BBA - Molecular Basis of Disease 1865 (2019) 1019–1030

Contents lists available at ScienceDirect

BBA - Molecular Basis of Disease

journal homepage: www.elsevier.com/locate/bbadis

Animal models of portal hypertension☆

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ARTICLE INFO

Keywords:
Portal hypertension
Liver disease
Cirrhosis
Animal model
PPVL
NCPH
BDL
CCl_4
TAA
NASH
MCD
HFD

ABSTRACT

Chronic liver diseases ultimately lead to cirrhosis and portal hypertension (PHT). Indeed, PHT is a major cause of severe complications, while medical treatment is limited to non-selective beta blockers. Sophisticated animal models are needed to investigate novel treatment options for different etiologies of liver disease, effective anti-fibrotic agents as well as vasoactive drugs against PHT. In this review, we present some of the most common animal models of liver disease and PHT – including pre-hepatic, intra-hepatic and post-hepatic PHT in rodents. Methodology for induction, considerations for disease etiology, advantages and limitations and practical issues of these animal models are discussed. The appropriate and sensible use of animal models in preclinical research supporting the 3R concept of replacement, reduction and refinement is highlighted.

1. Introduction

1.1. Liver disease and cirrhosis

Liver cirrhosis as the common advanced or end-stage fibrosis is caused by various etiologies of liver disease [1]. As evident from the HEPAHEALTH project [2], the burden of liver disease, especially due to liver cirrhosis, continues to grow in Europe with death rates ranging up to 60/100,000 inhabitants. The main etiologies of liver cirrhosis include viral hepatitis, toxic cirrhosis by excessive alcohol consumption and non-alcoholic steatohepatitis (NASH), which is closely linked to the metabolic syndrome [3]. The pathomorphological changes in liver fibrosis are defined by replacement of severely damaged functional liver tissue (i.e. hepatocytes) by scar tissue (fibrosis), mostly made up of collagen fibers.

1.2. Histological pathology in different etiologies of liver disease

However, the pattern of histological abnormalities varies according to the type of liver injury, i.e. the etiology of liver disease. Even if cirrhosis is considered the common end-stage resulting from repetitive or continuous liver injury, there are distinct patterns and specific differences evident in liver histology, thus also suggesting distinct and etiology-specific underlying molecular mechanisms driving liver injury and disease progression. For example, in alcoholic liver disease, micro- and macrovesicular steatosis together with eosinophilic Mallory-Denk bodies can be found [4,5]. In cholestatic liver diseases, the associated injury of the biliary tree leads to bile duct proliferation and pronounced portal inflammation and subsequent periportal fibrosis [6]. Some of these distinct histological disease patterns can be recapitulated by specific animal models. Typical histological images of some etiology of human cirrhosis and respective animal models are shown in Fig. 1. Briefly, there are similar histopathological patterns between human alcoholic cirrhosis and rat models intoxicated by carbon tetrachloride (CCl4) and as well between human primary biliary cholangitis (PBC) cirrhosis and the rat model of bile duct ligation (BDL).

1.3. Portal hypertension

The injury and inflammation in various liver diseases does not only result in fibrosis of the liver but also affects the hepatic microcirculation, i.e. progressive remodeling of the liver sinusoids. The resulting distortion of the hepatic vasculature increases the intrahepatic vascular resistance (IHVR) as the first trigger for the development of portal

☆ This article is part of a Special Issue entitled: Animal Models in Liver Disease edited by Peter Fickert and Martin Wagner.

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https://doi.org/10.1016/j.bbadis.2018.07.018
Received 22 April 2018; Received in revised form 14 July 2018; Accepted 16 July 2018
Available online 25 July 2018

