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REVIEW

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Experimental models of fatty liver diseases: Status and appraisal

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Abstract

Fatty liver diseases, including alcohol-associated liver disease (ALD) and nonalcoholic fatty liver disease nonalcoholic fatty liver disease (NAFLD), affect a large number of people worldwide and become one of the major causes of end-stage liver disease, such as liver cirrhosis and hepatocellular carcinoma (HCC). Unfortunately, there are currently no approved pharmacological treatments for ALD or NAFLD. This situation highlights the urgent need to explore new intervention targets and discover effective therapeutics for ALD and NAFLD. The lack of properly validated preclinical disease models is a major obstacle to the development of clinical therapies. ALD and NAFLD models have been in the development for decades, but there are still no models that recapitulate the full spectrum of ALD and NAFLD. Throughout this review, we summarize the current *in vitro* and *in vivo* models used for research on fatty liver diseases and discuss the advantages and limitations of these models.

INTRODUCTION

Alcohol consumption and metabolic syndrome are both very common in the population and often coexist. Both conditions are associated with a wide range of health problems, with alcohol-associated liver disease (ALD) and nonalcoholic fatty liver disease NAFLD being the most commonly occurring hepatic consequences.[1,2] Approximately 2.5 billion people worldwide drink alcohol, and 300 million of them suffer from alcohol use disorder. Every year, ~25 million individuals suffer from alcohol-induced compensatory cirrhosis, resulting in 750,000 deaths, or 1% of all deaths.[3,4] NAFLD prevalence has increased dramatically over the past few decades due to high caloric intake and inactive lifestyles.[5] Both alcohol and obesity increase the risk of liver cancer, fibrosis, and death associated with liver disease. ALD and NAFLD are now global public health and economic problems.

Simulating and understanding human disease often involve the use of models based on cells and

Abbreviations: ALD, alcohol-associated liver disease; ALT, alanine aminotransferase; AST, aspartate aminotransferase; Adh1, Alcohol dehydrogenase 1; Aldh2, aldehyde dehydrogenase 2; ApoE, apolipoprotein E; APAP, acetaminophen; BAC, blood alcohol concentration; CCl4, carbon tetrachloride; CXCL1, chemokine (C-X-C motif) ligand 1; CYP2E1, cytochrome P4502E1; CD, choline-deficient diet; CDAA, Choline-deficient L-amino-defined diet; CDAHFD, choline deficiency, L-amino acid–defined high-fat diet; DEN, diethylnitrosamine; eIF2α, eukaryotic translation initiation factor 2α; ER, endoplasmic reticulum; FFAs, free fatty acids; HCC, hepatocellular carcinoma; HFD, high fat diet; HFFC, high-fat, high-fructose, and high-cholesterol diet; HFHC, high-fat, high-cholesterol; HSCs, hepatic stellate cells; IL-8, interleukin 8; IRE1α, inositol requiring protein 1α; JNK, c-Jun N-terminal kinase; KCs, Kupffer cells; LDL, low density lipoprotein; LDE diet, Lieber-De Carli ethanol diet; LESCs, liver sinusoidal endothelial cells; LPS, lipopolysaccharide; MCD, methionine-deficient and choline-deficient diet; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; PERK, Protein kinase R -Like Endoplasmic Reticulum Kinase; PPAR-α, peroxisome proliferator-activated receptor α; STAM, streptozotocin + HFD-treated mice; STZ, streptozotocin; TAA, thioacetamide

animals. Various animal models have been developed to study ALD and NAFLD over the years; however, they are not capable of accurately replicating human drinking patterns and mimicking human alcohol-associated liver injury.[6] Moreover, animal models of NAFLD exhibit similar problems since NAFLD is a multisystem disease that affects the liver, kidneys, and cardiovascular system. It is, therefore, extremely difficult to develop a comprehensive experimental model that can simulate the multifactor pathogenesis of NAFLD.[7]

Finding the "ideal" experimental model will be essential to advancing research on pathogenesis and developing new treatment strategies for liver diseases. As part of this review, we aim to summarize the current animal and *in vitro* models used in ALD and NAFLD research, and discuss their advantages and disadvantages with respect to human disease conditions.

