other metabolic diseases.\textsuperscript{7–9} NASH is the pathologically more severe form of NAFLD and is characterized by hepatocellular ballooning, active hepatocellular necrosis, and liver inflammation with the presence of steatosis; moreover, it is associated with more rapid progression of fibrosis and cirrhosis.\textsuperscript{10,11} Given the rapid growth in NAFLD prevalence, further research on the exact pathogenic mechanisms and potential drug treatments for NASH is imperative. Established animal models have vividly highlighted the important aspects of each stage of NAFLD and provide significant clues to the critical molecular events during NAFLD development, which opens up new opportunities for treatment of NAFLD in humans. This review will summarize the pathogenesis and molecular basis of NAFLD and discuss the benchmark animal models that recapitulate the histopathology and pathophysiology associated with human NAFLD.

Histopathology of NAFLD and NASH
Intracytoplasmic lipid accumulation in the form of triglycerides is an iconic feature of NAFLD. Liver biopsy followed by histological analysis is the gold standard for confirming the presence and activity of NAFLD, which is histologically diagnosed when hepatic triglyceride accumulation occurs in more than 5% of hepatocytes.\textsuperscript{12} Grading and staging systems for NAFLD consider a wide spectrum of histopathology features. In particular, semiquantitative scoring assesses 4 major histological features: steatosis (0–3), hepatocellular ballooning (0–2), inflammation (0–3), and fibrosis (0–4).\textsuperscript{13,14} The size of fat droplets can differ; macrovesicular steatosis is the predominant pattern seen in NAFLD and is characterized by large vacuoles that occupy the whole cytoplasm and push the nucleus to one side of the cell. Some NAFLD patients, however, present with multiple small lipid vacuoles in the cytoplasm and the nucleus remains unmoved, which is termed “microvesicular steatosis.”\textsuperscript{15} Hepatocellular ballooning, which refers to cells with swollen and rarefied cytoplasm, is a distinguishing feature of progression to NASH.\textsuperscript{16} Hepatocellular ballooning is often associated with Mallory-Denk bodies, which result from the clumping of cytokeratins and subsequent ubiquitination.\textsuperscript{17} Inflammation is another remarkable feature of NASH development. Lobular inflammation and portal inflammation can both present in NASH. Lobular inflammation, which is characterized by the presence of small clusters of inflammatory cells near ballooned hepatocytes, reflects the dysregulation of cytokine and chemokine expression in the fatty liver.\textsuperscript{10,14,18} Portal inflammation is
common and usually mild in NASH patients. Increased portal inflammation is associated with many clinical and pathologic features of progressive NASH and may be considered a marker of aggravation and advanced disease.\textsuperscript{19} NASH patients often develop a typical “chicken-wire” fibrosis surrounding individual or groups of hepatocytes that is termed “pericellular fibrosis.” This finding reflects progression of NASH and it can further spread to the portal areas and subsequently lead to septal fibrosis and even cirrhosis.\textsuperscript{20} Representative haematoxylin and eosin (H&E)-stained sections of human NASH and murine steatohepatitis are shown in Figure 1.

**Pathogenesis and molecular basis of NAFLD and NASH**

The pathogenesis of NAFLD and the factors that promote progression from simple steatosis to NASH are complex. Lipid accumulation in hepatocytes and its interplay with inflammatory responses, cellular stress, and cell death are believed to be the major factors contributing to NAFLD development.\textsuperscript{21–24} Genetic factors and intestinal dysbiosis are also crucial.\textsuperscript{25}

Steatosis occurs whenever the rate of import or synthesis of lipid by hepatocytes exceeds the rate of export or degradation.\textsuperscript{26,27} Triglyceride is the most conspicuous type of lipid in the livers of NAFLD patients, so steatosis can be graded according to the extent of triglyceride accumulation. However, the triglycerides are not hepatotoxic compared with the other types of lipids that accumulate in the fat liver (including fatty acids, diacylglycerol, oxysterols, cholesterol, and phospholipids), so steatosis grade or severity does not predict hepatic injury, inflammation, or fibrosis.\textsuperscript{28,29} Overnutrition and particularly IR

