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REVIEW

Alcoholic liver disease: Utility of animal models

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Abstract

Alcoholic liver disease (ALD) is a major cause of acute

and chronic liver injury. Extensive evidence has been accumulated on the pathological process of ALD during the past decades. However, effective treatment options for ALD are very limited due to the lack of suitable *in vivo* models that recapitulate the full spectrum of ALD. Experimental animal models of ALD, particularly rodents, have been used extensively to mimic human ALD. An ideal animal model should recapitulate all aspects of the ALD process, including significant steatosis, hepatic neutrophil infiltration, and liver injury. A better strategy against ALD depends on clear diagnostic biomarkers, accurate predictor(s) of its progression and new therapeutic approaches to modulate stop or even reverse the disease. Numerous models employing rodent animals have been established in the last decades to investigate the effects of acute and chronic alcohol exposure on the initiation and progression of ALD. Although significant progress has been made in gaining better knowledge on the mechanisms and pathology of ALD, many features of ALD are unknown, and require further investigation, ideally with improved animal models that more effectively mimic human ALD. Although differences in the degree and stages of alcoholic liver injury inevitably exist between animal models and human ALD, the acquisition and translational relevance will be greatly enhanced with the development of new and improved animal models of ALD.

Key words: Steatohepatitis; Cirrhosis; Hepatocellular carcinoma; Alcoholic liver disease; Reactive oxygen species

Core tip: Alcoholism is now considered a global health issue. Although significant progress has been made in our understanding of the mechanisms and pathology of alcoholic liver disease (ALD), many features of ALD remain unidentified - requiring further investigation with improved animal models that more effectively emulate human ALD. In this Review, we provide an update on the prevalence, current and emerging experimental models, as well as the pathophysiology of ALD.

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INTRODUCTION

Alcohol has been part of human culture for thousands of years. Excessive alcohol consumption is the oldest form of liver injury known to civilization. Currently, alcohol abuse is an important global health problem with a significant socioeconomic burden in most societies. The term alcoholic liver disease (ALD) comprises a range of disorders including simple steatosis, steatohepatitis, cirrhosis, and end-stage hepatocellular carcinoma (HCC).

Animal models are frequently used to emulate and understand the underlying mechanisms of human disease. However, over the last decades, a great variety of animal models for ALD have been developed with different outcomes. To find the "ideal" experimental model for ALD would greatly help the study of the pathogenesis and thus development of new therapeutic strategies for the treatment of ALD. However, most models do not recapitulate the full spectrum of human ALD. A clinically-relevant model should induce certain characteristics, including: severe steatosis, hepatocellular damage and hepatic infiltration.

This review provides an overview of the pros and cons of the most frequently used experimental models of ALD, and the advances and implementation of new animal models that show great potential. We also discuss the recapitulation of pathological events in such models that commonly occur in human ALD.

PATHOPHYSIOLOGY OF ALD

Alcohol abuse has a long history although it was not until the 20th century when it was studied with a scientific perspective. In 1965, pioneering work by Lieber and colleagues[1] identified the hepatotoxic function of alcohol, instead of the malnutrition effect, previously assumed. ALD is now recognized as a complex disease induced by alcohol abuse with a broad spectrum of liver diseases. These range from simple steatosis to more severe forms of injury, including steatohepatitis, cirrhosis and HCC[2,3].

Following absorption in the gastrointestinal tract (GI), only 2% to 10% of total ingested ethanol is directly eliminated through the lung, the kidney and sweat in an unchanged form[4]. Most ethanol will undergo metabolic processing in the liver (Figure 1). First, ethanol (C_2H_6O) is oxidized and transformed into acetaldehyde (C_2H_4O) in hepatocytes. This step is mainly achieved by the enzyme alcohol dehydrogenase (ADH); although alternative minor pathways are involved including the catalase enzyme pathway (which has low expression in the liver), and the microsomal ethanol oxidation system (MEOS) - which depends on cytochrome P450 (CYP450) enzymes, particularly cytochromes P450 2E1 (CYP2E1)[4]. Successive oxidation reactions take place: Acetaldehyde loses hydrogen, and is metabolized to acetate (C_2H_3O), under the catalysis of acetaldehyde dehydrogenase (ALDH). Major reactions in this process require the coenzyme nicotinamide adenine dinucleotide (NAD^+) for transferring hydrogen, and the amount of reducing equivalents (NADH) is increased as a result. It has been reported that the change in NAD^+/NADH ratio favours hepatic triglyceride accumulation and fatty acid synthesis[4].