Current experimental models of ALD

The pathophysiology of ALD is complex. Excessive alcohol consumption can lead to cytotoxic metabolites, reactive oxygen species accumulation, and lipid peroxidation, manifested by hepatocyte necrosis, apoptosis, lipid deposition, inflammation, and advanced fibrosis.[8,9] Despite rodents' natural aversion to ethanol, researchers have found that ethanol can be incorporated into a liquid diet to overcome natural aversion, leading to a high blood alcohol concentration (BAC).[10] Since then, numerous studies have identified a variety of mechanisms that lead to ALD, such as acetaldehyde toxicity, reactive oxygen species, cytochrome P4502E1 (CYP2E1) activation, mitochondrial dysfunction, lipid metabolism disorder, and the gut-liver axis.[8,9,11–13] Most ALD studies are currently conducted on rodents (Table 1); however, the current rodent models are only capable of simulating the early stages of ALD (mainly steatosis and mild to moderate steatohepatitis).[14] Furthermore, rodents have a catabolic rate 5 times faster than humans.[15] Due to this, rodents suffer less damage than humans after having been exposed to alcohol. In addition to differences in alcohol metabolism, the innate immune system also plays a significant role in the pathogenesis of ALD.[16] For example, the proportion of neutrophils and lymphocytes in the blood of mice and humans varies greatly: neutrophils constitute 50%–70% of the white blood cell count in humans (10%–25% in mice). These differences remain largely unknown in terms of their pathophysiological consequences.[17] Some studies have used methods such as inhalation or i.p. injection in addition to alcohol intake through diet, but these methods are far from the natural drinking patterns of people with ALD.

Binge drinking model

A high-dose single alcohol exposure mouse model is suitable for assessing the effects of a single binge episode in humans.[18] Single alcohol gavage rapidly increases BAC in a dose-dependent and time-dependent manner, with peak blood alcohol levels at 1–2 hour after alcohol administration, and BAC significantly decreased 6 hours later (<50 mg/dL) and 9 hours to baseline.[18–21]. High BAC allows more alcohol to enter the portal circulation so that the liver experiences the most severe alcohol-induced tissue damage.[6] Researchers have recently used a simple binge model to determine how alcohol damages the liver. A low dose of alcohol gavage (<5 g/kg) does not induce dramatic chemistry parameters and morphological cellular changes; however, a high-dose alcohol exposure (7 g/kg) causes a significant elevation of serum alanine aminotransferase and aspartate aminotransferase levels.[21]

Based on the findings, the alcohol-associated liver injury was maximized 9 h after 7 g/kg alcohol administration; nevertheless, it is not recommended to provide >8 g/kg alcohol dose due to high mortality.[21] After a high-dose single ethanol exposure, the mRNA expressions of *Gadd34*, *Bak*, *Bim*, and *Ero1b* were significantly elevated together with the elevation of endoplasmic reticulum (ER) stress–related proteins (Protein kinase R -Like Endoplasmic Reticulum Kinase, eukaryotic translation initiation factor 2α, inositol requiring protein 1α, and c-Jun N-terminal kinase) and proapoptotic proteins. Serum alanine aminotransferase levels were higher in alcohol dehydrogenase 1 (*Alcohol dehydrogenase 1*) deficient mice, which do not convert ethanol to acetaldehyde, than in WT mice after a single ethanol binge. In agreement with this finding, hepatocyte death and hepatic ER stress–related proteins were higher in *Alcohol dehydrogenase 1* KO mice than those in WT mice after high-dose alcohol intoxication. Binge drinking–associated nonoxidative alcohol metabolism has recently been implicated in the pathogenesis of alcohol-induced liver injury through the production of fatty acid ethyl esters, esterification products of alcohol, and free fatty acids (FFAs).[22] Binge drinking–mediated nonoxidative metabolites cause ER stress, adipocyte mortality, and lipolysis, all of which contribute to a single binge–associated liver injury.

Chronic rodent ALD models

Voluntary drinking model

In voluntary drinking patterns, rodents have access to food and drinking water containing ethanol [0%–40% (v/v)], which is similar to human drinking patterns. Because rodents have a natural aversion to ethanol, their consumption of alcohol is relatively low, and their high rate of ethanol metabolism ensures that ethanol does not