![Figure 1. Histopathological features of human and murine nonalcoholic fatty liver disease (NAFLD) and nonalcoholic steatohepatitis (NASH) as determined by haematoxylin and eosin (H&E) staining. (a) Healthy human liver; (b) simple liver steatosis in human; (c) human NASH with hepatocellular ballooning and inflammation; (d) healthy murine liver (C57BL/6 mice fed a normal diet); (e) liver of obese ob/ob mice fed a normal diet that spontaneously developed liver steatosis; and (f) liver of C57BL/6 mice fed a methionine- and choline-deficient (MCD) diet for 4 weeks; mice developed steatosis with notable inflammation. Scale bars, 20 μm](image-url)
are closely associated with the etiology of steatosis and provide the initiating and propagating damage for liver injury and resultant inflammation.\textsuperscript{30,31} Fatty acid accumulation, in turn, exacerbates IR and hyperinsulinemia, leading to further steatosis and inflammation.\textsuperscript{32–34} Overnutrition increases free acid influx from diets and consequent uptake by the liver, resulting in increases in \textit{de novo} lipogenesis in the liver. Overnutrition also induces chronic inflammation and promotes IR.\textsuperscript{35,36} IR is tightly associated with lipid accumulation in the liver and subsequent steatosis. IR promotes increased efflux of free fatty acids (FFA) from adipose tissues and overwhelsms FFA uptake by the liver because insulin cannot suppress adipose tissue lipolysis via hormone-sensitive lipase when IR occurs.\textsuperscript{36} IR promotes lipid accumulation in the liver primary by mediating uptake of FFA via the scavenger receptor CD36 and uptake of free cholesterol (FC) via CD36 and oxidised low-density lipoprotein (ox-LDL).\textsuperscript{37} IR-associated hyperinsulinemia and hyperglycaemia promote hepatic \textit{de novo} lipogenesis by upregulating the key lipid synthesis regulator sterol regulatory element-binding protein isoform 1c (SREBP-1c) and the glucose metabolism regulator carbohydrate response element-binding protein (ChREBP), respectively.\textsuperscript{38} In addition, hyperinsulinaemia can directly suppress \(\beta\)-oxidation of FFA.\textsuperscript{39} More importantly, IR-associated hyperinsulinemia is implicated in driving the accumulation of cytotoxic lipid species such as FC in the liver and activating the c-Jun N-terminal kinase (JNK) signaling pathway, resulting in mitochondrial damage and hepatocyte injury in a process called “lipotoxicity.”\textsuperscript{25,40} Molecules released from damaged hepatocytes further promote changes in signaling pathways that regulate cellular stress (such as oxidative stress and endoplasmic reticulum stress) and inflammatory responses, thus perpetuating hepatocellular injury and subsequent cell death and promoting NAFLD development.\textsuperscript{41–43}

An increase in the production of pro-inflammatory cytokines, such as tumor necrosis factor (TNF)-\(\alpha\) and interleukin (IL)-6, and activation of toll-like receptors (TLR) and the NLRP3 inflammasome are critically involved in pathophysiology of various aspects of NASH.\textsuperscript{44,45} TLR4 links to the activation of nuclear factor (NF)-\(\kappa\)B and macrophage recruitment in steatohepatitis.\textsuperscript{46} The NLRP3 inflammasome, which is highly expressed in liver, is associated with IL-1\(\beta\) release.\textsuperscript{47} These elements and their interaction perpetuate liver damage, inflammation, and fibrosis, resulting in progression of NASH (Figure 2).

**Dietary and genetic animal models of NAFLD and NASH**

**High-fat diet**

The high-fat diet (HFD) model is a good simulation of the modern Western diet. The main calorie intake (energy) of HFD is derived from fat (45% to 75%). Animals fed with HFD can replicate the major histopathology and pathogenesis seen in human NAFLD. With long-term HFD feeding, animals develop obesity, IR, and hepatic damage.