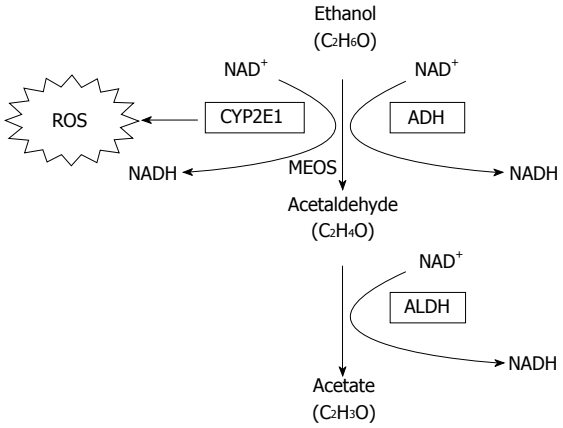

Figure 1 Alcohol metabolism in hepatocytes. Ethanol is oxidized to acetaldehyde through action of the enzyme alcohol dehydrogenase and cytochrome P450 isoenzyme 2E1 a major component of the microsomal enzyme oxidation system. Acetaldehyde is subsequently metabolized to acetate by acetaldehyde dehydrogenase. In this process coenzyme nicotinamide adenine dinucleotide is reduced to coenzyme nicotinamide adenine dinucleotide reduced. The metabolism of ethanol increases generation of reactive oxygen species, including hydroxyethyl, superoxide anion and hydroxyl radicals, which contribute to oxidative stress and also can react with other cellular molecules, forming adducts (proteins, lipids or DNA). ADH: Alcohol dehydrogenase; CYP2E1: Cytochrome P450 isoenzyme 2E1; MEOS: Microsomal enzyme oxidation system; ALDH: Acetaldehyde dehydrogenase; NAD^+: Nicotinamide adenine dinucleotide; NADH: Nicotinamide adenine dinucleotide reduced; ROS: Reactive oxygen species.

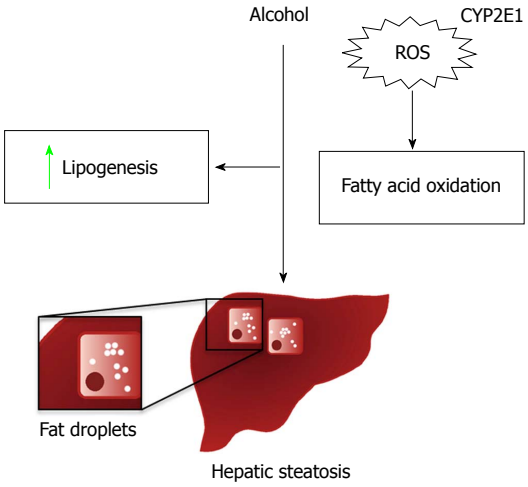

Figure 2 Alcohol induces fatty liver disease. Alcohol causes the accumulation of fat droplets in hepatocytes increasing the lipogenesis and decreasing the fatty acid oxidation. CYP2E1: Cytochrome P450 isoenzyme 2E1; ROS: Reactive oxygen species.

Another feature of alcohol metabolism is the generation of reactive oxygen species (ROS), which are largely regulated (and which can be exacerbated) by the CYP2E1 family[5]. These active radicals are usually produced by the mitochondria, endoplasmic reticulum (ER) or Kupffer cells (KCs). They rapidly form a variety of active metabolites which can further contribute to oxidative stress in hepatocytes[6]. Last but not least, acetaldehyde, the major metabolite of ethanol, is a powerful hepatotoxin. Multiple studies indicate acetaldehyde-induced liver injury *via* mechanisms that promote glutathione depletion, ROS toxicity and lipid peroxidation[7-9] (Figure 1).

Thus, ethanol metabolism can lead to direct biochemical changes in hepatocytes, including cytotoxic metabolites, accumulation of ROS and lipid peroxidation. Importantly, all of these effects can further trigger complex pathological responses that eventually cause damage in the liver. Patterns involved in alcohol-induced liver injury include inflammation, different types of cell death (mainly apoptosis and necrosis), steatosis, fibrogenesis, and even liver regeneration (Figure 2).

Statistically, only about the 35% of ALD patients go on to develop ALD with liver fibrosis. Alcohol-induced damage in liver significantly increases the production of cytokines, chemokines, other soluble mediators and components of the innate immune system[10,11]. This pro-inflammatory environment causes the activation of hepatic stellate cells (HSCs) and myofibroblasts, increasing the production of extracellular matrix (ECM) proteins, which can subsequently induce fibrogenesis in the liver[12]. HSC is the main source of ECM proteins but also a critical target in alcoholic liver fibrosis. Acetaldehyde and adducts such as malondialdehyde (MDA) or 4-hydroxynonenal (4-HNE) directly affect HSC activation and collagen-I genes *via* different signalling cascades[13]. Another crucial mechanism of alcohol-promoting liver fibrosis is associated with endotoxin and immune responses. Studies have shown correlation between alcohol administration, endotoxin in blood and KCs[14]. In the intestine, alcohol impairs tight junctions (TJs) - increasing gut permeability between epithelial cells, thus allowing the gut-derived bacterial endotoxin, lipopolysaccharide (LPS), to enter the liver *via* the portal vein[15]. It is common to see increased levels of serum LPS in ALD patients. KCs, the principal immune cells in the liver, are involved in this process. Several studies have shown that increased LPS levels induced by alcohol stimulate KCs to generate ROS and cytokines. These inflammatory mediators subsequently activate HSCs *via* a Toll-like receptor 4 (TLR4) signalling pathway, which eventually results in enhanced, chronic production of ECM proteins - and promotion of fibrogenesis[16,17]. Additionally, HSCs are also enriched with TLR4 that directly bind, and thus activate through LPS signalling[18]. To summarize, alcohol-stimulated liver fibrosis is a result of a robust immune response involving many types of liver cells and different signal transduction pathways. Fibrosis can develop into alcoholic cirrhosis, which is an advanced stage of liver fibrosis (occurring in 8%-20% of heavy drinkers) - this event is a significant risk factor for HCC. Such pathophysiological transitions will certainly reveal unique mechanisms, requiring more detailed studies and more realistic models[19,20].