TABLE 1 Summary of rodent models of alcohol-associated liver disease

Models	Characteristics	Pathological features			Advantages and disadvantages
		ALT and AST levels	Inflammation	Fibrosis and cirrhosis	
Binge drinking model	Single alcohol gavage	++	+	No	Simple with no mortality rate; mild levels of steatosis and inflammation
Voluntary drinking model	Oral alcohol in drinking water	+	+	No	Easy to perform; low BAC; no liver fibrosis
Lieber-DeCarli liquid diet model	Chronic ethanol feeding (4–12 wk)	++	+	No	Easy to perform; short-term or long-term feeding with no mortality rate; no liver fibrosis
Tsukamoto-French intragastric infusion model	Intragastric infusion (8–12 wk)	+++	++	++	High BAC; mild liver fibrosis; difficult to perform; requirement for intensive medical care; high mortality rate
NIAAA/Gao-Binge model	LDE diet plus a single binge (10 d) or multiple binges (8 wk)	+++	+++	+ (multiple binges)	Time efficient; significant steatosis and neutrophil infiltration; mild liver fibrosis
"Second or multiple-hit" model	LDE diet + Second hit: LPS, CCl4, DEN, APAP (4–12 wk)	+++	++	+++	Induction of end-stage liver injuries; toxic components; complex etiology
Genetically engineered model	CYP2E1 transgenic mice; Aldh2 knockout mice; etc.	NA	NA	NA	Research status is not mature

Abbreviations: APAP, acetaminophen; BAC, blood alcohol concentration; CCl4, carbon tetrachloride; DEN, diethylnitrosamine; LDE diet, Lieber-De Carli ethanol diet; LPS, lipopolysaccharide.

cause significant liver damage to them.[15,23] Only mild steatosis was observed in mice exposed to low concentrations (10%–20%) of ethanol-containing drinking water.[24,25] In addition, rats that drank water with a similar concentration of ethanol for 177 days did not exhibit any significant liver damage.[26] There is a clear lack of satisfaction with this level of liver damage by researchers.

Lieber-DeCarli liquid diet model

There is no doubt that the Lieber-DeCarli model is one of the most commonly used rodent models in ALD research, which is an important advancement in this field. By feeding the animals only liquid feed containing ethanol, the model overcame the rodents' aversion to ethanol.[27] An animal that is fed an increasing liquid ethanol diet (from 1% to 5%, w/v) for 1 week, followed by a continued 5% ethanol diet for 4–12 weeks, will exhibit a distinct phenotype of liver damage.[28,29] As a result, this model is suitable for all laboratories due to its simplicity of operation, low price, short time frame, and low animal mortality. Although the model did not cause severe liver damage, it is an effective tool for studying the early stages of ALD.[30]

Tsukamoto-French intragastric infusion model

To maintain high BAC in animals and cause liver fibrosis and cirrhosis, in 1984, researchers developed a new feeding model of direct infusion through a surgically implanted intragastric tube, known as the Tsukamoto-French model.[31,32] The use of this method to deliver ethanol to rats induced steatosis, apoptosis, fibrosis, and mixed inflammation that are very similar to the inflammatory responses seen in human ALD.[30] A gastric infusion model was also established in mice and proved to be successful.[33] Since the Tsukamoto-French model can achieve higher BAC levels and more severe liver damage, it becomes a useful tool for studying advanced ALD. In addition, the controllability of feed composition allows a method to be used for the study of ALD, which is associated with a number of pathogenic factors.[34] It should be noted, however, that this model is limited in its application due to the need for more complex surgery and more expensive equipment.

National Institute on Alcohol Abuse and Alcoholism (NIAAA)/Gao-Binge model

The long-term consumption of alcohol is one of the major factors leading to steatosis and steatohepatitis. Chronic plus binge mimics ALD patients' drinking pattern of "long history of alcohol consumption and recent binge drinking." The Gao group created the original model in mice in 2010 and named it the NIAAA model[35] that is also called the Gao-Binge model later.[36–38] Mice were fed an alcohol liquid diet for 10 days and then force-fed with acute ethanol (5 g/kg). Nine hours later, the mice were euthanized for liver injury examination. An increase in hepatic oxidative stress and the production of proinflammatory cytokines were observed in this model, as well as significant steatosis and injury to the liver.[35,39] Through a variety of mechanisms, a single overdose of ethanol results in neutrophil-mediated liver injury in chronically ethanol-fed mice, including increased expression of E-selectin, activation of natural killer T cells, the release of ER stress-dependent mitochondrial DNA-enriched particles, and activation of neutrophils.[39–43] The NIAAA-/Gao-Binge model has been now widely used in the field due to its simple procedure and excellent reproducibility.

In 2015, the team improved the ethanol chronic plus binge model. The combination of single or multiple ethanol binges with long-term chronic ethanol feeding in mice resulted in greater neutrophil infiltration and liver damage.[44] There are several histological and molecular characteristics of advanced clinical alcohol-associated steatohepatitis, which are outlined in this model. In addition to being flexible and easy to use, this model is suitable for exploring the pathological mechanism of hepatitis.