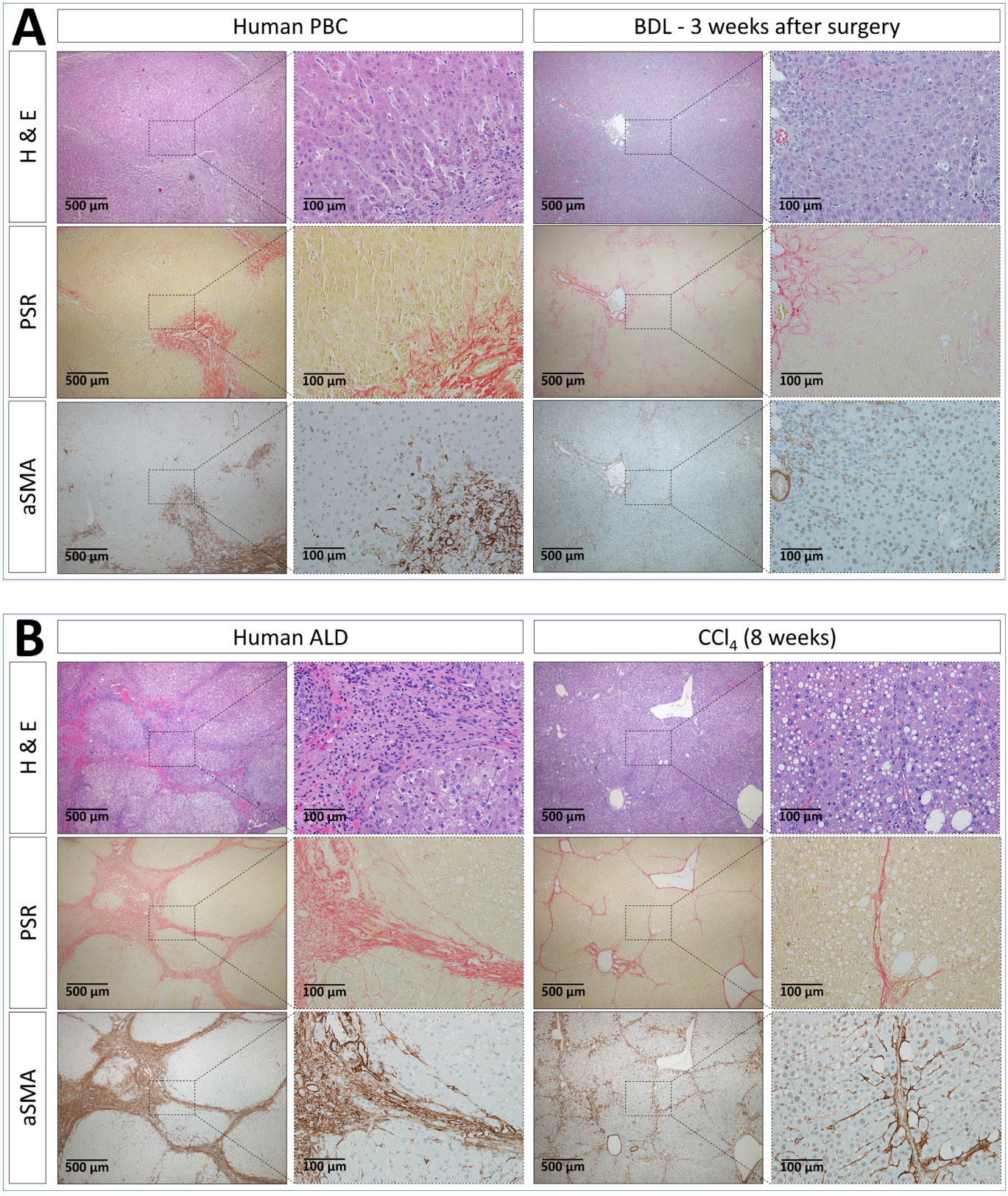

Fig. 1. Typical histological pathology in [A] primary biliary cholangitis (PBC) versus BDL and [B] alcoholic liver disease (ALD) versus CCl_4.

hypertension (PHT) in cirrhosis [7]. Next, to the increased IHVR, PHT is characterized by an increased splanchnic blood flow [8,9] which arises from vasodilatation of arterial splanchnic vessels and the hyperdynamic circulatory syndrome (HCS) [10]. In addition, portosystemic collateral networks develop in an attempt to decompress the portal venous system, on the one hand by the generation of new vessels (neo-angiogenesis) and on the other hand by reperfusion and dilatation of pre-existing vessels [11,12]. With the increasing splanchnic blood inflow decreasing systemic vascular resistance, there is a consequent increase in heart rate and cardiac output aiming to compensate a decrease in

arterial blood pressure [13,14]. Ultimately, PHT increases the risk for severe complications such as variceal bleeding, ascites formation, and hepatic encephalopathy due to portosystemic shunting. Thereby, PHT significantly contributes to morbidity and mortality in patients with cirrhosis and most often, liver transplantation is the only curative treatment. Thus, research on the molecular mechanisms contributing to the portal-hypertensive syndrome and investigation of potential novel treatment options is of significant clinical relevance [1].

PHT can be classified by the anatomic location of the main site of resistance to the portal venous blood flow: prehepatic versus intrahepatic versus post-hepatic PHT. Pre-hepatic (non-cirrhotic) PHT is mostly caused by portal vein thrombosis while intrahepatic (sinusoidal) PHT caused by liver cirrhosis is by far the most common cause of PHT in patients [15]. Cirrhotic and intrahepatic portal hypertension can be induced by BDL, intoxication by CCl_4, intoxication by thioacetamide (TAA), or by metabolic triggers (e.g. high fat diet, HFD or methionine choline deficient diet, MCD).

The most common animal model for pre-hepatic portal hypertension is the partial portal vein ligation (PPVL). An animal model for portal hypertension of non-cirrhotic idiopathic portal hypertension (NCPH) was recently established by Klein et al. [16]. Animal models for post-hepatic PHT such as for the Budd-Chiari syndrome are not widely used and are not well-validated [17–20]. Nevertheless, it is possible to mimic this disease by a gradual obstruction of the supra-hepatic inferior vena cava in rats as described by Orloff et al. [21].

These animal models are used to model disease progression of portal hypertension and/or cirrhosis, to decipher specific molecular pathways and pathophysiology and to assess novel treatment strategies in different disease etiologies. Therefore, the severity of liver disease (such as hepatic fibrosis) or hemodynamic readouts [22] are pivotal parameters to be investigated in these models, while considering the specific timelines and disease course of each animal model. Furthermore, different development courses of portal pressure (PP) after induction of the animal model should be considered by example of BDL and PPVL model, i.e. slowly increasing PP versus PP peak-point at the beginning with slight decrease to a lower level over time, respectively (Fig. 2).