HISTORY OF EXPERIMENTAL MODELS

The use of animals as models for scientific study is

a very old practice of human civilization. Acquiring knowledge and experience from his predecessors, Galen of Pergamum (2nd century BC), a Roman physician, greatly improved techniques for dissection and vivisection of animals, and further used them to study cardiovascular and neural anatomy extensively[21].

However, landmark findings in anatomy and physiology in ancient times were largely based on observation, inference and extrapolation of animal physiology to humans.

A Flemish anatomist, Vesalius (1514-1564), a physician and surgeon, was also a pioneer in animal modelling. He compared the similarity and differences between human body and other animal species, overturning the work of Galen - dogma which held for nearly 2000 years. He also recognized the value of animal experiment in teaching and performed vivisection of animals for medical students at his courses. Among the list of new, animal experimentalists, were scholars such as William Harvey (1578-1657). Using results from elegant and sophisticated experiments on live animals, Harvey published his revolutionary work *De Motu Cordis* in 1628, in which he described the anatomic and functional properties of the heart and vascular system from many species with remarkable accuracy[22,23].

The 20th century has witnessed unprecedented advances in biological and medical science. Innovations such as the invention of antibiotics, new diagnostic methods and surgical techniques, chemo- and radiotherapy for cancer, and improved vaccination. Saving millions of lives and significantly increased the average life expectancy. In the past few decades, the use of animal modelling increased dramatically - further supporting the development of medical science. Currently, animal species (model organisms) frequently used in laboratories include: rodents (mouse and rat), zebrafish, swine, rhesus, guinea pig, rabbit, cat, and dog. However it is mammalian rodent models that are the most frequently used - several important advantages: (1) Rodents are highly-resistant to successive in-breeding, with less genetic variability between individual animals (and generations); (2) Their short lifespan and a fast rate of reproduction allows more rapid accumulation of data; (3) Rodents are small in size and easy to handle for most experimental procedures; and (4) Costs are low per animal in terms of initial purchase cost, housing and maintenance. Altogether, rodents provide a model system for study of alcohol effects on mammalian physiology, are amenable to a tremendous array of experimental questions, and are highly-efficient in both time and budget.

Transgenics has become an important tool for generating animal models of human disease. Transgenics involves the addition of foreign genetic information (nucleic acids) to animals, often for specific inhibition of endogenous gene expression. However, despite the generation of several transgenic and knockout models, the development of relevant models has theoretical and technical challenges. Indeed, many ALD-associated genes of interest have not been fully identified and gene addition or inactivation can yield inconclusive results. Some models relevant to ALD are transgenic mice for human CYP2E1 and $p47^{phox}$ NADPH oxidase-deficient mice[24].

CURRENT EXPERIMENTAL MODELS OF ALD

Early attempts of studying ALD with animal models began in the 1950s - using primarily rodents (mice, rats, hamsters, guinea pigs), and primates. An early study using six animal species in parallel was designed to detect their voluntary consumption of alcohol[25]. The data interestingly suggested that golden hamster had a clear preference for alcohol solution (about 88% of their total liquid intake amount), while all other species significantly prefer water over alcohol (with rabbits as the only exception that consumed both drinks at comparable levels). In another study, baboons receiving alcohol-containing diet for 3-4 years, all developed severe hepatic injury (liver fibrosis or cirrhosis), which closely resembled all the pathological stages of human ALD. Thus primates are considered an ideal animal model of studying ALD[26]. However, ethical issues, and the now very tight regulatory controls on the use of primates, as well as high cost and time, prevent the use of primates for the study of ALD in most laboratories. Thus, although rodents (mainly mice and rats), are still the preferred animal species to mimic ALD in human, such models fail to display the complete disease spectrum of human ALD[27,28].

Various hypotheses have been proposed to explain the disparity in liver injury between human and rodents after ethanol exposure. Notably, most rodents have a natural aversion to alcohol and tend to consume ethanol only for calories rather than for craving. In addition, the catabolic rate in rodents is 5 times faster than in humans[29]. These characteristics lead to less damage in rodents, after alcohol exposure, than humans. In addition to effects of alcohol metabolism, difference in the innate immune systems must be carefully considered as immune responses and the pattern of inflammation all play a critical role in the pathology of ALD. For example, the balance between neutrophils and lymphocytes in the blood differs greatly in mice and humans: neutrophils account for 50%-70% of total leukocytes in human blood (10%-25% in mice); whereas lymphocytes comprise 75%-90% of leukocytes in mouse blood, compared with 30%-50% in humans[30]. The physio-/pathophysiological consequences of these differences remain largely unknown. Studies have however demonstrated that mice exhibit greater resistance against endotoxin-induced inflammation, thus experiments usually require a higher ethanol challenge to create the extent of damage comparable with humans[31].