In addition, efforts have been made to develop ALD models of chronic plus binge in rats. Compared with long-term ethanol feeding alone or multiple alcohol binges, this pattern resulted in higher plasma alanine aminotransferase levels, steatosis, inflammation, and neutrophil infiltration.[45,46]

"Second or multiple-hit" model

During the chronic feeding phase of the Lieber-DeCarli diet, other hepatotoxins (eg, lipopolysaccharide, carbon tetrachloride, diethylnitrosamine, and acetaminophen) can be added to cause further liver damage.[47–49] These models provide a useful way to study the effects of ethanol on the occurrence and progression of severe liver injury, such as liver cirrhosis or HCC. Nevertheless, it is clear that there are some differences between liver damage caused by ethanol combined with second or more liver poisons and liver damage caused by ethanol itself, which researchers need to be aware of.

Genetically engineered model

Researchers have confirmed that many genes play important regulatory roles in the occurrence and development of ALD. CYP2E1, for example, is an important contributor to oxidative stress, hepatotoxicity, and DNA damage in ALD. Cederbaum et al transferred humanized CYP2E1 into mice and found that liver damage was significantly increased after 3 weeks of ethanol feeding.[50]

Other studies have found that aldehyde dehydrogenase 2 (*aldehyde dehydrogenase 2*) deficiency in mice promotes alcohol-associated cancers.[51] Although the use of genetically engineered mice as a single standard model for the study of ALD is not yet mature, it deserves further investigation in the future as an important tool for exploring new mechanisms and developing potential drugs for the treatment of advanced ALD.

Other experimental models of ALD

Nonrodent models

In the study of ALD, nonhuman primates have long been considered to be ideal animal models. ALD studies using nonhuman primates are, however, not feasible in most laboratories due to reasons such as high costs and ethical concerns.[52]

Zebrafish is an effective vertebrate model for studying liver diseases. Its basic physiological processes are similar to those of humans, and its genetic similarities are high.[53] Alanine aminotransferase and triglyceride levels were increased in adult male zebrafish treated with 0.2% ethanol (v/v) water for 4 weeks.[54] In addition, higher concentrations of ethanol (2%) can significantly increase zebrafish liver damage and lipid accumulation.[55] Therefore, despite the differences in physiology, zebrafish still has certain advantages in ALD model research because of their high reproduction rate, economy, easy maintenance, convenient genome editing, and fewer ethical constraints.[56]

In vitro models

In vitro ALD studies have contributed to our understanding of key pathogenic processes and molecular mechanisms, including 2-dimensional monolayer cell culture models, 3-dimensional cell culture models, and liver microarrays. The 2-dimensional monolayer cell cultures as *in vitro* models have been widely used for many years. After incubation with 100 mM ethanol for 24 hours, lipid accumulation and increased mRNA expression of lipid synthesis–related genes were observed in AML-12 cells, HepG2 cells, and mouse primary hepatocytes.[57,58] The complex structure of the liver, however, cannot be fully simulated by monolayer cell culture.[59]

Organoid technology is an *in vitro* system for replicating tissue in a 3-dimensional format *in vitro* within a culture apparatus.[60] More recently, Wang et al developed an *in vitro* organoid construction method that can reproduce the typical phenotype of ALD pathophysiology by integrating human fetal liver mesenchymal cells into expandable hepatic organoids.[61] To mimic ALD in humans, the researchers also used liver chips of hominid cells.[62] The liver microarray consists of primary hepatocytes, Liver sinusoidal endothelial cells (LSECs) and Kupffer cells. After stimulating the chip with 0.08% ethanol and lipopolysaccharide for 48 hours, lipid accumulation, mitochondrial reactive oxygen species, and the release of proinflammatory factors were significantly increased.[63] As the liver model on the chip can be used to reconstruct the 3-dimensional structure on a microscopic scale, key characteristics of liver cells in an alcohol environment can be reproduced.[59]

CURRENT EXPERIMENTAL MODELS OF NAFLD

In NAFLD, there is a series of diseases characterized by asymptomatic steatosis that progresses to steatohepatitis (NASH) and eventually fibrosis.[64] The "multiple hits" hypothesis may be more appropriate since liver cells are required to undergo numerous molecular and metabolic changes to undergo these changes, including oxidative stress, lipid peroxidation, and inflammation.[65] However, as there are currently no approved drug treatments for NAFLD, a deeper understanding of NAFLD pathology is required to identify promising pharmacological targets.[7,66] Despite the use of a large number of animal models, it continues to be a challenge to select those that are most closely related to human pathology and translate the results obtained into clinical applications.[15]