The classic HFD model was established in male rats and involved feeding a diet with 71% fat, 11% carbohydrates, and 18% protein for 3 weeks. A standard diet containing 35% fat, 47% carbohydrates, and 18% protein was used as the control; this diet has the same fat content as the average US diet. Rats fed HFD developed steatosis, IR, mitochondrial dysfunction, and mononuclear inflammation, accompanied by increased hepatic TNF-\(\alpha\) and cytochrome P4502E1 (CYP2E1) induction.\textsuperscript{48} Another frequently used HFD animal model was established in mice. Male mice (C57BL/6...
Male mice (C57BL/6 strain) that received the same HFD for up to 16 weeks became obese and showed steatosis, hepatocyte ballooning, increased serum glucose, and decreased adiponectin, indicating hyperglycaemia and IR. Similarly, our group found that male C57BL/6 mice fed a HFD (60% fat, 20% carbohydrates, and 20% protein) for 12 weeks developed steatosis (Figure 3a and 3b).

HFD diets can replicate the hallmark features of altered metabolic parameters seen in human NAFLD but the degree of hepatic pathology is not as severe. Increasingly, studies use additional elements in the HFD to more closely mimic human NAFLD, such as diets supplemented with fructose, cholesterol, or both.

**High-fat, high-fructose diet**

A significantly increased consumption of calories from fructose-rich foods has been confirmed to be closely associated with development of human NAFLD and severity of fibrosis. Male mice (C57BL/6 strain) that were fed a high-fat, high-fructose (HFHF) diet—that is, a HFD (58 kcal% fat) supplemented with 42 g/L of carbohydrates (mixed at a ratio of 55% fructose and 45% sucrose by weight) in drinking water—for 16 weeks developed more severe hepatic oxidative stress, increased hepatic macrophage aggregation, and exacerbated liver fibrosis compared with mice fed HFD without carbohydrate supplementation. However, both groups showed gains in body weight and body fat mass and increased steatosis, fasting glucose, and IR, indicating that fructose consumption is required for NAFLD progression.

Fructose can promote de novo lipogenesis in liver, inhibits β-oxidation, and induces hepatic insulin, which result in rapid development of intrahepatic lipid accumulation. Excessive consumption of fructose also promotes intestinal bacterial overgrowth and leads to hepatocellular damage, thus triggering NAFLD progression.
High-fat, high-cholesterol diet

High cholesterol intake can induce dyslipidemia and IR, and it has been recognized as a critical factor associated with hepatic inflammation and NAFLD progression in both animal models and humans.\(^{56-58}\)

Male mice (C57BL/6 strain) fed a high-fat, high-cholesterol (HFHC) diet (15% fat and 1% cholesterol) for 30 weeks became obese and developed more profound hepatic steatosis and inflammation, as well as typical perisinusoidal fibrosis compared with mice fed a single HFD or high-cholesterol diet, both of which resulted in increased hepatic steatosis with little inflammation and no signs of fibrosis. Mice fed with HFHC diets also showed hypercholesterolaemia and a significant reduction in serum adiponectin levels.\(^{57}\)

Obese \(foz/foz\) mice (deficient in the \(Alms1\) gene) fed a HFD containing different percentages of cholesterol (0.0%, 0.2%, or 2.0%) for 24 weeks showed different outcomes. Mice fed with 2.0% cholesterol had higher hepatic cholesterol content and much higher alanine aminotransferase (ALT) levels than other groups, suggesting that increased accumulation of free cholesterol is associated with NAFLD progression.\(^{59}\)

Cholesterol increases hepatic oxidative stress and promotes hepatic apoptosis, macrophage recruitment, and fibrogenesis, thus triggering NAFLD progression.

Methionine- and choline-deficient diet

Feeding animals a methionine- and choline-deficient (MCD) diet is a commonly used nutritional model for NASH. MCD diets are usually highly enriched in sucrose (40%) and moderately enriched with fat (10%) but deficient in methionine and choline, which are essential for hepatic \(\beta\)-oxidation and production of very-low-density lipoprotein (vLDL).\(^{60}\)

Depriving animals of methionine and choline causes notable steatosis, inflammation, hepatic ballooning, reactive oxygen species
(ROS)-mediated liver damage, and fibrosis.\(^2\)