1.4. Need for animal models

Appropriate disease models are essential to understand the underlying pathophysiology and to develop novel treatment modalities. In this regard, animal models of cirrhosis and PHT are used to decipher molecular pathways in basic research and to investigate potential novel treatment strategies in preclinical studies. There is an immense effort on replacement and complementary methods to reduce animal testing. Unfortunately, in-vitro or other non-vital methods are currently not sufficient to recapitulate the complexity of liver diseases and their interaction or impact on the physiology and pathophysiology of the circulatory and vascular system and thus, the whole body. Approaches to replace animal models by in-vitro methods range from liver perfusions, co-cultured isolated cells of multiple cell types to 3D models or bioreactors [23]. This is supporting the 3R's of Russel and Burch [24]. The need of special equipment and the considerable costs have to be considered. However, the cellular components used in most of these complementary methods originate from animals and thus, animal donors would be required in any case. Replacement of animals in the field of liver diseases is only at the beginning. Especially for the investigation of novel treatment strategies against comprehensive diseases like liver cirrhosis, the side effects on other organs are exceedingly important to evaluate the efficacy and safety to human health. On top, there is a wide range of etiologies for liver cirrhosis and portal hypertension with partially unknown molecular pathways involved. Thus, up to date animal models seem necessary in order to study the clinical entity of cirrhosis and PHT from bench to bedside, until equivalent in-vitro methods are available.

1.5. Expected clinical picture in animal models of fibrosis and portal hypertension

Animal models of liver fibrosis/cirrhosis will show a disturbed intrahepatic microcirculation with increased intrahepatic vascular resistance, consequently hyperdynamic circulation and formation of portosystemic collaterals. Progressive deterioration of liver function may occur at different rate depending on the model but is usually less severe than in patients with cirrhosis, e.g. less coagulopathy and only modest signs of hepatic encephalopathy. The resulting liver disease usually depicts a non-painful clinical picture characterized by increased portal pressure, lower mean arterial pressure and splanchnic hyperperfusion. Furthermore, jaundice may develop (especially in models with obstructive cholestasis, such as bile duct ligation) associated with yellowish coat or ears and intensively colored urine.

Severe stages of liver cirrhosis can be identified by the appearance of ascites (i.e. an excessive accumulation of fluid in the abdomen) and confusion caused by poor liver function in combination with slight indisposition and feeling of pressure in the abdominal area. Most animal models are used at maximum to the point when ascites develops to prevent animals from pain, stress or discomfort.

1.6. Evaluation of the portal hypertensive syndrome

The essential two main pillars to evaluate the portal hypertensive syndrome in animal models are the portal and hepatic hemodynamic parameters in consideration of all clinical side effects, next to the assessment of liver fibrosis. Additional analysis of blood parameters and the liver tissue are related to the field of interest in each research issue.

The invasive hemodynamic characterization in rats is well described

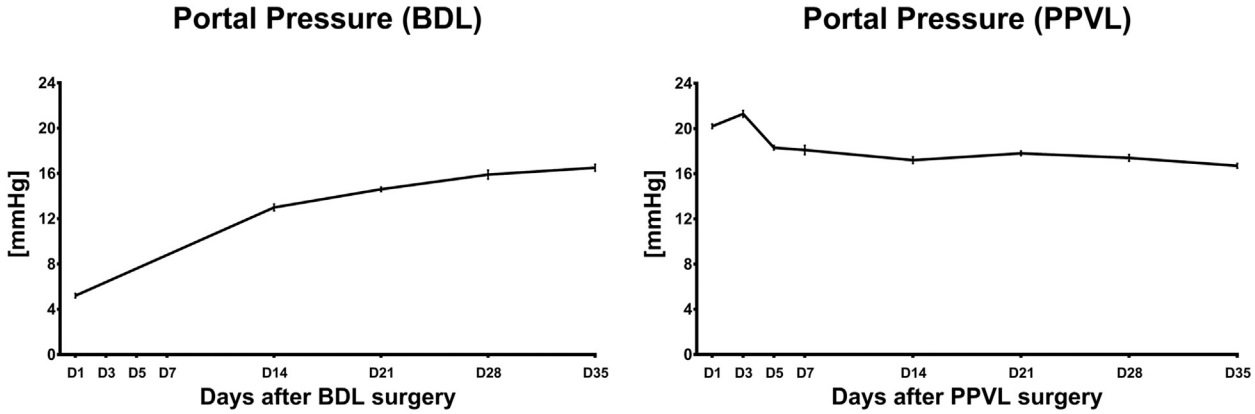

Fig. 2. Portal pressure at BDL and PPVL model, adapted from Königshofer et al. [22].

Table 1
Advantages, disadvantages, reversibility and timelines of animal models in [A] cirrhotic & non-cirrhotic portal hypertension and [B] of cirrhosis by metabolic liver injury.

	Model	Advantages	Limitations	Timeline [genetically engineered]	Species - strain [sex]	Reversibility
[A]						
Non-cirrhotic	**PPVL**	(+) Well established (+) High reproducibility (+) Development of Hyperdynamic circulation after 7 days (+) Short timeline (+) Easy surgery	(−) Surgical intervention (−) Immediate (artificial) onset of PHT	1–7 d, 7 d [32]	Rat – Sprague Dawley [m]	Yes
	gOIVC	(+) Experimental model for Budd-Chiari syndrome (+) Short timeline (+) High rate of reproducibility	(−) Surgical intervention (−) Limited long-term experience with the model (−) Animals survive only up to 8 weeks	2 d–4 w [21]	Rat – Lewis [m]	n/a
	NCPH	(+) Novel and promising animal model (+) Short timeline	(−) Multiple surgical interventions (−) Great experience needed (−) Missing long-term experience with the model (−) Expensive	3 w [16]	Rat – Sprague Dawley [m]	n/a
Cirrhotic	**BDL**	(+) Well established (+) Short timeline (+) High rate of reproducibility	(−) Surgical intervention (−) Adhesions and scar tissue at liver hilus (−) Possible development of biliomas (−) High mortality after week 5 (−) Not suitable for drug testing that is eliminated through the biliary route	2–5 w 4 w [32,121] 5 w [122]	Rat – Sprague Dawley [m] Rat – Sprague Dawley [m]	Depends on the type of surgery used for induction and the severity level of fibrosis/cirrhosis
	CCl_4	(+) Well established (+) No special equipment needed (+) No surgical intervention (+) Various application routes (oral, i.p., inhalation) (+) Easy adaptation of liver disease progression	(−) Potential for organ injury due to repetitive injections (−) Divergent sensitivity to CCl4 across animal strains (−) High workload during induction period	8–16 w 8 w [123–125] 10 w [126,127] 12 w [128] 15 w [129] 16 w [130]	Rat – Sprague Dawley [m] Rat – Sprague Dawley [m] Rat – Wistar [m] Rat – Sprague Dawley [m] Rat – Sprague Dawley [m]	Depends on severity level of fibrosis/cirrhosis
	TAA	(+) Well established (+) No special equipment needed (+) No surgical intervention (+) High rate of reproducibility (+) Various application routes (drinking water, i.p.)	(−) Potential for organ injury due to repetitive injections (−) High workload during induction period	6–18 w 6 w [131] 10 w [132,133] 12 w [131,134,135] 18 w [131]	Rat – Wistar [m] Rat – Wistar [m] Rat – Wistar [m] Rat – Wistar [m]	Depends on severity level of fibrosis/cirrhosis