The response to alcohol and the development of

ALD in humans varies considerably between individuals, and ethnic groups. Besides familiar risk factors such as age, diet, and smoking - genetic differences among individuals or ethnic groups are also of great significance. A genotyping study in Asian populations, showed that approximately 50% of Chinese and Taiwanese have low ALDH activity compared with Western nations, due to different genetic polymorphisms in the ALDH2*2 allele[32]. Similarly, diverse outcomes after receiving alcohol application are also seen in different rodent strains. In one case study, mice from 14 commercially acquired inbred strains received ethanol diet (up to 27 mg/kg body weight per day) with an intragastric enteral feeding model for 28 d; all strains exhibited comparable caloric intake and blood alcohol concentration (BAC) levels after the feeding[33]. Interestingly, mice from strains NZW/LacJ, C57BL/10J, FVB/NJ, BALB/cByJ showed severe liver injury compared with mice from WSB/EiJ, PWD/PHJ, C3H/HeJ, AKR/J strains. These results indicated that the marked difference in sensitivity to alcoholic liver injury, was strongly dependent on the mouse strain. Thus, careful consideration of the strain/ desired traits and experimental outcomes should be undertaken when considering an experimental model of ALD. In another study, rats from three different strains (Long Evans, Sprague Dawley, Fisher 344) were fed with an isocaloric liquid diet containing ethanol (equivalent to 37% of the total caloric intake) for 8 wk[34]. All three strains exhibited equally increased BAC, but significantly varied in body weight, alanine aminotransferase (ALT), triglycerides and cholesterol levels. Notable differences were also found in proinflammatory parameters including TNF-α, IL-6 and interleukin-1beta (IL-1β), indicating different degrees of hepatic inflammation after alcohol administration among the three strains. Moreover, dramatic variations were detected between the three strains in some critical enzymes of ethanol metabolism including ADH1, ADH2, AHD3, Catalase, and CYP2E1, suggesting the inequality of alcoholic liver damage may be partly due to different rates of ethanol metabolism between all strains.

Numerous models employing rodent animals have been established to investigate the effects of acute and chronic alcohol exposure on the initiation and progression of ALD (Table 1). To achieve the desired animal model of alcohol disorder, consideration should be given to such factors as amount, route and duration of ethanol given to the animal, and, as mentioned above, the particular animal strain. The amount and duration of ethanol applied to the animal should be sufficient to maintain both a consistently high level of BAC, and long enough to create an acute or chronic injury. In addition, the route of alcohol delivery also plays a critical role in determining the effect of a model. Studies of alcohol administration using vapour inhalation, intravenous, or intraperitoneal injection have been widely reported. These approaches can overcome the unwillingness to imbibe alcoholic beverage in most rodents, and accurately control the amount of ethanol absorbed. However, despite the high level of BAC in rats and mice, these models fail to mimic the natural alcohol "drinking" *i.e., via* the oral route, ingestion and subsequent metabolic processes in humans. As a result, such models are more frequently employed in the field of addiction and behavioural studies, rather than in studies related to alcoholic-induced liver damage[35-37].

Focusing on the effects of alcohol on the GI and liver, rodent models of oral alcohol ingestion have been developed and extensively utilized. By engaging the rodents in "voluntary drinking", these approaches largely replicate the overall process of human drinking habits as well as the general effects of alcohol on liver and intestine. Patterns of alcohol exposure in humans include both short-/ and long-term drinking. Whilst acute liver injury occurs even after 4-5 acute or binge episodes, within a period of several hours, chronic damage accumulates over many years of continuous ethanol consumption. In rodents, gastric intubation is commonly used to administer ethanol dosages of 4-6 g/kg body weight to induce acute hepatic injury. One study using this approach, followed by LPS injection, demonstrated that acute ethanol administration exacerbated hepatic damage caused by endotoxin[38]. Compared with acute animal models using only one or a few gavages, chronic models of alcohol feeding typically last 4-12 wk, usually with a specially designed diet. As acute and chronic alcoholic injury in the liver share remarkable overlap in their pathology, an increasing number of studies combine both models (chronic plus binge model), to better emulate current drinking patterns in humans.

Lieber-DeCarli liquid diet

One of the earliest and most successful diets designed specifically for studying the effect of alcohol consumption *in vivo* is the Lieber-DeCarli liquid diet. It was first introduced by Lieber *et al*[39] in 1963 in response to the need for a more accurate *in vivo* research model for ALD. In a previous study, rats received a 15% (v/v) solution of ethanol instead of drinking water for 177 d. Afterwards, no obvious liver injury (including steatosis and fibrosis) were found in the ethanol-only feeding groups but mainly in groups fed also with a diet of nutritional deficiency[40]. The investigators suggested that the damage to liver after alcohol consumption was a consequence of malnutrition. Thus, it was widely accepted that alcohol alone has no hepatotoxic risk.

