Rodent Models of Nonalcoholic Fatty Liver Disease

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Abstract

Background: Nonalcoholic fatty liver disease (NAFLD) is a continuous diseases spectrum associated with obesity, type 2 diabetes, insulin resistance, and hyperlipidemia. Simple hepatic steatosis may progress to nonalcoholic steatohepatitis (NASH), even fibrosis and cirrhosis, and finally hepatocellular carcinoma. In recent years, NAFLD has become a public health concern with increasing prevalence. However, the mechanisms underlying the pathogenesis remain incompletely understood, and few effective therapeutic approaches are available. **Summary and Key Messages:** A myriad of different rodent models has been developed to elucidate pathophysiology of NAFLD/NASH and guide therapeutic strategy. To date, no single rodent model can display the whole disease spectrum and metabolic features associated with human NASH, but can imitate particular characteristics. In this paper, we review the most commonly used dietary, genetic, and chemical rodent models for NAFLD referring to their advantages and disadvantages. Also, we illustrate the status of latest treatment strategy using various NAFLD rodent models. We hope to provide critical guidance for researchers to select appropriate animal models.

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Introduction

Nonalcoholic fatty liver disease (NAFLD) is a clinico-pathological syndrome characterized by liver fat deposition and hepatocyte steatosis without excessive alcohol consumption [1, 2]. It represents a continuous disease spectrum encompassing simple steatosis, nonalcoholic steatohepatitis (NASH), fibrosis, and cirrhosis, which can further progress to hepatocellular carcinoma (HCC) [3]. As one of the histological phenotype of NAFLD, NASH is characterized by cellular injury such as hepatocellular ballooning, inflammatory infiltrates, and tendency to fibrosis or cirrhosis [4, 5]. NAFL, another histological phenotype of NAFLD, is a less aggressive disease only with steatosis [4]. The mechanisms underlying the pathogenesis and progression of NAFLD/NASH remain incompletely understood. It has been proposed that NAFLD/NASH may actually be the result of a consequence of “2-hit” process involving; first, the advance of steatosis and lipid accumulation of liver cells induced by insulin resistance (IR) and, next, the inflammation and necrosis of liver cells caused by oxidative stress and lipid peroxidation on the basis of the first hit [6]. In recent years, the classical “2-hit” hypothesis is now being modified by a “multiple parallel hits” hypothesis such as gut- and adipose tissue-derived factors [7].

NAFLD is now emerging as a common medical problem due to its high incidence and the difficulty of

treatment. The latest epidemiology shows that NAFLD has become the second largest liver disease after viral hepatitis, the incidence of which is 20–30% [8, 9], and the incidence of obesity in the population is up to 57.74% [10]. In addition, as a hepatic manifestation of metabolic syndrome [11], NAFLD is associated with visceral overweight or obesity, dyslipidemia, hyperinsulinemia or type 2 diabetes, and arterial hypertension, which is associated with an increased risk of mortality [12]. Given all these considerations, it is urgent to further explore the mechanism and the progression of the disease in order to develop methods for better targeting and therapeutic strategies for individual patients. However, clinical research on the mechanisms of NAFLD/NASH development is constrained by ethical considerations, particularly with respect to collecting tissue samples from patients and administering drugs to patients. Furthermore, the occurrence and progression of NAFLD/NASH can take a long period of time even several decades, which limits the quality of data. Therefore, animal models are necessary to elucidate the pathophysiological mechanisms and therapeutic targets of NAFLD/NASH.

The ideal animal model needs to reflect correctly both the pathophysiology and histopathology of human NAFLD/NASH. It should have the following characteristics: (1) recapitulates as many aspects of human NAFLD as possible such as: lesions have a certain development process and covers all disease spectrum of NAFLD; metabolic characteristics similar to those of humans including dyslipidemia, adiposity, and IR; (2) stable and reliable; (3) reproducibility, high success rate, low mortality; and (4) modeling method is simple and feasible. Thus far, no single animal model has encompassed the whole spectrum of human NASH progression, but could simulate particular characteristics. In this paper, we review the most frequently used animal models for NAFLD referring to their advantages and disadvantages and elaborates the mechanism of NAFLD pathogenesis in each model (Fig. 1), so that provide critical guidance for researchers to select corresponding animal models. We also illustrate the status of latest treatment strategy using various NAFLD rodent models focus on current research hotspots, such as the correlation between intestinal microecology and NAFLD. Research findings about endocrine-related potential therapeutic targets for NAFLD of our research team were also described. Mice and rats are the most common animals for NAFLD modeling, thus constitute the main focus in this review.

Dietary Models

Amounts of overnutrition-based models have attempted to mimic the hepatic histological features as well as the metabolic disturbance similar to human NAFLD [13–15]. However, some diet patterns are quite different from what is known in humans. For instance, most human diets are not deficient in choline. The animal models of NAFLD are mainly based on various types of diet such as high fat, high fructose, cholesterol and cholate, methionine and choline-deficient (MCD) diet, choline-deficient L-amino acid-defined (CDAA) diet, and so on. These diets often include one or a combination of >1 different dietary patterns to induce simple steatosis and steatohepatitis.