(*continued on next page*)

Table 1 (*continued*)

	Model	Advantages	Limitations	Timeline [genetically engineered]	Species - strain [sex]	Reversibility
[B]						
Cirrhosis by metabolic liver injury	**NAFLD/ NASH**	(+) No surgical intervention (+) High rate of reproducibility (+) Various transgenic models (+) Models for partial components of the NASH syndrome	(−) Currently there is no 'perfect' complete NASH model available (−) Potential for organ injury due to repetitive injections (−) Special and artificial diets (−) Expensive (−) Metabolic profile is different to human NASH (MCD)	*CDHFD* (10–12 w) 10 w [111,136] 12 w [110] ***MCD*** (1–8 w) 1–2 w [104,137] 5–8 w [116,138,139] ***HFD*** (3–9 w) 3 w [140] 9 w [141] ***Western diet*** (6 m – 16 w) 6 w [113] [ob/ob; db/db; fa/fa] 16 w [142] 6 mnth [112] ***ApoE*$^{-/-}$ & *Western diet*** 7 w [114] ***DIAMOND*** 4–14 w [115] **[ob/ob]** 10 w (MCD) [104,143] **[db/db]** 4–8 w (MCD) [104,144,145] 4–8 w (MCD) [145] **[fa/fa]** 16 w [146]	 Rat – Wistar [m] Rat – Wistar [m] Mouse - C57BL/6 [m] Mouse - C57BL/6 [m] Rat – Sprague Dawley [m] Mouse - C57BL/6 [m] Mouse - C57BL/6 [m] Mouse - C57BL/6 [m] Mouse - C57BL/6 [m + f] Mouse – C57BL/6 Mouse – B6/129 [m] Mouse - C57BL/6J [m] Mouse - C57BL/6J [m] Rat – Sprague Dawley [m] Rat – Zucker [m]	Depends on severity level of fibrosis/cirrhosis Depends on severity level of fibrosis/cirrhosis Depends on severity level of fibrosis/cirrhosis Depends on severity level of fibrosis/cirrhosis Depends on severity level of fibrosis/cirrhosis Depends on severity level of fibrosis/cirrhosis No No No

in literature [22,25]. Briefly, the primary outcome parameter reflecting the severity of portal hypertension is portal pressure (PP), best measured by a direct cannulation of the portal venous system as compared to the indirect measurement of hepatic venous pressure gradient (HVPG) in patients. There is no clear definition of a physiological cut-off value for PP in animal models, since there are several factors during the induction period or sham interventions that may take influence on it. However, the physiological PP in healthy animals is at maximum 5 to 6 mmHg. Thus, a negative and positive control group is always necessary to be included to confirm the successful establishment of a model. Further essential parameters that may impact on PP have to be recorded for an optimal interpretation of the results, including the mean arterial pressure and the heart rate as main determinants of the systemic circulation. Additionaly, the splanchnic/mesenteric arterial blood flow should be determined since it is influenced by the severity of the hyperdynamic circulation.

Application of radioactive microspheres [11] and nowadays safer non-radioactive colored tracer microspheres [25] to the prehepatic vascular field are the gold standard to detect formations of portosystemic collaterals and disturbed intrahepatic microcirculation. The portal vein blood flow is used to calculate the intrahepatic venous resistance by vascular parameters, leading to a secondary confirmation of the fibrosis levels in cirrhotic animals.

For primary assessment of the second main readout, the level of liver fibrosis is determined by techniques that enable reliable results, while a heterogenous/singular method of assessment is not recommended. Therefore, multiple spatial apart lying slices of a whole liver lobe are stained by picro-sirius red to assess histological the collagen proportional area in help of digital image analysis [26]. In the same setting it is possible to quantify alpha-SMA-positive hepatic stellate cells which are responsible for fibrotic tissue accumulation in the liver by immunohistochemistry staining. Another technique is represented by the measurement of hydroxyproline content in liver tissue of a whole liver lobe [27,28]. By combining these parameters the structure of the liver and the content of fibrosis is estimated.

Other clinical signs that occur during the timeline of the model such as ascites are monitored by physical examinations within a fluid thrill test or abdominal ultrasound examination.

1.7. Animal species, strains and sex

The animal species used for animal models of cirrhosis and portal hypertension depends on the main readout parameters of each study.