In 1960s, in a series of studies, Lieber *et al*[1,41-43] designed a diet containing ethanol and other nutritional components. They demonstrated that when rats received adequate diet, the absorption of alcohol was insufficient to cause significant liver damage, due to their natural aversion to ethanol. This aversion can be overcome when rats had access only to an ethanol-containing liquid diet formula but with no other food or drink. In this case, the daily intake of ethanol in rats can reach 12-18 g/kg, which was two to three times more than that achieved from drinking the ethanol-only

Table 1 Comparison of experimental models of alcoholic liver disease

Models	Animal model	Characteristics	Advantages and disadvantages
Lieber-DeCarli liquid diet[27,28]	Rat/mice	Chronic ethanol feeding (4-12 wk)	Easy to perform Marked elevation of ALT Short term feeding with no mortality rate No liver fibrosis
	Rat/mice	Chronic ethanol feeding + single/multiple binges (4-6 wk)	Easy to perform Marked elevation of ALT and marked steatosis Long term feeding + multiple binges with a high mortality rate No liver fibrosis
	Rat/mice	+ Second hit: DEN, LPS, CCl_4, APAP (4-12 wk)	Easy to perform Marked elevation of ALT and marked steatosis Long term feeding + multiple binges + injection with a high mortality rate Liver fibrosis
Ethanol *ad libitum* feeding[27,28]	Mice	Oral alcohol in drinking water (10 d/1-2 wk)	Easy to perform Minimal elevation of ALT and mild steatosis Short-or long-term feeding with no mortality rate No liver fibrosis
The Tsukamoto-French model[27,28]	Rat/mice	Intragastric infusion (2-3 mo)	Difficult to perform Requirement for intensive medical care Marked elevation of ALT and steatosis Long-term feeding with a high mortality rate Mild liver fibrosis
The NIAA model[47]	Mice	LDE + single ethanol binge	Cost and time efficient High blood alcohol levels Liver injury Inflammation Fatty liver
	Rat /mice	LDE + 3 ethanol binges	Cost and time efficient Increased blood alcohol levels Augmented liver injury Increases in ERK1/2
Ethanol + CCl_4 treatment[106]	Mice	4% ethanol liquid diet + 2 times IP CCl_4 injection per week (8 wk)	Easy to perform Toxic components Elevated acetaldehyde levels Liver fibrosis

Lieber-DeCarli liquid diet with different variants, ethanol *ad libitum* feeding and the Tsukamoto-French and the NIAA model. ALT: Alcoholic liver disease; DEN: Diethylnitrosamine; LPS: Lipopolysaccharide; CCl_4: Carbon tetrachloride; APAP: Acetaminophen; LDE: Lieber-De Carli ethanol diet; IP: Intraperitoneal.

solution. Notably, higher BACs were also observed (100 to 150 mg/dL)[39,44,45]. Using this approach, in seminal work, Lieber *et al*[46] observed significant steatosis in the liver and concluded that alcohol alone is a pathological factor that can induce liver disease. In the next decade, they further detected that this process was influenced by other factors such as gender, dietary fat, the essential nutrients methionine and choline, and vitamin A. These findings opened a new era for ALD research. The liquid diet formula in these studies later became known as the Lieber-DeCarli ethanol (LDE) and Lieber-DeCarli control (LDC) diets, and are now a standard experimental model for the study of ALD[47].

The Lieber-DeCarli diet is an isocalorically-controlled liquid diet in that the total caloric content (0.6-1.0 cal/mL) in the diet remains unchanged, while specific components vary to serve different groups and experimental objectives. The LDC diet, often used for pair-fed control groups, is formulated from several key parts of nutrition: Casein (consisting of methionine and cystine), contributes 18% of total calories; fat, derived from olive and corn oils, makes up 35% of total calories; fat-soluble vitamins (A, D, E, K) and water-soluble vitamin B_{12}, minerals and fiber; the remaining formula (dextrin and maltose mixture) provided the majority of energy (47% of the total calories)[42,44,45]. In the ethanol-containing formula (LDE diet), an amount equal to 36% of total calories of the dextrin and maltose mixture is removed and replaced by isocalorically measured alcohol[42,44,45]. Of note, when applying the LDE diet, the amount of ethanol in the diet should be increased gradually during a primer period of approximate five days, in which the concentration of ethanol increases from zero to the final concentration (in most studies 50 g/L). This short priming period allows the animal to adapt to the ethanol-contained diet gradually, thus ensuring the effect of

subsequent formal feeding.

The feeding period using the LDE model usually varies from 4 wk to 12 wk in mouse and 1-9 mo in rat[48-58]. In most studies, there was a marked elevation of serum ALT and aspartate aminotransferase (AST), with a 6-fold average increase in hepatic triglycerides[46]. Moreover, varying degrees of hepatic steatosis was widely observed in the experimental group. However, no other major hepatic pathological changes, particularly severe forms such as fibrosis, have been reported with the LDE diet feeding model, including long feeding periods of up to nine months in rat[58]. A possible explanation for this limitation is that the LDE diet can only maintain a relatively low BAC in animals, compared with other feeding models such as patients with advanced stage ALD[27].