High-Fat Diet

High-fat diet (HFD) is widely used to building NAFLD animal models. This group of diets consists of a variety of regimens with fat content varying between 45 and 75 kcal% [16]. Lieber et al. [17] reported a rat model using HFD (71% of calories from fat, 11% from carbohydrates, and 18% from proteins). Rats developed IR, marked panlobular steatosis, inflammation, and fibrogenesis [17–20]. The mice-fed HFD showed similar results around 16 weeks [21]. Feeding C57BL/6 mice via an implanted gastrostomy tube with HFD up to 85% in excess of standard intake for 9 weeks, obesity, IR was observed as well as the histopathological features similar to NASH in human, with inflammatory infiltrate and perisinusoidal fibrosis, but no ballooning [22]. Their plasma alanine aminotransferase (ALT) levels showed 9- to 10-fold increases. Overall, HFD model displays features similar to NASH in human, but the hepatic pathological consequence is not severe. The composition of HFD is similar to that of Western people. It can be used as animal model to simulate fatty liver disease induced by western dietary.

Recently, Ejima et al. [23] developed novel dietary model of NASH named steatohepatitis-inducing HFD (STHD-01) with a 5% cholesterol and 40% fat content, which is rich in high saturated fatty acids and cholesterol. Hepatic steatosis and steatohepatitis induced by STHD-01 take a short period. Hepatic triglycerides and cholesterol accumulated in large amounts only on day 1 during STHD-01 feeding and gradually increased over time, and steatohepatitis is developed within only 1 week. At 36 weeks, perivenular-pericellular fibrosis and a slender, chicken-wire pattern were observed in the liver tissue of STHD-01-fed mice, as well as extensive fibro-

Fig. 1. Main mechanism of NAFLD pathogenesis in each rodent model. NAFLD is caused by the imbalance of production, accumulation, and transport of triacylglycerol, which is mainly manifested in the increase of dietary fat, lipogenesis, adipose tissue lipolysis, impaired β oxidation and VLDL synthesis. In addition, intestinal dysbacteriosis, IR, and oxidative stress also play an important role in the pathogenesis of NAFLD. The figure shows the mechanism of NAFLD pathogenesis in each rodent model. $\uparrow$ denotes enhanced

and increased, $\downarrow$ denotes impaired and reduced. HFD, high-fat diet; CCL₄, carbon tetrachloride; SREBP-1c, sterol regulatory element-binding protein 1c; PTEN, phosphatase and tensin homolog; MCD, methionine and choline deficient; CDAA, choline-deficient L-amino acid-defined; VLDL, very low-density lipoprotein; AOX, acyl-coenzyme A oxidase; PPAR- α , peroxisome proliferator-activated receptor- α ; IR, insulin resistance; ApoE, apolipoprotein E; TNF, tumour necrosis factor.

sis of the entire liver and even benign tumors in some mice. Ejima et al. [23] also found that STHD-01 augmented the tumorigenic potential of diethylnitrosamine. STHD-01 model is suitable for screening and evaluation of drug candidates for steatohepatitis, fibrosis, and tumor formation.

It should be noted that the results vary with species and strain. Rats seem to be more susceptible to HFD than mice, which manifest as a shorter time is needed to induce a more severe histological manifestation in rats [24]. Sprague-Dawley rats susceptible to steatohepatitis development when fed with HFD [25]. Whereas hepatic steatosis and NASH were not induced in Wistar rats with long-term high saturated fat feeding [26]. Moreover, it

was reported that BALB/c male mice had more severe hepatic lipid deposition than C57BL/6J male mice when fed an HFD [27].

High-Fructose Diet

Fructose-rich foods – primarily in the form of corn syrup in soft drinks – are being highly consumed by humans, and this has been linked to the development of obesity and NAFLD [14]. Fructose consumption promotes de novo lipogenesis, oxidative stress, and IR [28]. In addition, it has been reported that ingestion of fructose promoted intestinal bacterial overgrowth, which result in increased gut-derived endotoxin levels in the portal blood, thereby promoting the activation of Kupffer cells and he-

patic inflammation [29]. Compared with glucose and sucrose, fructose induces greater hepatic fat accumulation [30]. Mice fed 30% fructose-supplemented drinking water result in a 3- to 4-fold increases in the levels of hepatic triglycerides, a significant increase in body weight and simple steatosis [31]. In male Wistar rats, consumption of water sweetened with 20% fructose lead to a significant obesity, hypertension, hyperglycemia, and hypertriglyceridemia after 8 weeks. These rats also show deposition of lipid vacuoles but no ballooning degeneration and inflammatory cells [32]. Kohli et al. [14] fed C57BL/6 mice a high-fat, high-carbohydrate diet (58% of calories from fat) combined with drinking water containing 55% fructose. After 16 weeks, these mice developed a phenotype of obesity, IR, and fibrosing NASH and showed increased hepatic oxidative stress, macrophage infiltration, and fibrosis when compared to mice fed the HFD only [14]. High fat, fructose, and cholesterol diet (40% fat, 42 g/L final concentration fructose, 0.2% cholesterol) model recapitulated the features of IR, steatosis, hepatocellular ballooning, and progressive fibrosis [13]. In summary, adding fructose to an overfed diet can promote liver fibrosis, hepatocellular ballooning, and adipose tissue inflammation. However, it is worth noting that the hepatic phenotype of NASH cannot be produced by using a high fructose diet alone when administered ad libitum.