Rodents, most often mice and rats, are used mainly. The husbandry and the maintenance costs of housing mice are space-saving and cheaper. Mice are used more often if organ harvesting for evaluation of liver fibrosis and hepatic inflammation is the main focus of the study and if no complex surgical interventions are needed for induction of the disease model. Since mice are able to reproduce quickly and have a large offspring they are the species of choice used for genetical engineering and associated basic research. However, in mice surgical interventions are more challenging and less reproducible due to the small animal size, and a high degree of technical expertise is needed. Thus, for complex surgical studies rats are used more often, since reproducibility is much higher and mortality is lower.

In consequence, the evaluation of complex hemodynamic readout parameters is most commonly done in rats. Of note, these rationales are in line with the 3Rs.

Strains comprise differences within each species as well. For example, there are interstrain differences in the development of diet-induced hepatic necroinflammation and fibrosis between C57BL/6, BALB/c and C3H/HeN mice [29,30]. On top of that there are not only strain- but as well gender-specific differences as for example in the development of steatosis in rats [31]. Nevertheless, even if research on both sex is desirable, male animals are mostly used due to a longer period of body weight gain accompanied by steady growth rate which enables a better health monitoring, as well as to eliminate potential additional confounding factors such as the more complex female hormonal status.

Therefore, there are no general recommendations as to which species, strain and sex should be used for fibrosis and portal hypertension research. However, the considerations summarized above should guide the reader/experimenter in the decision to choose not only the most suitable disease model, but also the optimal species/strain and gender for an individualized experimental setup.

For further assistance for choice of the model, strain and species, we have extracted and summarized information from previous animal studies on fibrosis and portal hypertension in Table 1.

1.8. Study design and planning an experiment with animal models

Attention should be paid to time management, manpower, feasibility, daily workload, premises or even public holidays interfering with a planned experiment with animal models. Time management is flexible and easily manageable at short timelines. Models induced by surgical interventions are more labor-intensive and need more comprehensive post-surgical observation, thus limiting the number of experiments per day.

The best way to avoid infeasible accumulation of daily work is to plan time-delayed study groups. As support and rough visualization example timelines for each following animal model are presented in Fig. 3. The minimum number of animals used per study group is specified by biometric statistical analyses depending on the primary outcome parameter supporting both the gain of sufficient data for evaluation and the 3Rs.

2. Animal models

2.1. Prehepatic portal hypertension: partial portal vein ligation (PPVL)

Partial portal vein ligation (PPVL) is a validated model for non-cirrhotic hypertension by creating an artificial, calibrated stenosis at the level of the portal vein. Briefly, a median laparotomy is performed, then the intestine is carefully, temporarily, and partly excavated from the peritoneal cavity for a better view on the portal vein. Subsequently, a blunt-tipped needle is placed along the isolated portal vein and a ligature (3–0 silk in rats) is placed around both the needle and the portal vein. By removing the blunt-tipped needle a stenosis of at portal vein is created by the size of the needle diameter. For the negative control group (sham operated animals) the portal vein is isolated and manipulated in a similar way but not ligated. Different diameter sizes of blunt-tipped needles are used in various studies from 18G [14] up to 21G [32] that will also yield in different degrees of prehepatic PHT. However, the most commonly used needle diameter for this animal model is a 20G needle with an outer diameter of 0.889 mm [11,25,33–35]. The PPVL model induces a maximum increase in portal pressure immediately on the day of surgery. Subsequently, there is a slight decrease of portal pressure, paralleled by the formation of portosystemic collaterals that compress the portal venous system, while hyperdynamic circulation develops being most pronounced after 7 days [36]. This model develops portal hypertension without fibrosis, cirrhosis or other injuries connected to the liver. This method gives an opportunity to study hemodynamic parameters of the portal hypertensive syndrome, effects of splanchnic blood inflow modulation on PP [37,38], and the development of portosystemic shunting [38,39]. The development of a portosystemic collateral vascular network is significantly more pronounced in this model as compared to most cirrhotic models [40]. Thus, this model is especially useful for the study of hyperdynamic circulation and mechanisms involved in the formation of portosystemic collaterals and shunting, e.g. angiogenesis. PPVL rats rarely develop ascites, while there is mild sodium retention caused by ligation of the portal vein. Main advantages of this well-established