Hepatic steatosis can be seen within 1 to 2 weeks of MCD induction.\(^60,61\) Moreover, mice fed with MCD diets developed extensive necro-inflammation as early as 2 weeks, and the typical chicken-wire fibrosis can be seen as early as 6 weeks after MCD induction, similar to the presentation in human NASH.\(^62,63\) MCD feeding also increases serum ALT levels and induces ballooning degeneration of hepatocytes in mice.\(^64\) Similarly, our group found that male C57BL/6 mice fed with MCD diets developed steatosis and inflammation at 4 weeks (Figure 3c). The severity of steatohepatitis in MCD-fed mice is associated with impaired hepatic adiponectin action and adipogenic transformation of hepatocytes.\(^64\) The responsiveness that develops in mice fed the MCD diet depends on sex and strain. For example, C57BL/6 mice exhibited more pronounced release of transaminases than did DBA/2J mice, whereas long-term MCD induction caused more severe liver injury, even hepatocarcinogenesis, in DBA/2J mice, but did not result in carcinogenesis in C57BL/6 mice.\(^61,65\) The notable inflammation observed in MCD-induced steatohepatitis is associated with increased macrophage infiltration in the liver, activation of the NF-κB signaling pathway, and concomitant increases in downstream pro-inflammatory cytokines, such as TNF-α, monocyte chemoattractant protein (MCP)-1, transforming growth factor (TGF)-β, and IL-6.\(^66-68\) MCD diets also promote induction of adhesion molecules, such as intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1, which are essential for polymorph recruitment.\(^68-70\)

Animal models using MCD diets can replicate the hallmark pathological features of severe human NASH more closely than other dietary-based animal models. The steatosis, inflammation, and fibrosis induced by MCD diets develop more quickly than with HFD and other Western diet models. Cellular stress, such as endoplasmic reticulum (ER) stress, oxidative stress, and auto-phagocytic stress, is more pronounced in the MCD model than in other dietary-based NAFLD models.\(^71\)

However, the MCD diet model has obvious disadvantages. Mice fed MCD diets always exhibit significant loss of body weight and the liver decreases proportionally in size, which go against effects seen in overweight and obese individuals with NAFLD.\(^2,72\) In addition, the metabolic profile in the MCD model is opposite to that seen in NAFLD patients: serum levels of triglyceride, insulin, leptin, and fasting glucose are dampened, whereas serum adiponectin is not decreased.\(^72\) Therefore, \(db/db\) (deficient in leptin receptor activity) or \(ob/ob\) (deficient in leptin) mice are often used in the MCD model to better imitate human NASH. Findings suggest that \(db/db\) mice fed with MCD diet for 4 weeks show remarkable hepatic inflammation and fibrosis.\(^73\)

\(ob/ob\) and \(db/db\) mice

Leptin-deficient (\(ob/ob\)) mice, which carry an autosomal recessive mutation in the leptin gene, develop spontaneous liver steatosis under normal chow feeding. \(ob/ob\) mice are grossly overweight and show the altered metabolic parameters seen in human NAFLD, such as hyperinsulinaemia, hyperglycaemia, and IR.\(^60,74,75\) However, \(ob/ob\) mice are resistant to hepatic fibrosis, given that leptin is essential for the hepatic fibrogenic response to liver injury.\(^76\) In addition, the \(ob/ob\) mouse model is limited to spontaneous steatohepatitis unless secondary insults are added (such as a HFD or MCD diet or administration of small doses of lipopolysaccharide endotoxin).\(^77\) Our group found that male \(ob/ob\) mice (C57BL/6 strain) fed MCD diets developed
| Model | Diet formula (kcal%) | Obesity | Insulin resistance | Steatosis | Steatohepatitis | Fibrosis |
|-------|----------------------|---------|---------------------|-----------|-----------------|---------|
| Methionine- and choline-deficient diet (MCD) | 40% sucrose, 10% fat, methionine (−), choline (−) | No | Hepatic insulin resistance | Yes | Yes | Yes |
| High-fat diet (HFD) | 45% to 75% fat. Classic model is 71% fat, 18% protein, and 11% carbohydrates | Yes | Yes | Yes | Yes, but mild | Yes, but mild |
| High-fat, high-fructose diet (HFHF) | HFD supplemented with fructose (usually 23 g/L in drinking water) | Yes | Yes | Yes | Yes, but mild | Yes, but mild |
| High-fat, high-cholesterol diet (HFHC) | Approximately 1% cholesterol fed in conjunction with HFD (usually 15% to 40% fat) | Yes | Yes | Yes | Yes | Yes |
| ob/ob mice | — | Yes | Yes | Yes | No\(^1\) | No\(^1\) |
| db/db mice | — | Yes | Yes | Yes | No\(^1\) | No\(^1\) |
| foz/foz mice | — | Yes | Yes | Yes | No\(^1\) | No\(^1\) |