Cholesterol and Cholate

Cholesterol in diet is an important risk factor for NASH because it makes the liver sensitive to tumour necrosis factor- and Fas-induced steatohepatitis [33]. The presence of cholic acid promotes the absorption of cholesterol and fat and inhibits the conversion of cholesterol to bile acids, thereby reducing the removal of cholesterol and raising the cholesterol levels, particularly non-high-density lipoprotein cholesterol [34]. The cholesterol and cholate diet containing 1.25% cholesterol and 0.5% cholate induces progressive formation of steatosis, inflammation, and fibrosis over 6–24 weeks: steatosis and inflammation (after 6 weeks), hepatocellular ballooning and fibrosis (after 24 weeks) [35]. In addition, the investigated animals show increased levels of ALT, after 6 weeks. The addition of 60% fat (cocoa butter) to the diet accelerated the development of abovementioned histopathological features. Moreover, the cholesterol and cholate diet contributes to the presentation of oxidative stress, dyslipidemia, and the activation of stellate cell in the liver. Taken together, it means that atherogenic diet can replicate the pathophysiological findings of human NASH. However, the mice fed this diet did not show systemic IR, only showed the hepatic IR. On the

other hand, the mice fed this diet lost 9% of their body weight and showed smaller fat pads and lower triglyceride levels compared with mice fed standard chow. In general, although the cholesterol and cholate diet replicates the histopathology in human NASH, the metabolic status differs.

MCD Diet

MCD diet has a high sucrose content and moderate fat content (40% sucrose, 10% fat), but deficient in methionine and choline that are essential nutrients in hepatic β -oxidation and the production of very low-density lipoprotein (VLDL) [36]. Furthermore, choline deficiency leads to an impaired hepatic VLDL secretion, resulting in hepatic lipid deposition, oxidative stress, and changes in cytokines and adipokines, culminating to liver injury [37]. The primary advantages of the MCD diet are that it replicates NASH histological features observed in human NASH in a relatively shorter feeding time than other dietary models. MCD diet fed mice occurred increasing serum ALT level and steatohepatitis at day 10, and then perisinusoidal fibrosis develops after 8–10 weeks [38]. Nevertheless, the MCD model does not exhibit any other metabolic features that are similar to human NAFLD, including obesity, peripheral IR, and hyperlipidemia. In contrast, MCD diet fed animals show significant weight loss, low fasting blood glucose, low peripheral insulin sensitivity, low serum insulin and leptin levels, decreased blood triglyceride and cholesterol, and unchanged or increased serum adiponectin level [39–43]. So, it is inadequate to study the multisystemic disease entity in all aspects of NAFLD, but generally adequate to study the intrahepatic events and the pharmacological treatment of NASH. To narrow the metabolic differences between MCD models and human, adding MCD diet to ob/ob or db/db mice is frequently used.

Like HFD model, the species, strain, and gender also may influence the severity of NASH in MCD model. By comparing the effects of MCD diet between male and female Wistar, Long-Evans, Sprague-Dawley, and C57/BL6 mice rats, Kirsch et al. [44] discovered that the male gender and Wistar strain were related to the greatest degree of steatosis in rats. Moreover, male C57/BL6 mice showed the maximum lipid peroxidation, ultrastructural injury, inflammation, and necrosis and the most similar characteristics of NASH.

CDAA Diet

CDAA diet is similar to the MCD diet because of their shared deficiency in choline. The difference is that mixture of L-amino acids replaces an equivalent and corre-

sponding proteins [45]. Similar to MCD diet, CDAA diet fed mice have increasing lipid synthesis, oxidative stress, inflammation, and resulting in liver fibrosis [36]. Animals fed CDAA diet require a longer time than MCD diet for these histological changes to progress, but the degree of NASH perhaps slightly more severe, as well as a larger increase in ALT levels [37]. Like the MCD diet, the metabolic profile of CDAA diet model is opposite of the typical human NASH, they does not show hepatic IR, nor do they grow fat or have changes in peripheral insulin sensitivity [46, 47]. It was reported that CDAA diet combined with HFD can bring about rapid development of NASH with fibrosis (6–9 weeks), without substantial weight loss, but it still fails to elicit features of the metabolic syndrome [48]. Therefore, the CDAA diet should not be used for examining the metabolic profile of the disease.

Genetic Models

To date, a great variety of genetic models have been developed to better understand NASH. The nature that specific gene needs to be altered renders these models different from humans who do not have these alteration of specific genes. The common feature of most of these models is that genetic alterations lead to significant hepatic lipid accumulation without obvious inflammation and fibrosis. It is frequent to add stimulus, such as a modified diet, to induce the histopathological and biochemical features of NASH in these models. We introduced the most commonly used genetic models from 3 aspects: fat synthesis, liver fat transport, and lipid oxidation.

Promoting Fat Synthesis

Leptin Deficiency (The ob/ob Mice)

Leptin, which is an adipokine secreted predominantly by white adipose tissue, plays a vital role in the regulation of energy balance by operating on the hypothalamic ventral median nucleus. It exerts a marked anorexic effect and increases energy expenditure [38]. The ob/ob mice own a spontaneous mutation in the leptin gene, which renders them leptin-deficient. The ob/ob mice are inactive, hyperphagic, overly obese, and display hyperlipidemia, hyperglycemia, hyperinsulinemia, and IR [49]. Moreover, fat is reallocated from adipose tissue to the liver and other nonadipose tissues due to the deficiency of leptin. This reallocation induces the accumulation of fat in the liver which further leads to hepatocyte lipotoxicity and lipoapoptosis. The ob/ob mice develop hepatic ste-

atosis but rarely progress to severe liver damage or spontaneous steatohepatitis including inflammation and fibrosis [50]. On the contrary, ob/ob mice have characteristics of preventing fibrosis [38]. Therefore, an additional stimulus is needed such as lipopolysaccharide endotoxin, HFD, MCD diet to induce features of NASH [51].