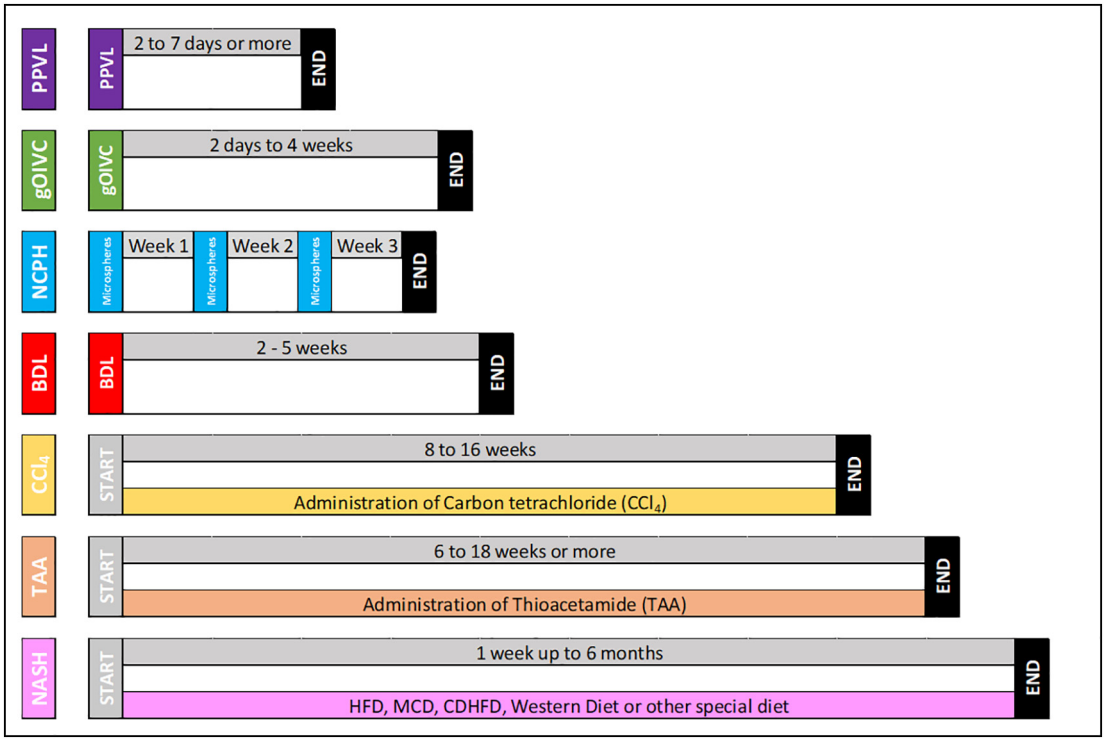

Fig. 3. Timelines for different animal models.

model [41] include the straightforward surgery procedure and its high reproducibility. However, morbidity due to surgery-related complications and even mortality might occur (Table 1).

2.2. Post-hepatic portal hypertension: gradual occlusion of the inferior caval vein (gOIVC)

For the gradual occlusion of the inferior vena cava (gOIVC) as described by Orloff [21] the inferior vena cava (IVC) is encased by an ameroid constrictor. This ameroid constrictor consists of an inner ring of hygroscopic casein that is surrounded by a stainless steel sheath. Both rings have a small opening to ensure affixation around blood vessels. Additionally, they are moveable into each other for fixation by full closure of the ring-constrictor. The hygroscopic casein plastic ring takes up body fluid over time and thus, a successive constriction of the IVC is initiated. Within the first weeks, the closure of the ring occurs more rapidly, then more slowly, and finally, there is nearly complete constriction at week four producing a severe hepatic outflow block. This outflow block results in post-hepatic portal hypertension, severe ascites and hepatomegaly similar to the Budd-Chiari syndrome. While there are clear advantages and disadvantages (Table 1), there is limited long-term experience with the gOIVC model. There might likely be room for modification and adaptation. Still, the gOIVC represents an appropriate model to study pathophysiology and molecular mechanism related to post-hepatic portal hypertension and respective treatment options.

2.3. Idiopathic portal hypertension: repetitive microsphere injections

The disease of non-cirrhotic portal hypertension (NCPH) is poorly characterized but is histologically most often characterized by microthrombotic lesions in small portal venules [42]. Briefly, these microthrombotic lesions can be created in animals by repetitive injections of microspheres (15 μm diameter) into the ileocolic vein according to the methodology described by Li et al. [43] and refined by Klein et al. [16] For this model, a weekly laparotomy is necessary to get access to the ileocolic vein, to insert a PE-50 catheter that is fixed at the ileocolic vein by 5–0 silk prior to application of the microspheres. Application of microsphere is performed weekly for 3 weeks. Table 1 summarizes the advantages and disadvantages of this animal model.

2.4. Cirrhotic portal hypertension: bile duct ligation (BDL)

The most commonly used method to induce obstructive cholestasis in rodents is surgical bile duct ligation (BDL) that mimics secondary biliary cirrhosis [44]. A laparotomy is performed and the common bile duct and its lobular branches are ligated and subsequently resected. During the following time period animals develop advanced fibrosis, jaundice, and portal hypertension [44] – similar to the symptoms observed in human biliary cirrhosis [45]. Sham operations are performed by pulling silk of a similar diameter as used for ligations around the bile duct branches, which then gets removed without a ligation or dissection to serve as a healthy ('negative') control. The BDL model is useful for understanding the pathogenesis of hepatic inflammation and fibrosis in cholestasis. Different types or approaches of the surgery are being developed in order to study particular disease settings, such as common bile duct ligation – a commonly used and well-described model in rats [46], or partial BDL to model acute cholestasis [47]. The most refined and precise surgical technique is a microsurgical operation technique by ligation of each bile duct branch as proximal to its liver lobe as possible [48]. There are also techniques, such as re-anastomosis of the previously ligated bile duct branches or biliodigestive anastomosis of the gall bladder (in mice) with the small intestine, which allows the BDL model to be reversible [49,50]. Development of marked portal fibrosis caused by slow increase of PP takes between 4 and 6 weeks. The severity of PHT reaches a mild level after 2 weeks and severe level after 4 weeks, with an incidence rate of 30–60% of hyperdynamic circulation and portosystemic shunting [51,52]. The BDL model is also reported as an animal model for development of the hepatopulmonary syndrome [53].

Main advantages of this model are high reproducibility and relatively short induction time to induce cholestatic liver injury as

compared to other cirrhotic models. However, there are also complications mostly related to the surgical procedure - dropouts during the anesthesia, surgery or post-operative complications such as infections [54]. One very common post-operative complication is the formation of biliary cysts, so-called biliomas [55]. These cysts may also have an impact on hepatic perfusion and portal pressure when developing next to the portal vein, close to the liver hilus and after gaining a certain size. Preventative techniques such as the injection of 10% formalin [56] or Ethibloc® [57] into the bile duct or a ligation and resection of the bile duct branches to each liver lobe by microsurgery have been proposed to overcome this limitation [58,59].