\(^1\)Although these mice do not develop steatohepatitis and fibrosis spontaneously, additionally feeding with MCD diets or HFD can promote development of steatohepatitis and fibrosis (not in ob/ob mice)
steatosis and inflammation at 4 weeks (Figure 3d). db/db mice are homozygous for the autosomal recessive diabetic gene (db), which encodes a point mutation in the leptin receptor and leads to defective leptin signaling. db/db mice are obese and diabetic and develop macrovesicular hepatic steatosis accompanied by hyperglycaemia and hyperinsulinaemia. db/db mice are obese and diabetic and develop macrovesicular hepatic steatosis accompanied by hyperglycaemia and hyperinsulinaemia. Unlike ob/ob mice, db/db mice exhibit normal or elevated levels of leptin but are resistant to its effects. Similarly, db/db mice do not spontaneously develop inflammation or show features of NASH without further insulin. The ob/ob and db/db mice are good genetic models of NAFLD because they develop pronounced hepatic steatosis and show the significant altered metabolic characteristics seen in human NAFLD. db/db mice can also be used to study the progression of steatosis to NASH in the presence of secondary insults such as a MCD diet. However, congenital leptin deficiency or leptin resistance caused by gene mutations is not prevalent in obese humans or NASH patients, so the ob/ob and db/db mice models are limited in their ability to reflect the genesis of human obesity or NASH.

foz/foz mice

Obese foz/foz mice, which carry a mutated Alms1 gene, spontaneously develop hepatic steatosis, obesity, diabetes, and IR, and show significant upregulation of cholesterol levels. HFD feeding can accentuate transition of simple steatosis to steatohepatitis by aggravating metabolic abnormalities, resulting in severe hepatocyte ballooning, inflammation, and fibrosis, accompanied by significant decreases in adiponectin levels and increases in cholesterol levels. However, despite upregulation of hepatic triglyceride content, serum triglyceride levels remain unchanged in foz/foz mice, even those fed with HFD. All foz/foz mice are obese but the severity of NASH is strain dependent. Serum ALT levels and NAFLD activity score were higher (worse) in foz/foz C57BL6/J mice than in foz/foz BALB/c mice fed with HFD. Moreover, HFD-induced fibrosis was severe in foz/foz C57BL6/J mice but absent in foz/foz BALB/c mice.

To date, diverse animal models have been proposed to mimic particular characteristics of human NAFLD, such as the American lifestyle–induced obesity syndrome (ALIOS) model, the diet-induced animal model of nonalcoholic fatty liver disease (DIAMOND) model, and the Ldlr−/− mice model. Recently, a murine NASH model was proposed that showed rapid progression of extensive fibrosis and hepatocellular carcinoma. The model used a Western diet, which contained high fat, high fructose, and high cholesterol, combined with a low weekly dose of intraperitoneal carbon tetrachloride (CCl4). This model captures the progressive stages of human fatty liver disease, from simple steatosis to inflammation, fibrosis, and cancer. It is important to choose the appropriate animal model to meet the research purpose (Table 1).

Conclusion

NAFLD is becoming a worldwide issue because of changes in lifestyle and resultant overnutrition. Lipid accumulation in the liver and its interplay with inflammation, oxidative stress, cell death, and autophagy is considered a major process of NAFLD progression. However, the exact mechanisms of NAFLD progression remain largely unknown. The use of animal models to replicate the important aspects of NAFLD progression provides significant clues to the critical molecular events that occur during NAFLD development and suggests a number of therapeutic targets for future treatment of NAFLD. Nevertheless, none of established animal models are
perfect and it is important to choose the appropriate animal model to meet the research purpose.

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