Actually, leptin mutations have been reported in humans. Nevertheless, the plasma concentration of leptin in NASH patients is normal or elevated compared to healthy controls, which represents a defect of this model [52]. On the other hand, this model is not suitable for studying the mechanisms behind the progression of inflammation and the late stages of NASH, but has become one of the most commonly used genetic models for studying the steatosis in NAFLD and related systemic metabolic changes [16].

Leptin Receptor Deficiency (The db/db Mice or fa/fa Rat)

The db/db mice possess a spontaneous mutation in the leptin receptor gene that brings about defective leptin signaling. Thus, these mice have normal or elevated levels of leptin, but the mutation confers resistance to the effects of leptin. Highly similar to ob/ob mice, db/db mice are inactive, hyperphagic, overly obese, and display hyperlipidemia, hyperglycemia, hyperinsulinemia, and IR and develop hepatic steatosis but no steatohepatitis and fibrosis. They also need additional stimulus such as MCD diet to develop the symptoms of steatohepatitis [53]. In addition, a recent study reports that iron overload in db/db mice can induce progress of simple steatosis to steatohepatitis and fibrosis [54]. An analogous mutation exists in rats and is described as fa/fa (also known as Zucker rats). Fa/fa rats display similar features to both ob/ob and db/db mice [37].

Phosphatase and Tensin Homolog Knockout Mice

As a tumor suppressor gene, phosphatase and tensin homolog (PTEN) negatively regulates several signaling cascades such as phosphatidylinositol 3-kinase and serine-threonine protein kinase B (Akt) pathway, which regulate apoptosis, cell proliferation, and tumor formation. PTEN knockout mice spontaneously develop steatosis, steatohepatitis, and fibrosis [55]. These mice exhibit micro- and macrovesicular lipid vacuoles, and then ballooning degeneration, inflammatory cell infiltration, and perisinusoidal fibrosis, which are similar to human NASH, were observed at 40-week-old. However, PTEN deficiency results in liver insulin hypersensitivity with decreased fasting glucose levels [56].

Sterol Regulatory Element-Binding Protein 1c Transgenic Mice

As an insulin-regulated transcription factor that principally regulates lipid synthesis, sterol regulatory element-binding protein 1c is mainly expressed in liver and adipocytes [57]. Transgenic mice overexpress sterol regulatory element-binding protein 1c in adipose tissue and develop IR and marked hepatic steatosis (8-day-age). Besides steatosis, they also spontaneously develop steatohepatitis manifests as lobular inflammation, ballooned hepatocytes, and pericellular fibrosis at 20 weeks [58]. Although this model recapitulates many liver histological features of human NASH, it has decreased body fat, which is in striking contrast to the human condition. These mice seem to be suitable for the lipodystrophy-associated steatohepatitis model.

KK-Ay/a Mice

KKAy/a mice are established by crossing KK mice with yellow obese mice (Ay) mouse. These mice carry a heterozygous mutation in the agouti gene, which impairs hypothalamic appetite and results in hyperphagia and obesity [59, 60]. Although they develop hyperglycemia, hyperinsulinemia, IR, and liver steatosis, significant steatohepatitis does not occur spontaneously. Additional stimulus such as MCD diet can induce steatohepatitis [61].

Inhibition of Lipid Oxidation

Peroxisome Proliferator-Activated Receptor- α Knockout Mice

Peroxisome proliferator-activated receptor- α (PPAR- α) is a key regulatory factors that can regulate transcription of genes involved in mitochondrial, peroxisomal, and peroxisomal fatty acid oxidation systems. Mice with a knockout of PPAR α do not accumulate fat in the liver under conditions of normal feeding. But they develop severe hepatic steatosis when subjected to fasting for 24 h or HFD but does not progress to NASH [62, 63]. However, when feeding PPAR- α null mice with MCD diet, they develop more severe steatohepatitis than that in wild-type mice fed MCD diet only, which means that null PPAR- α aggravates steatohepatitis caused by methionine and choline deficiency [64].

Acyl-Coenzyme A Oxidase Knockout Mice

Acyl-coenzyme A oxidase (AOX) catalyzes the first and rate-limiting reaction of peroxisomal β -oxidation of very-long-chain fatty acids. AOX null (AOX $-/-$) mice thus show defective peroxisomal β -oxidation of very-long-chain fatty acids, which start to accumulate in the

liver and lead to steatohepatitis without marked fibrosis [36]. Microvesicular fatty change in hepatocytes began to appear at 7-days-old and extensive steatosis developed at 2-month-old [65]. Interestingly, a compensatory increase in fatty acid oxidation occurs by 6–8 months of age, and hepatic steatosis is then completely reversed by hepatocyte regeneration [65]. Despite of the absention of fibrosis, AOX $-/-$ mice develop adenomas and carcinomas by 15-month-old [36, 65].