A variety of animal models are available for studying metabolic diseases, but rodents are the most commonly used (Table 2). They are more likely to develop obesity, type 2 diabetes, and NAFLD.[67]

Mouse models of NAFLD

Dietary models

Animal models of NAFLD and NASH rely on various types of diets, such as choline-deficient diets (CDs), high-fat diets (HFDs), and high-fructose and high-cholesterol diets, which, alone or in combination, induce varying degrees of steatosis and steatohepatitis.[7]

The methionine and choline-deficient diet (MCD) or choline-deficiency, L-amino acid–defined diet is widely used in animals because it can cause significant liver damage and fibrosis. Combined with the above model, choline deficiency, L-amino acid–defined high-fat diet (CDAHFD) is another widely used model in NAFLD/NASH studies. The most commonly used formulation of CDAHFD is a high-fat, CD consisting of 0.1% methionine and 45% fat. After 6 weeks of feeding, CDAHFD models showed liver steatosis, liver injury, inflammation, and liver fibrosis. This model can better simulate human NAFLD pathology. Since most animal models of CD

TABLE 2 Summary of rodent models of NAFLD

Rodent model	Obesity	Insulin resistance	Steatosis	Inflammation	Fibrosis	HCC
Dietary						
MCD	Weight loss	No	++	+++	+++	No
CDAA	No	No	++	++	++	Yes
CDAHFD	No	No	+++	++	++	Yes
HFD	Yes	Yes	+++	+	+	No
HFHC	Yes	Yes	+++	++	+	No
High-fructose diet	No	Yes	++	+	No	No
HFFC	Yes	Yes	+++	++	++	No
Genetic						
ob/ob	Yes	Yes	+++	+	No	No
db/db	Yes	Yes	+++	+	No	No
fa/fa	Yes	Yes	+++	+	No	No
ApoE$^{-/-}$	Yes	Yes	+++	++	+	NA
PPAR-α$^{-/-}$	No	No	++	++	+	No
CD36$^{-/-}$	No	Yes	+++	No	No	No
Chemical						
Tetracycline	No	No	+++	+	++	NA
CCl_4	No	No	++	++	+++	Yes
TAA	NA	NA	++	++	++	Yes
Combined models						
STAM	No	Yes	+++	++	++	Yes
ob/ob + MCD	Yes	Yes	+++	+++	+++	No
HFD + CCl_4	No	No	+++	++	+++	Yes

Abbreviations: CDAA, Choline-deficient L-amino-defined diet; CDAHFD, choline deficiency, L-amino acid–defined high-fat diet; HFD, High-fat diet; HFFC, high-fat, high-fructose, and high-cholesterol diets; HFHC, high-fat, high-cholesterol; MCD, methionine-deficient and choline-deficient diet; STAM, streptozotocin + HFD-treated mice; TAA, thioacetamide.

do not exhibit weight gain or changes in glucose tolerance, they are considered to be distinct from the clinical pathogenesis of the disease.[68,69]

A high-calorie diet with excessive nutrients (mainly fats and sugars) is more similar to the progression of human disease, with differences in the phenotype ultimately induced by different amounts of fat (40%–60% of calories from fat) and different sources of fat (eg, using vegetable oils from coconut or soybeans rather than animal fats from lard or milk).[70,71] The "traditional" HFD diet usually contains only moderate levels of cholesterol (0.2%) and relies on sucrose as the main source of carbohydrates. The main disadvantage of HFDs is that they need to be used for long periods of time (over 1 year); otherwise, they do not induce significant inflammation and fibrosis in the liver.[7,66] Therefore, to overcome this limitation, increasing the cholesterol content in the HFD feeding regimen for 16 weeks resulted in more significant liver damage and fibrosis. This high-fat, high-cholesterol model promotes NASH progression by activating TAZ in the liver, a transcriptional regulator that promotes fibrosis.[72,73] Henkel et al [74] recently extended these findings, demonstrating that adding 0.75% cholesterol to a plant-based HFD significantly increases the level of inflammation and liver fibrosis at the histological level, and increased dietary fructose intake in addition to cholesterol is an important risk factor for NAFLD in humans.[75] Models of high-fat, high-fructose, and high-cholesterol diets are often used to establish animal models of metabolic syndrome. The high-fat, high-fructose, and high-cholesterol diets diet typically consists of 40% fat, 20% fructose, and 2% cholesterol, which was formerly known as the amylin liver NASH diet.[76] This model has great utility in developing new therapeutic targets for NASH and new drugs to improve extrahepatic complications. In practice, a growing number of studies are currently using a HFD rich in high levels of fructose and cholesterol (such as the western diet) to induce NASH within a reasonable time frame (~4–6 mo).[77]