Many attempts have since been made to elevate the effect of LDE diet - to induce more sever forms of liver injury, in order to overcome its limitations. The general aim would be better mimicking the pathogenesis of ALD in human, in particular its advanced forms. It is not rare for physicians to observe that advanced alcoholic hepatitis (AH) occurs in patients who have a long history of chronic drinking, but also have one or several more recent heavy binge drinking experiences[59,60]. In this context, the chronic-binge ethanol feeding rodents model, which combines a chronic feeding period using LDC diet and one or multiple binges has been introduced and widely accepted[28]. To perform this model, the LDE diet (5% v/v) is given for four weeks to create chronic liver injury as described above. In addition, single or multiple binges are applied by intragastric gavage twice a week during the chronic feeding phase. For gavage, absolute ethanol is diluted to 32% (v/v) in tap water and the recommended dosage of alcohol is calculated at 5 g/kg body weight[61]. It has been reported that in this model, the BAC in rodents can reach 200 to 500 mg/dL, with remarkable elevation of transaminases in serum and significant steatosis in liver[60,62].

Besides binge drinking, other hepatotoxins can subsequently be added during the chronic feeding phase of the LDE diet to provide a "second hit" and increase liver damage, such as: diethylnitrosamine (DEN), LPS, carbon tetrachloride (CCl_4), or acetaminophen (APAP)[63-66]. These studies have expanded the use of the LDC diet and provided useful insight into the effects of ethanol on the initiation and progression of severe liver injuries such as cirrhosis or HCC.

Ethanol ad libitum feeding

The *ad libitum* alcohol feeding model was one of the earliest animal models used for ALD study in rodents[40]. Alcohol is administrated in tap water serving as the only source of drinking water for animals, whilst animals have free access to a standard rodent chow diet. The *ad libitum* feeding model is simple to perform, and easy to manipulate the precise concentration of ethanol in the water. The "voluntary" consumption of alcohol with the normal diet mimics the typical drinking pattern in humans; *i.e.,* intermittent alcohol use with ordinary food intake. Partly due to its great flexibility, protocols used in different studies have varied considerably. The concentration of ethanol solution varied from 10%-40% (v/v), and the period of alcohol administration used in different groups can range from 8 wk, and up to 70 wk, without significant mortality[67-70]. In most studies, the *ad libitum* feeding model is sufficient to induce liver damage with clear steatosis and elevation of ALT and AST, but without more advanced lesions of fibrosis or cirrhosis[68,70,71].

Despite its convenience, *ad libitum* feeding method has limitations compared to other ALD animal models. Noticeably, rodents show strong natural aversion to alcohol, as they tend to drink less ethanol than expected[25]; whilst the rate of alcohol metabolism in rodents is much faster than in humans. These factors prevent the rats or mice from achieving high BAC consistently after chronic ethanol *ad libitum* feeding. The relatively low levels of BAC may be one of the main reasons for some misconceptions of early ALD studies[40]. Mice receiving ethanol *ad libitum* of 20% (v/v) alcohol solution for eight weeks reached BACs between 50-70 mg/dL[67]. Whereas only moderate increase in serum ethanol (to 90 mg/dL) was reported in an early study, where rats were given 40% ethanol solution daily, up to 29 wk[68]. High BAC (up to 150 mg/dL) was also reported indicating wide variations of BAC after ethanol *ad libitum* application[72]. Unlike the LDC diet, which is a nutrition-balanced diet ensuring equal calories in the presence or absence of alcohol content, it is very challenging to evaluate the nutritional status when applying *ad libitum* feeding.

Although the ethanol *ad libitum* feeding model is useful as a "standalone" model of mild alcoholic liver injury, an increasing number of studies combined it with other stressors to stimulate inflammation, fibrosis or HCC in liver. Noticeably, consistent long-term feeding can be substituted by *ad libitum* feeding for long-term periods of time due to its low mortality rate. In one study, 15 different mouse strains were tested with ethanol *ad libitum* from 8 wk to 78 wk[69]. More recently, secondary factors have been introduced including other dietary models such as the high-fat diet and high-fructose diet, to evaluate whether such dietary factors potentiate chronic alcohol-induced liver injury[70,73]. Other studies combining ethanol *ad libitum* feeding model with well-known hepatic stressors like DEN, diallyl disulphide (DADS), phenobarbital, and CCl_4 - typically induced advanced liver injury, including inflammation, fibrosis and HCC[67,72,74,75]. In summary, ethanol *ad libitum* feeding is a simple and reproducible approach to introduce alcohol in rodents, is amenable to the introduction of secondary hits - and is thus widely used by many laboratories for ALD study.