Impeding Fat Transport

CD36-Deficient Mice

CD36, also known as fatty acid translocase, plays an important role in promoting uptake and intracellular trafficking of fatty acid [66]. The CD36 knockout mouse exhibits decreased fatty acid uptake and utilization by skeletal muscle, which enhances lipolysis in adipose tissue and increases plasma free fatty acid (FFA), thereby increasing fatty acid delivery to liver [67]. They develop steatosis with inflammation, elevated circulating fatty acid and triglyceride levels, and hepatic IR [50].

Apolipoprotein E Knockout Mice

Apolipoprotein E (ApoE) is a high-affinity ligand for lipoprotein receptors existed on all lipoproteins except for LDL [68], which plays an important role in the uptake of chylomicron and VLDL residues [69, 70], and secretion of VLDL in liver [71]. ApoE knockout mice fed normal chow only exhibit steatosis [72]. When feeding ApoE knockout mice with a western diet rich in cholesterol and fat, they develop metabolic syndrome (obesity, high fasting glucose levels) and show characteristics of human NASH including steatosis, hepatocellular ballooning, and fibrosis 7 weeks later, as well as increased free hepatic cholesterol levels and portal hypertension [73]. ApoE knockout mice receiving western diet possess the characteristics of NASH and metabolic syndrome, making it a valuable tool in NASH research.

Chemical/Pharmacological Models

The pathogenesis of NAFLD is complex, and environmental factors such as chemical/pharmacological substances play an important role. Chemical factors commonly used to induce NAFLD are streptozotocin, carbon tetrachloride (CCL₄), and tetracycline. However, the use of a chemical drug alone does not induce an ideal NAFLD model, which is why it is frequently combined with dietary models when modeling NAFLD.

Streptozotocin

Low doses of streptozotocin (intraperitoneal or subcutaneous administration) in newborn animals lead to a chemical inflammation and destruction of the pancreatic islets, thus inducing diabetes [74, 75]. This method combined with HFD can establish a model for NAFLD. In this model, newborn (2 days after birth) C57BL/6J mice were given streptozotocin (200 µg), and then, surviving mice were started on HFD at 4-weeks-old. These mice developed simple steatosis at 6-week-old, NASH with inflammatory foci and ballooning at 8-week-old, progressive pericellular fibrosis at 8–12 weeks, and even multiple HCC at 20-weeks-age [75]. In addition, the levels of transaminase and fasting glycemia are elevated at 6-weeks-old. This model recapitulates several important histological features of human NAFLD and is also relevant to oxidative stress. But streptozotocin recreating beta cell a function rather than a systemic inflammatory insulin resistant milieu, which is different from the human state [21].

There was also a similar model that mice were given streptozotocin followed by HFD. Compared to exclusively HFD-fed mice, streptozotocin-treated group shows more fibrosis in central vein, portal tract areas, and perisinusoidal space, although there is no significant differences in levels of transaminase, degree of steatosis, or inflammation, accompanied by weight loss [74].

Carbon Tetrachloride

CCl₄, as the first chemical used to establish a toxic fatty liver model, is a hepatotoxic substance that can cause acute liver injury. By producing activated oxygen-free radicals, CCl₄ accelerates the process of the fatty degeneration, fibration, and leads to the destruction of the hepatocellular structure and function. The metabolites of CCl₄ stimulate Kupffer cells to release proinflammatory cytokines, which further aggravates liver damage. In mice, peritoneal injection of CCl₄ every 2 weeks induces extensive liver damage with degenerated, ballooned, and necrotic hepatocytes, as well as increased levels of transaminase and triglyceride [76, 77]. Similar results were observed in mice with intragastric administration [78]. The model can easily and quickly cause fatty liver, but model animals are susceptible to death by poisoning. In addition, the pathogenesis, evolution of disease, and histomorphological changes are different from those of human fatty liver. On the other hand, CCl₄ induces fibrosis, but no obesity, nor IR. Thus, the combination of a different diet, such as HFD, CDAA diet with CCl₄ can be used when establishing NAFLD models [79, 80]. HFD-fed obese mice combined with multiple administration of

CCl₄ develop inflammation, apoptosis, and fibrosis in the liver [79]. Under these circumstances, CCl₄ potentiates the effect of HFD on NASH and fibrosis development [81]. Low-dose intraperitoneal injections of CCl₄ to CDAA diet-fed C57BL/6 mice induce marked features of NASH compared with CDAA diet alone and develop HCC [80].

Tetracycline

Tetracycline deposited in hepatocellular mitochondria inhibits the replication or transcription of mitochondrial DNA, which causes VLDL secretion to be blocked, thus inducing hepatocellular fatty degeneration. Yin et al. [82] gave a single oral dose of 1 g/kg tetracycline to male ICR mice. Histopathological evaluation showed microvesicular steatosis after 24 h, accompanied by increased cholesterol and triglyceride biosynthesis and a decreased in β-oxidation of fatty acids. Ito et al. [83] used diet of tetracycline combined with HFD (60 cal% fat) to establish the model based on “2 hit theory.” The mice fed this combinational diet developed multifocal inflammatory cellular infiltration in the livers and thus caused mild steatohepatitis. In addition, plasma ALT levels were significantly elevated. The tetracycline model has advantages such as short molding time, low cost, lower toxicity than CCl₄. It can simulate the fatty liver disease induced by some specific environmental factors.