Genetic models

At present, many transgenic animals are used to construct NAFLD models. Leptin deficiency (ob/ob) mice and leptin receptor deficiency (db/db) mice are the most commonly used genetic models of NAFLD. Due to the deficiency of

leptin or its receptors, they have hyperappetite and develop severe obesity, hyperlipidemia, hyperglycemia, and insulin resistance but rarely progress to steatohepatitis and fibrosis.[78,79] In addition, there are many other genetically engineered animal models, such as apolipoprotein E-deficient mice, CD36-deficient mice, peroxisome proliferator-activated receptor-α knockout mice, Zucker (fa/fa) rats, and Otsuka Long Evans Tokushima Fatty rats.[80–84]

In addition to lipid metabolism–related genes, microRNA knockout mice also yielded inspiring results. Genetic deletion of miR-223 induced a full spectrum of NAFLD in long-term HFD-fed mice, including steatosis, inflammation, fibrosis, and HCC.[85,86] The current models, however, are based on assumptions about mechanisms and have one major limitation: each specific mutation in a single gene is not usually found in humans. Synchro-knockout animals may provide a better tool for NASH construction in the future.

Chemical models

Tetracycline, carbon tetrachloride (CCl_4), thioacetamide (TAA), and streptozotocin (STZ) are the most common chemical models used to study NAFLD.[69,70,87] Overall, the chemical model represents a faster, more severe approach to studying liver injury, but the onset and progression of the disease bear less similarity to human NAFLD than do dietary or genetic models.

Several HFD-based NAFLD models can develop spontaneous HCC driven by NASH after a sufficient feeding period (~1 y). HFD feeding in combination with the use of certain toxins/carcinogens can significantly accelerate the development of experimental liver cancer.[88,89] The stelic animal model is one of the most widely used NASH-HCC models. In this model, neonatal animals were treated with a single low dose of STZ at 2 days after birth. The incidence of tumors is very high at week 20.[90] STZ treatment can induce a type 2 diabetes-like phenotype with hyperglycemia and insulin resistance, but stelic animal model mice did not show significant obesity.[90,91] Patients with NAFLD typically develop insulin resistance along with overweight and obesity, which is in stark contrast to what is observed in this study.

Combined models

Commonly used dietary models do not usually spontaneously develop severe liver damage, so “second strikes” are often supplemented to achieve progression to NASH, fibrosis, or HCC. The same is true for genetic models, such as ob/ob mice, which often need to be combined with MCD to induce NASH.[67] Obese mice do not readily develop NASH by feeding HFD in contrast to the high tendency of obese individuals with fatty liver to develop NASH.[92,93] Therefore, it is reasonable to speculate that the inflammatory and/or fibrotic factors that behave differently between mice and humans may contribute to the resistance of mice to the development of NASH. Several studies have demonstrated that neutrophils could be the factor that may explain the different behaviors between mice and humans in terms of NASH development. The livers of NASH patients exhibit a marked infiltration of neutrophils compared with fatty liver.[94,95] However, obese mice do not show the sign of hepatic neutrophil infiltration and resist to NASH development. It is interesting to note that the number of circulating neutrophils in mice is ~25% that of humans.[96] In addition, there exist apparent differences in the characteristics of neutrophil-recruiting chemokines (eg, chemokine (C-X-C motif) ligand 1 (CXCL1) and interleukin 8) between mice and humans.[97].

A study conducted in HFD-fed mice demonstrated that hepatic overexpression of CXCL1 resulted in the progression of steatosis to NASH.[98] In this study, neutrophil infiltration was markedly increased in the liver after CXCL1 overexpression, and signs of the liver injury became evident. In addition, CXCL1 overexpression and neutrophil infiltration elevated hepatic oxidative stress through p47phox-dependent oxidative burst, which resulted in the activation of the ASK1-p38 axis that relays the oxidative stress signal to the cell death signal.[99] p38 is also known to stimulate the transcription of ER stress–related factors, such as CHOP.[100,101] In line with these findings, Hwang et al[97] showed that the hepatocyte-specific deletion of *p38a* attenuated CXCL1-induced NASH progression in mice. Besides hepatocyte death, other parameters for NASH, such as inflammation and fibrosis, were also significantly increased at 2 weeks after CXCL1 overexpression.[98] The expression profiles of genes involved in inflammation and fibrosis were similar to those of NASH patients, and histological analysis of the liver revealed the predominance of the inflammatory and fibrotic features in the CXCL1-induced NASH model.