The Tsukamoto-French intragastric infusion model

Although oral alcohol administration including *ad libitum* feeding and ethanol-containing LDC diet has proved a

convenient and effective way to apply alcohol in rodents, it has several limitations. Generally, the average BAC of rodents received oral alcohol administration is usually observed below 150 mg/dL, compared with human levels. Moreover, liver steatosis is the major pathological change in studies conducting oral ethanol application (without a second stressor), where no fibrosis or cirrhosis is found. To overcome these limitations, a new feeding model of direct infusion through a surgically implanted intragastric cannula was developed in 1984, also known as Tsukamoto-French (TF) model[76,77].

Compared with other feeding models of oral administration, the TF infusion model has several distinct advantages. By circumventing the natural aversion to alcohol that generally exists in rodent animals, the TF model removes the barrier on the amount of alcohol that is consumed by the animals. An early study employed liquid diet with alcohol (reaching as high as 49% of total calories) with 30-d infusion. Rats developed severe hepatic steatosis and focal necrosis with a high average BAC (216 mg/dL), and highly elevated ALT and AST levels[78]. More importantly, the TF model also allows easy manipulation of the food content in order to create the desired model of liver damage. When progressively increased ethanol intake (32%-47% of total calories), combined with high fat diet (25% of total calories as fat), fibrosis started to develop in rats within 30 d of feeding, and was observed in all animals after 120 d of feeding[79]. Furthermore, this group also showed that by adding carbonyl iron (0.25% w/v) into the high fat/ethanol-containing diet - by the end of 16 wk most mice developed fibrosis, to different extents, whilst 2 out of the 20 mice developed liver cirrhosis[80].

Even after only 4 wk of intragastric infusion, the average BAC in mouse experiment can reach as high as 300-350 mg/dL, and peak BACs above 400 mg/dL can occur. This reflects a substantially greater level of alcoholic intoxication, achieved by the TF infusion model, over other alcohol feeding regimes[81]. Altogether, in rodents the TF model produces a sequence of liver damage that closely resembles human ALD, *i.e.,* progressive steatosis, fibrosis, cirrhosis with focal necrosis and immune cell infiltration[82].

There are however several potential drawbacks of the TF model. First, the implantation of intragastric tube requires high technical and surgical competences in small animal handling and surgery. Extensive and stringent post-operative care is also essential as early contamination can increase mortality. In addition, the post-operative maintenance work can be a challenge, as the infusion cannula is usually kept *in situ* often for 2-3 mo. The open access nature of the cannula increases the possibility of infection and irritation that may affect the results or result in death. Therefore, animal health and welfare, physiological signs and any pathological changes demand close monitoring. These stringencies make the TF an expensive model that cannot be performed by all laboratories. However, rats with implanted cannula (TF model) and daily food infusion have been kept and maintained for as long as 6 months, indicating once achieved successfully, the intragastric infusion can be a reliable model for investigating experimental dietary conditions in ALD[83].

As a feeding model initially designed for studying ethanol intake in rodents, the TF model has actually produced results with more severe alcoholic liver injury than in other alcohol administration methods. Additionally, the TF model has also been employed in studies focusing on obesity-associated disorders such as non-alcoholic fatty liver disease (NAFLD)[84]. To sum up, the TF rodent model is an effective and reliable approach for ALD study as well as studies related to other metabolic complications associated with diet.

The National Institute on Alcohol Abuse and Alcoholism NIAA model

The group of Gao *et al*[28] developed a chronic-plus-binge alcohol feeding mouse model in 2013 (Table 1). This model mimics acute-on-chronic alcoholic liver injury in patients. The model consists of 5 d of adaptation to the liquid diet. Subsequently, mice are fed a LDE containing 5% (v/v) ethanol for 10 d. A single dose of ethanol (5 g/kg body weight) is given at day 11 and 9 h later animals are euthanased. This model specifically triggers high levels of alcohol in blood, liver injury, fatty liver and inflammation.

The NIAA model has since then been modified: A single binge (5 g/kg) or repeated intragastric infusions of alcohol (5 g/kg, 32% v/v, 3 doses, 12-h intervals) were added following chronic feeding with the LDE diet (5% v/v, 4-7 wk). The advantage of this modification is that the binge increases the neutrophil infiltration in mice[47].

COMPARISON OF HUMAN AND MURINE ALD

Although there are several mouse models of ALD, differences exist between human and mouse in mild and early forms of ALD.

In human ALD, serum liver function tests and liver histology analyses reveal high concentrations of the enzymes ALT and AST, steatosis, ballooning of hepatocytes, neutrophil infiltration and Mallory-Denk hyaline inclusions in the liver[28]. Nevertheless, mouse models of mild and early ALD do not reflect the observed human pathology at each stage.

The model of *ad libitum* feeding with the LDC ethanol diet in mice for 4 wk, results in only mild steatosis and minor elevation of serum ALT, with low-level inflammation[85-89]. Twelve weeks of stepwise feeding with the LDC diet containing ethanol shows fatty liver, but mild elevation of ALT in serum.