Therapeutic Strategy using NAFLD Models

The purpose of constructing NAFLD mouse model is not only to explore the pathogenesis of NAFLD but also to uncover potential therapeutic targets. The number of pharmacotherapy has been tested in a variety of NASH rodent models for their therapeutic capacity. Next, we briefly illustrate latest drug therapy using various NAFLD rodent models, focusing on microbiome-targeted therapy (MTT), insulin-related therapy, antioxidant agents therapy, and farnesoid X receptor (FXR) agonist therapy.

Microbiome-Targeted Therapy

There is a growing evidence that intestinal microecology is associated with the pathogenesis of NAFLD. For instance, compared with healthy people, the intestinal permeability and the prevalence of small intestinal bacterial overgrowth of NAFLD patients were significantly higher compared with healthy people [84, 85]. The study of 52 patients with NAFLD showed that the anti-actinomycescomitans IgG antibody titer was significantly

higher than that in healthy subjects. And further experiments in mice have shown that infection with actinomycetemcomitans could promote the pathogenesis of NAFLD by altering the intestinal microflora and glucose metabolism in C57BL/6J mice [86]. Another study discovered that infants born to obese mothers were more susceptible to NAFLD because of abnormal gut microbiota [87].

The mechanism of intestinal microecology affecting the pathogenesis of NAFLD is complex and not sufficiently elucidated. The disruption of intestinal epithelial barrier in NASH leads to increased translocation of bacterial products into the portal vein circulation and resultant bacterial endotoxin such as lipopolysaccharide can stimulate the production of inflammatory cytokines and increase oxidative stress in the liver, leading to liver injury [88]. In addition, intestinal microflora is considered to be related to NAFLD by the effect on proinflammatory signaling, caloric salvage, and host energy metabolism [89, 90]. Maybe the production of short-chain fatty acids, metabolism of bile acids and choline, ethanol production, amino acid biosynthesis, and metabolism are all regulated by gut microbiota, which facilitate the pathogenesis of NAFLD [91], but this postulated mechanisms have not been fully confirmed.

Based on the correlation between intestinal microecology and NAFLD, the hypothesis of MTT for NAFLD was proposed. MTT mainly include fecal microbial transplant, intestinal specific antibiotics, prebiotics, and probiotics [91]. By transplanting healthy intestinal microbiota into the intestines of NASH mice, the portal vein pressure of NASH mice decreased to normal [92]. There are several studies about the effect of antibiotic administration on intestines microbiota and liver. Antibiotic therapy could lead to changes in intestinal microbiota [93], while improving glucose tolerance and liver lipid accumulation [86, 94]. Komazaki et al. [86] treated mice fed with actinomycetes with norfloxacin, after 12 weeks, glucose tolerance was improved, and lipid accumulation in liver was partially suppressed compared with mice in control group. A recent animal experiment shows that oral administration of ferulic acid could significantly reduce the formation of NAFLD and decreased serum levels of total cholesterol, triglycerides, and LDL cholesterol levels, as well as alter the composition of gut microbiota [95]. Probiotics are effective for NAFLD treatment in rodent models through improving intestinal microecology, reducing endotoxin production, and restoring gut-barrier functions. Such as, by improving the integrity of intestinal tight junctions, probiotics MIYAIRI 588 treatment

attenuates hepatic steatosis, fibrosis in mice fed CDAA diet, and inhibits hepatic GST-P expression, a marker for hepatocellular in the early stage of hepatoma [96]. One small randomized controlled trials (RCTs) of probiotics for NAFLD revealed that treatment with bifidobacterium longum combined with fructo-oligosaccharides could reduce NASH activity and serum ALT levels [97]. Probiotics are safe and well tolerated, but it should be used for a long time because the intestinal flora is likely to return to its original state after the intervention. MTT is widely used in animal models, but it is unlikely to be used in clinical practice at present due to the lack of high-quality, large-scale clinical intervention trials to evaluation MTT values in patients with NAFLD.

Insulin-Related Therapy

IR is a key character of NAFLD; therefore, the therapy targeting this pathway has been a research hot spot. Insulin-related therapy includes insulin sensitizers such as metformin and thiazolidinediones and newer agents such as glucagon-like peptide-1 (GLP-1) receptor agonists and dipeptidyl peptidase 4 inhibitors.

In various NASH rodent models, metformin has shown the beneficial effect on liver lipid deposition, steatohepatitis, and fibrosis. For example, metformin treatment for 8 weeks ameliorated MCD + HFD-induced hepatic steatosis, steatohepatitis, and fibrosis and even reversed steatosis and inflammation [98]. Furthermore, metformin could prevent liver tumorigenesis in C57BL/6 mice induced by long-term HFD [99]. However, in clinical studies, metformin has been shown to improve only transaminase levels and hepatic steatosis. Its effect on inflammation was weak, and only 1 study showed an improvement in fibrosis [100]. Therefore, most guidelines do not recommend the use of metformin in treating NASH. Similarly, a number of studies showed pioglitazone could exert an inhibitory effect on hepatic steatohepatitis and fibrosis in mice of NAFLD [101–103]. But many clinical studies of thiazolidinediones differ with respect to histological and other outcomes. As for newer agents, treatment of Exendin-4 (GLP-1 analogue) alleviated steatohepatitis of db/db mice-fed MCD through inhibiting hepatic FFA influx and oxidative stress, suggesting GLP-1 analogue can be a therapeutic options in patients with NASH [104]. The treatment of dipeptidyl peptidase 4 inhibitors such as linagliptin and alogliptin also showed the similar curative effect for NAFLD in various NASH animal models [105–107]. High-quality RCTs are required to prove a protective effect of those newer agents in NAFLD.