Lean NAFLD models

Despite the fact that NAFLD is often associated with obesity, about 20% of people with the disease are not overweight, a condition called “lean NAFLD.”[102] Whether late clinical outcomes of lean NAFLD are worse is still controversial[103,104;] thus, people are increasingly interested in understanding how lean NAFLD develops. Currently, lean NAFLD animal models are based on common NAFLD animal models, such as high-fat and high-fructose diets and choline-deficiency diets. Velázquez et al[105] found that the high-fat-high-fructose diet for 3 months caused hypertriglyceridemia and hepatic lipid deposition in SD rats

but no inflammation, ER stress, or oxidative stress. Furthermore, rats fed the high-fat-high-fructose diet did not gain weight or become obese.[106] CDs, such as MCD and choline-deficiency, L-amino acid–defined diet, are believed to be unable to reproduce the clinical features of insulin resistance in obese patients, but they have certain advantages in the lean NAFLD model. Moreover, these diets can be combined with high-fat and high-fructose diets to shorten the modeling time or aggravate the injury. Farazi et al[108] induced lean NASH-HCC in mice using a choline deficiency + high trans-fat + sucrose/ fructose + cholesterol diet.[107]

In recent years, genetically engineered animals have also made progress in inducing lean NAFLD models. Wang et al[109] found that mice with adipocyte-specific deletion of prohibitin 1 showed lean NAFLD phenotype after 3 months of HFD. The mechanism may be that the interferon pathway is significantly inhibited and the bone morphogenetic protein 2 pathway is significantly upregulated in the liver of $Phb1^{adipo-/-}$ mice fed HFD. In addition, patients with lean NAFLD had higher serum levels of secondary bile acids and FGF19, and showed changes in the gut microbiome.[110] Fan et al conducted a study revealing that *Escherichia fergusonii*–derived small-RNA 23487 was significantly increased in nonobese rats with NAFLD and downregulated hepatic peroxisome proliferator-activated receptor-α expression.[111] These studies suggest that intestinal flora imbalance may also be one of the factors to be considered in the construction of a lean NAFLD model. Although NAFLD has been extensively studied, the pathogenesis of lean NAFLD remains poorly understood. Developing stable animal models of lean NAFLD and clarifying its pathophysiology will be our next objective.

Other animal models of NAFLD

Wistar or Sprague-Dawley rats are usually selected for the rat NAFLD model, and the modeling method is similar to that for mice.[67] In addition, hamsters are becoming an increasingly popular choice for studying NAFLD. Since hamsters carry more cholesterol as low density lipoprotein than wild-type mice and rats, their liver cells produce only apoB-100, which is more similar to human lipoprotein metabolism.[112] Compared with mice, hamsters appear to be more responsive to the HFC diet, manifested by increased plasma alanine transaminase, aspartate transaminase, and low density lipoprotein levels and more severe hepatic steatosis, inflammation, and fibrosis development.[113,114] In terms of cholesterol and lipoprotein metabolism, guinea pigs are a good alternative animal model for diet-induced NASH.[115] One problem is that they do not gain weight significantly after being fed the HFC diet. Thus, clinical research may be limited to nonobese people with NAFLD using this model.

The anatomical and metabolic makeup of pigs is similar to that of humans, and mini pigs fed choline-deficiency, L-amino acid–defined diets induce steatohepatitis within 8 weeks. However, these nonrodents do have some drawbacks, such as involving more complex genetic methods and being less economically suitable for widespread laboratory use than rodents.[116]

In vitro cell culture models of NAFLD

The source of the cells used in the *in vitro* models is usually hepatocyte lines, primary hepatocytes, or hepatocellular-like cells. Two-dimensional cell culture models include monocultures or cocultures with other cells, such as hepatic stellate cells, to induce steatosis by adding FFAs, to the cell medium. Due to the absence of key liver nonparenchymal cell interactions, the results differ significantly from animal models.[117]