An alternative to the BDL model is the knockout of MDR2 (Abcb2) in a genetically modified mouse model which is of special interest for experimental studies on cholestasis and biliary injury, as the model recapitulates several features of human biliary fibrosis similar to primary sclerosing cholangitis [60]. These mice lack excretion of phosphatidylcholine into bile which results in sterile inflammatory cholangitis with portal inflammation and ductular proliferation. Starting at birth, these animals go on to develop cholangitis with biliary fibrosis within 3 to 6 months characterized by early onset of severe portal hypertension. Ultimately, the MDR2$^{-/-}$ model will also develop tumors at the age of 4 to 6 months, mostly hepatocellular carcinoma [61].

2.5. Cirrhotic portal hypertension: Carbon tetrachloride intoxication (CCl_4)

Carbon tetrachloride (CCl_4) intoxication is a very common method to induce toxic liver cirrhosis. This model mimics a similar situation as in humans' toxic fibrosis, including inflammation, liver cirrhosis and portal hypertension. CCl_4 is metabolized in hepatocytes, where it causes production of toxic trichloromethyl (CCl_3) radicals [62,63]. These radicals initiate lipid peroxidation and degradation of polyunsaturated fatty acids, thus resulting in membrane layer degradation and providing cellular inflammation, thus leading to tissue fibrosis and finally cirrhosis development [62,64]. The most common method to perform this model is periodic administration of CCl_4 intraperitoneally (i.p.). Weight adopted doses (0.5-1 mL/kg) of CCl_4 solution in olive/corn oil are administered 2–3 times per week and induce a liver cirrhosis in several timelines and [44,65]. Subcutaneous injections (s.c.) are less efficient, because the onset of cirrhosis development is prolongued to twenty weeks or even longer and there is an increased risk of tissue necrosis, while at least mortality rate is minimal [65,66]. Administration of CCl_4 via intraperitoneal injections (i.p.) is most commonly used but may cause increased mortality in case of high starting doses or high concentrations of CCl_4 (> 1:1, usually added to corn oil or olive oil). For proper i.p. injections, a clean, accurate and standardized procedure is absolutely necessary [65]. CCl_4 intoxication can also be achieved by oral gavage, however, if parallel oral treatments are necessary oral administration of CCl4 could have an impact on resorption and thus on pharmacokinetics and the resulting effect of the respective study drug. Alternative CCl_4 administration routes include inhalation that seems to be associated with a decreased mortality rate as compared to oral administration [44]. However, inhalation of CCl_4 increases the risk of ascites – which might be desired as an advanced model but also an unwanted side-effect (e.g. if intraperitoneal injections of compounds must be performed) [67].

Rats gavaged with CCl_4 develop less consistent fibrosis [66,68] and present increased mortality during the early induction period. Repeated inhalation of CCl_4 takes approximately 10–13 weeks to induce the model, and additional attention should be put on animals during inhalation to avoid respiratory arrest [65]. Moreover, utmost caution should be paid to the investigator's safety because of CCl_4 toxic effects, which is the greatest practical limitation for this particular method of CCl4 administration. First sights of fibrosis and scaring fibers were already detected after 2–3 weeks of CCl_4 treatment in rats and about 4 weeks in mice [69], when administrated intraperitoneally.

Regarding reversibility of the animal model, fibrosis induced by 6 weeks of CCl_4 intoxication might resolve within approximately 4 weeks (depending on the severity of fibrosis present at cessation of CCl_4 injury) after last injection [44,70–72]. Frezza E. et al. reported that intragastrical application of CCl_4 leads to hepatocellular carcinoma (HCC) development in rare cases of 30% after 30 weeks of administration in a rat model [147].

In regard to potential differences in the CCl_4 fibrosis response among different mouse strains, the resulting fibrosis seems to be more pronounced in inbred BALB/c strains, while C57BL/6 mice develop an intermediate level of liver fibrosis. The lowest fibrogenic response to CCl_4 has been reported in FNB/N mice [62,73].

Main advantages of the CCl_4 model are the considerable parallels to the clinical situation, its reproducibility and adaptability depending on study conditions. The efficacy of the CCl_4 model is influenced by diet and xenobiotics intake, which can be beneficial to control/adapt model settings [74]. A main negative effect of CCl_4, next to hepatotoxicity and tissue necrosis in a case of injections, is the toxicity on other organs, such as peritoneum, mucosa, respiratory tract and central nervous system [75] (Table 1).

2.6. Cirrhotic portal hypertension: thioacetamide intoxication (TAA)

The thioacetamide (TAA) model is an alternative way of inducing hepatotoxic liver damage. Hepatotoxicity of TAA is based on production of reactive oxygen species (ROS) [76,77]. Already a single dose of TAA induce liver necrosis, however, followed by regeneration in case injections are stopped [78,79]. Long-term administration of TAA cause liver fibrosis, cirrhosis and HCC [78,80–82]. TAA can be administrated via i.p. injection of concentrations between 150 and 200 mL/kg [44,83,84] or orally with drinking water by a concentration of 200 mL/kg [85,86]. Injections are performed 2–3 times per week, during a time period of 6–12 weeks. Oral administration requires a longer application period of 6–18 weeks [86,87], and similar to CCl_4, irritations of the gastrointestinal tract by oral administration of toxic TAA have to be considered.