Antioxidant Agents Therapy

Oxidative stress plays an important role in the pathogenesis of NAFLD. Therefore, powerful antioxidants become attractive therapeutic drugs. Vitamin E is a fat-soluble molecule and a powerful antioxidant that can protect cell membranes from oxidation. Protective effects of vitamin E on NAFLD have been extensively studied, including animal experiment and clinical trials. Vitamin E administration could reduce hepatic oxidative stress, inflammation, and fibrosis in mice and prevent the progress from simple steatosis to steatohepatitis [108]. Two recently published large RCT, PIVENS, and TONIC showed that vitamin E resulted in improvements in lobular inflammation, hepatocellular ballooning, and NAFLD activity scores [109, 110]. In fact, vitamin E is the treatment recommended for NASH in the European Guidelines of 2016 and the United States Guidelines of 2012 and 2017. Astaxanthin, an antioxidant carotenoid that inhibits lipid peroxidation more powerful than vitamin E, is more effective than vitamin E in preventing and treating NASH in rodent model [111]. Another antioxidant agent, ursodeoxycholic acid, has also showed the beneficial effects such as decreasing ALT levels and serum fibrosis marker [112]. Pentoxifylline is both an antioxidant and tumour necrosis factor- α antagonist. It attenuated steatohepatitis, decreasing both serum ALT levels and hepatic inflammation in mice fed a MCD diet [113]. Besides, a recent publication shows its preventive effects on NASH-related liver tumorigenesis in rodent model [114]. In a number of small NAFLD trials, 2 of which demonstrated the improvement in steatosis, inflammation, and ballooning [115, 116].

Farnesoid X Receptor Agonist Therapy

FXR is a nuclear receptor transcription factor expressed in the liver, which plays an important role in participating in lipid metabolism and regulating insulin sensitivity [117]. FXR agonists have been studied in the management of NASH both in humans and animal models. In an animal experiment of fa/fa rats, 6-ethylchenodeoxycholic acid (6E-CDCA, a potent activator FXR) treatment inhibited the expression of genes involved in lipogenesis, fatty acid synthesis, and gluconeogenesis in liver and reduced FFA synthesis in muscles [118]. In addition, Zheng et al. [119] uncovered a new class of nonsteroidal FXR agonist – altenusin treatment almost reversed the accumulation of hepatic lipid droplets and vesicular steatosis induced by HFD, while lowering blood glucose level and body weight. Furthermore, a phase IIb clinical trial with 6E-CDCA in NASH human patients showed histological

improvements after 72 weeks of treatment [120]. Phase III trials of 6E-CDCA are underway in order to make its clinical application a reality.

Endocrine-Related Potential Therapeutic Targets

Increasing evidence suggests that endocrine is associated with the pathology of NAFLD. For instance, hypothyroidism and subclinical hypothyroidism have been demonstrated to be a risk factor for NAFLD [121, 122]. Men are more susceptible to NAFLD than women despite equal exposure to major risk factors [123].

Our research team has taken a great deal of research on the correlation between endocrine and hepatic lipid metabolism and has made some important discoveries. We demonstrated that thyroid-stimulating hormone (TSH) could increase the hepatic triglyceride content in liver of mice via TSH receptor. Compared with the littermates, the hepatic steatosis induced by HFD in TSH receptor knockout mice was relatively reduced, even when fed chow diet [124]. In addition, we also found the relationship between prolactin (PRL) and hepatic triglyceride accumulation. PRL was demonstrated to significantly reduce the accumulation of triglyceride in the liver of female mice and has a protective effect on liver steatosis induced by HFD in male mice via PRL receptor. Subsequent studies showed a significant aggravation of liver steatosis in mice with PRL receptor blockade [125]. These findings suggest that TSH or hepatic TSH receptor, PRL or hepatic PRL receptor might serve as a potential therapeutic target for NAFLD. Besides, we also studied the relationship between follicle-stimulating hormone (FSH), TSH, and hepatic cholesterol. Our research found that blocking FSH could effectively prevent hypercholesterolemia caused by high cholesterol diet or FSH injection in mice via inhibiting hepatic cholesterol biosynthesis [126]. Another of our research proved that TSH can upregulate the expression of 3-hydroxy-3-methyl-glutaryl coenzyme A reductase (HMGCR) in liver, suggesting that TSH directly acts on liver may be one of the mechanisms leading to hypercholesterolemia [127]. Although the above 2 studies focused on cholesterol not paying attention to NAFLD, cholesterol homeostasis and cholesterol accumulation in hepatocytes are closely relevant to the pathogenesis of NAFLD [128], which might be a new strategy for preventing and treating NAFLD.

Others

In addition to the above strategies, other drugs were tested to be effective in various NASH rodent models.