The TK model induces severe steatosis, mild liver inflammation and mild fibrosis through continuous intragastric feeding. This model is very useful for the study of ALD pathogenesis (Figure 2), but, as

mentioned, it is expensive, has technical limitations and requires intensive medical care[77,78,90,91].

Acute gavage of a single dose or multiple doses' of ethanol induces only hepatic steatosis with a slight elevation in serum ALT and AST enzymes[89,92-94]. Administration of various concentrations of ethanol in drinking water given as the only water source for longer-term periods has been shown to cause immune abnormalities and mild steatosis, but has little effect on serum ALT/ AST levels and liver inflammation[69,71].

STRATEGIES FOR THE FUTURE: HUMANIZED RODENT MODELS

The development of animal models of ALD has led to remarkable progress in the study of ALD over the last 6 decades. However, many of the models described have intrinsic weaknesses and do not fully recapitulate each stage and facet of human ALD. Following ingestion, ethanol is processed through the classical drug disposition routes: Absorption, Distribution, Metabolism and Excretion (ADME). Multiple factors and systems participate in this complex process and can affect, directly and indirectly, the pathogenesis and final outcome of ALD. Due to obvious species differences in physiology and pathology between rodents and humans, translation of results from rats or mice to humans is problematic. However, next generation experimental animals having certain features of human physiology are being developed that can better resemble the effects of disease in the human body.

The concept of "humanized rodent models" refers to mice or rats engrafted with functional human cells and tissues. Human cell and tissue types used in the development of humanized rodents include: Immune cells, hepatocytes, skin tissue, pancreatic islets, uterine endometrium, and neural cells[95]. Humanized liver in experimental animals has become an attractive target due to the high regenerative potential of the liver. Early attempts using isolated hepatocytes in rodent models in the 1970s. shifted gradually from ectopic transplantation to in-liver engraftment[96-100]. However, one major difficulty preventing these models from becoming effective therapies, is that numbers of functional repopulated hepatocytes after transplantation are still insufficient[101]. In the 1990s, breakthroughs came with the introduction of several transgenic mouse lines. The first model was developed by Sandgren *et al*[102] with exclusive expression of a protease, urokinase plasminogen activator (uPA) in hepatocytes. Overturf *et al*[103] developed a mouse model which targeted disruption of fumarylacetoacetate hydrolase (Fah) - regulated by 2-cyclohexane-1,3-dione (NTBC). With their extraordinary capacity for repopulation of hepatocytes, the engraftment efficiency of transplanted hepatocytes in transgenic models was substantially enhanced compared with normal mice[104].

Humanized animal models offer a novel approach, with tremendous opportunity to explore ALD, and to produce more reliable and robust data, that will ultimately be easier to translate from bench to bedside. For example, Cederbaum *et al*[105] employed humanized CYP2E1 knock-in mice and discovered significantly elevated liver damage in this group after 3 wk of ethanol feeding, suggesting a major role of CYP2E1 in alcoholic steatosis and oxidant stress. However, there is still a long way before humanized rodent models can become a single, standard model for ALD study. Major challenges include the residual host innate immune system, as well as impaired differentiation and maturation of the human immune cell population due to, for example, differences between human and mouse cytokines. However, these drawbacks will not prevent humanized animal model from being viewed as a promising strategy for the future.

CONCLUSION

Alcoholism is now recognized as a major global health issue. Health and socio-economic consequences of alcohol consumption represent a heavy burden world-wide. Although significant progress has been made in gaining better knowledge on the mechanisms and pathology of ALD, many features of ALD are unknown, and require further investigation, ideally with animal models that more effectively mimic human ALD.

Nonetheless, the development of ALD models in rodent has also undergone a significant evolution in terms of representing different stages of human ALD. The early *ad libitum* model revealed liver damage after alcohol administration but have the major limitation of natural aversion in rodent. By adding isocaloric ethanol into the diet to keep nutritional balance, the development of the LDC diet successfully overcame the aversion issue and brought the study of ALD into a new era. The TF model then allowed more control of ethanol intake - effectively increasing liver damage following large amount of alcohol infusion. However, current ALD animal models fail to replicate the all-round spectrum of ALD in patients, particularly ALD in advanced stages. As mentioned, 20%-40% of heavy drinkers tend to develop ALD with severe alcoholic hepatitis, liver fibrosis and cirrhosis or even HCC - after 10 years of excessive alcohol consumption. In contrast, the major change in rodent after ethanol-only application with all models is restricted to hepatic steatosis, even with long-term feeding. Importantly, fibrosis or cirrhosis only appears when secondary insults to the liver have been employed.

In the future, an ideal model of ALD in rodent would effectively mimic, step-wise, each stage of how alcohol adversely affects the liver in humans. Key facets of such a model would address processes such as: Ethanol metabolism/ ADME, oxidative stress, ROS production and immune system activation. These are crucial events particularly in advanced fibrotic liver disease. Presently, rodent models remain useful tools for us to improve our knowledge of ALD. Although differences

in the degree and stages of alcoholic liver injury exist among rats, mice and humans' - data acquisition and translational relevance will be greatly enhanced with the development of new and improved animal models of ALD.

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