Functional multicellular hepatic globular cultures were established using mouse primary hepatocytes, hepatic stellate cells, LSECs, and Kupffer cells.[118] The development of steatosis and fibrosis in organoids can be triggered by FFAs and lipopolysaccharides [119–121] Liver-on-chip technology is based on microstructures and microfluid perfusion devices, aiming at building microfunctional livers.[122] Gori et al[123] cultured HepG2 cells with the addition of FFAs in a microfluidic apparatus. The microhydrodynamic characteristics of the apparatus preserved higher hepatocyte viability and less oxidative stress compared with static culture. In addition, Lee and colleagues created a “gut-liver chip” to better understand the entero-liver axis in the context of NAFLD. In the microfluidic chip, FFAs are absorbed through the intestinal layer, and chylomicron secretion is subsequently observed, coinciding with fat accumulation in liver cells.[124] Due to the use of standardized protocols and bioengineering manufacturing techniques, liver-on-chip technology is more repeatable than organoids.[125]

Despite the novelty of these techniques, various liver disease models have been developed, which represent the major pathogenic aspects of liver disease. The use of organoids for liver disease research or drug screening has the potential to significantly reduce the number of animal models used for the same purpose.[117] This reduction in the number of cell species, particularly the absence of immune cell involvement, clearly limits the viability of liver organoids for studying complex pathophysiological processes.[126] Thus, the current construction protocol still needs to be improved. The ultimate goal is to accurately characterize the complex liver microenvironment that progresses from NAFLD to NASH and even HCC.

ALCOHOL-ASSOCIATED AND METABOLIC FATTY LIVER MODELS

Obesity and alcohol consumption are the 2 leading causes of chronic liver disease and often coexist, which together increase the progression of liver fibrosis, cirrhosis, and HCC.[127–129] With obesity on the rise and the drinking culture continuing, it is expected that this type of "alcohol-associated and metabolic steatohepatitis" will become more prevalent among patients with chronic liver disease. A variety of preclinical experimental models have been developed by researchers to gain a deeper understanding of pathogenesis.

Combining classic HFD with ethanol consumption is the most common way to induce alcohol-associated and metabolic fatty liver in animals. Gopal et al[130] fed C57BL/6 mice a normal diet or HFD for 10 weeks, followed by control or the addition of 5% ethanol to the Lieber-DeCarli diet for an additional 4 weeks. A combination of HFD and alcohol increased lipid dysregulation, inflammation, and fibrosis in these models. There are also other dietary models available, including the high-fructose Lieber-DeCarli diet.[131] Referring to the NIAAA model, Chang et al[95] established high-fat-plus-binge/chronic drinking models, which demonstrated that HFD feeding combined with a single binge of alcohol induced a significant increase in serum alanine transaminase in mice with symptoms of severe steatohepatitis. High-fat feeding for a period of merely 3 days significantly exacerbated hepatic neutrophil infiltration and liver damage; on the other hand, ethanol can exacerbate liver damage if fed for a period of 3 months.[132] Using these models allows us to study the synergistic effects of HFDs and alcohol on the liver.

The experimental rodent models can also be combined with the genetic models of NAFLD and alcohol consumption. Robin et al[133] developed a model using ob/ob mice in which ethanol is administered daily through tube feeding. Compared with the control group, the combination of multiple binges of ethanol induced dysregulation of TNF-α signal transduction. The combination of other genetic model mice, such as the KK-Ay[134] and the fa/fa Zucker rats,[135] has also been studied. The development of new preclinical experimental models may help elucidate new mechanisms of fatty liver disease and the interaction between alcohol and metabolic abnormalities. In particular, feeding protocols should be developed, which closely mimic the food and alcohol consumption of clinical patients, and consider how these factors induce more severe liver disease outcomes.

CONCLUSIONS AND FUTURE RESEARCH DIRECTIONS

This review explores different *in vivo* and *in vitro* models used to study the pathophysiology of ALD and NAFLD in humans. Over the last few decades, the ALD/NAFLD model has contributed significantly to the advancement of chronic liver disease research. Using animal models, particularly rodent models, has improved our understanding of liver disease development.[67,136] Although there is no perfect animal model, some models are able to replicate several aspects of the development of ALD/NAFLD and have become very important tools in the search for therapeutic targets.

Future studies of ALD and NAFLD models may focus on the establishment of advanced liver disease models. Despite the differences in severity and stages of disease progression between humans and animals, it is expected that future animal models will gradually be able to simulate the various pathological features and stages of human ALD and NAFLD with the continuous improvement and development of animal models. For example, the researchers' focus on microbial composition may partly explain differences in disease progression between species. Advances in new technologies for *in vitro* models, such as organoids and livers on a chip, are expected to reduce the need for experimental animal numbers in toxicological research. Approaches taken from different angles can lead to a greater understanding of many aspects of disease.

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CONFLICTS OF INTEREST

The authors have no conflicts to report.

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