Table 1. Main features of commonly used rodent models of NAFLD/NASH

	Obesity	IR	Steatosis	Steatohepatitis	Fibrosis	Ballooning	Carcinoma
<i>Dietary models</i>							
HFD	Yes	Yes	Yes	Yes	Yes (slight)	No	No
Fructose diet	Yes	Yes	Yes	No	No	No	No
Cholesterol and cholate	Weight loss	Hepatic IR only	Yes	Yes	Yes	Yes	No
MCD diet	Weight loss	Hepatic IR only	Yes	Yes	Yes	No	No
CDAA diet	Weight loss	No	Yes	Yes	Yes	No	Yes
<i>Genetic models</i>							
ob/ob mice	Yes	Yes	Yes	No	No (resistant)	No	No
db/db mice or fa/fa rat	Yes	Yes	Yes	No	No	No	No
PTEN knockout mice	No	No	Yes	Yes	Yes	Yes	Yes
SREBP-1c transgenic mice	Weight loss	Yes	Yes	Yes	Yes	Yes	No
KK-Ay/a mice	Yes	Yes	Yes	No	No	No	No
PPAR- α knockout mice	No	No	Yes (steatosis occurs in the starved state)	No	No	No	No
AOX knockout mice	No	No	Yes	Yes (transient)	No	No	Yes
CD36 knockout mice	No	Hepatic IR only	Yes	Yes	No	–	No
ApoE knockout mice	No	No	Yes	No	No	No	No
<i>Chemical/pharmacological models</i>							
Streptozotocin + HFD	Yes	Yes	Yes	Yes	Yes	Yes	Yes
CCL ₄	No	No	Yes	Yes	Yes	Yes	Yes
Tetracycline	No	No	Yes	Yes	Yes	–	–

NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; IR, insulin resistance; HFD, high-fat diet; MCD, methionine and choline deficient; CDAA, choline-deficient L-amino acid-defined; PTEN, phosphatase and tensin homolog; SREBP, sterol regulatory element-binding protein; PPAR- α , peroxisome proliferator-activated receptor- α ; AOX, acyl-coenzyme A oxidase; ApoE, apolipoprotein E; CCL₄, carbon tetrachloride; –, no describe.

Our recent research found that cyclophilin D (CypD) activation can stimulate excessive opening of mitochondrial permeability transition pore, which leads to endoplasmic reticulum stress, resulting in liver steatosis. Our recent study found that liver lipid accumulation induced by HFD was attenuate in Ppif $^{-/-}$ (CypD deficiency) mice when compared with Ppif $^{+/+}$ (littermate counterparts) mice, and triglyceride level in liver was lower as well. Therefore, we proposed a hypothesis that CypD inhibitors have preventive and therapeutic effects on hepatic steatosis and validated it in mice. By intraperitoneal injection of cyclosporine A (an inhibitor of CypD) into HFD-fed mice, we found that cyclosporine A could inhibit mitochondrial ROS production induced by HFD in a dose-dependent manner and inhibit lipid accumulation and hepatic steatosis especially triglyceride levels [129]. It is suggested that cyclosporine A would be a new pharmacological method to improve NAFLD.

A recent study demonstrated that schizandrin A could alleviate NAFLD in male C57BL/6J mice induced by high-fat and high-cholesterol diet by regulating hepatic

oxidative stress, lipid metabolism, and fecal lipid excretion [130]. One publications proved the effect of nitro-oleic acid in experimental mice models of NASH. The administration of nitro-oleic acid inhibited hepatic triglyceride accumulation, as well as liver steatosis and fibrosis [131]. Sawada et al. [96] observed that administration with clinically equivalent doses of losartan (30 mg/kg/day) significantly ameliorated hepatic fibrosis and suppressed the activation of hepatic stellate cells in a rat model of NASH induced by CDAA. And in this model, combination of losartan and probiotics has synergistic inhibitory effect on hepatic fibrosis [96]. However, these findings need to be further verified in RCTs with large sample samples.

Conclusion

Rodent models are important tools for studying the pathogenesis and treatment of NAFLD. Over the years, many mouse models of NAFLD have been developed and

have provided valuable insights on the pathogenesis of NASH. However, not all mouse models can replicate the full spectrum of human NASH with regard to both the metabolic characteristics and histological pattern, but only mimic specific features. We described some of the most frequently exploited models in terms of diet, genetics, and chemical or pharmacological induction and explored the different characteristics and limitations of each model (Table 1) in this review.

On the whole, the method to build dietary models is relatively simple and convenient. Nevertheless, some diet patterns are quite different from what is known in humans even there is a wide diversity in type and composition of the diet. Genetic models have certain advantages in experimental duration but increase the experimental expenditure. And on the other hand, the mutations in specific genes associated with disease are mostly not found in patients with NASH, which makes them less relevant when compared to humans. With regard to chemical- or pharmacological-induced models, they simulate the high-risk factors in human living environment, but few people are indeed exposed to chemical or pharmaco-

logical factors, which make these models to be used in some specific settings.

In summary, various animal models contribute to the elucidation of NASH pathogenesis, while none of them are faultless. Researchers should continue to perfect the existing models and to develop novel models that more closely reflect the histopathology, pathophysiology, and metabolism of human NASH to facilitate the advancement of knowledge on the pathogenesis and effective treatment.

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Disclosure Statement

All authors have no conflict of interest to declare.

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