This model is presented with acute liver failure/advanced cirrhosis and fibrosis after 6 weeks at earliest, however, severe fibrosis is still present until 2 months after last application of long-term TAA, which is advantageous for studying fibrosis regression [44]. Nevertheless, after 18 weeks of TAA intoxication, the risk for cholangiocarcinoma significantly increases [88].

Main advantages of the TAA model are high tissue specifity of the TAA reagent, a long-time interval between first presence of tissue necrosis and advanced liver failure - adaptability to research requirements [77,89] and possible reversibility depending on the severity level of fibrosis/cirrhosis (Table 1).

2.7. Animals models of portal hypertension by metabolic liver injury

2.7.1. Cirrhotic portal hypertension: non-alcoholic fatty liver disease (NAFLD) and liver non-alcoholic steatohepatitis (NASH)

NAFLD emerged as a new global case of liver disease worldwide. A great number of patients with NAFLD develops non-alcoholic steatohepatitis (NASH), characterized by liver inflammation and cell damage leading to liver fibrosis and liver cancer in advanced stages [90]. The ideal animal model for non-alcoholic steatohepatitis (NASH) should resume all features of human NASH with hepatic steatosis, inflammation, hepatocellular ballooning and progressive fibrosis, ideally in association with a phenotype of insulin resistance, overweight/obesity and other features of the metabolic syndrome. However, the ideal NASH model is not yet established and currently topic of discussion. Still, with the increasing epidemic of obesity, NASH is becoming the most common liver disease worldwide and a leading cause for liver transplantation [91–93]. Different rodent models have been developed that reflect features of the metabolic syndrome and of the human NASH phenotype [94]. In these models of metabolic liver disease, substantial

strain- and gender-differences in disease severity may occur with heterogenous severity of hepatic necroinflammation and fibrosis. However, this is also an important feature of human NASH disease.

2.7.2. Genetic rodent models of NAFLD/NASH

Nowadays there are a few genetic models of NAFLD available: *Ob/ob* mice model (spontaneous mutation in a leptin gene) [95,96], *db/db* mice model (natural mutation in a leptin gene) [97], obese (fa/fa) Zucker Rat model (mutation of fat allele) [98,99], heterozygous Agouti gene (KK-Ay/a) mutation model [100], MC4R-KO mice model (melanocortin 4 receptor-deficiency) [101,102] and SREBP knock-out mice model (Sterol Regulator Element-Binding Protein 1c) [103]. All these models are used to display metabolic abnormalities like overweight, diabetic phenotype, insulin resistance and macro-vesicular steatosis. These symptoms do not lead to spontaneous progression of the liver disease and need additional side intervention by high-fat diet (HFD) or methionine-choline deficient diet (MCD) in animal models [104].

2.7.3. Diet induced animal models of NAFLD/NASH

High fat diet (HFD) models of NASH are developed by feeding rodents with chow containing an increased amount of fat (30–75% of total calories intake derives from saturated/unsaturated fatty acids). Animals present metabolic syndrome (development of insulin resistance), supported by steatosis and increased transaminases levels already after 4 weeks of HFD. Some strains of rodents are more resistant, for example 14 weeks of HFD results in lack of steatohepatitis in Wistar rats as compared to Sprague-Dawley rats [105,106]. Metabolic differences were also observed between different mice strains, sex and age - Balb/C mice presented higher lipid accumulation than C57BL/6J [107]. Another commonly used diet to induce NASH in rodents is the MCD. This diet is based on high intake of sucrose (40%) and low intake of fat (10%), with full elimination of methionine and choline. The MCD is associated with faster weight loss, development of steatosis and inflammation – occurring already after 2 weeks of feeding, without features of insulin resistance [85,108]. A detailed comparative analysis of different inbred mouse strains fed by MCD diet has shown variations in transaminase levels and level of liver fibrosis. Gender differences in NASH models by MCD diet appear for example as stronger development of NASH attributes in male Wistar, Long-Evans and Sprague Dawley rats. Strain differences are shown by less steatosis in C57BL/6 mice [109].

Refined NASH models are induced by feeding with a choline-deficient high-fat diet (CDHFD). An advanced NASH phenotype is recapitulated by combining CDHFD with intraperitoneal sodium nitrite ($NaNO_2$) injections to induce methemoglobinemia, ROS formation and development of characteristic NASH features [74,75,87]. The NASH features of steatosis, inflammation and liver fibrosis were induced within 8–10 weeks by Nakamoto et al. [110] and Takayama et al. [111].

Western Diet (or Fast food diet) is characterized by a high fat intake (approximately 40% of calories derived by fat with 2% from cholesterol) supplemented by high sugar intake (fructose in water). This diet was described as a long-term diet (6 months) but presents metabolic changes as obesity and insulin resistance corresponded with steatosis, NASH and fibrosis development [112]. However, Kanuri et al. showed massive steatosis and inflammation already after 6 weeks [113]. A western diet for seven weeks combined with Apoe$^{-/-}$ CB57BL/6 mice represents a novel and fast mouse model for NASH and metabolic syndrome. While having the biggest advantage of displaying all characteristic features of NASH such as hepatomegaly, abnormal glucose tolerance, liver fibrosis and inflammation, the only limitations come up with the availability of species suited for an optimal evaluation of the hemodynamic parameters of portal hypertensive syndrome [114].

2.7.4. Diet induced animal model of non-alcoholic fatty liver disease (DIAMOND)

This model derived from a stabel isogenic inbreed strain out of two commonly used laboratory mouse strains - 129S1/SvImJ and C57BL/6J fed with HFD. Animals display features of metabolic syndrome such as obesity and insulin resistance with fast development of steatosis, steatohepatitis, progressive fibrosis and HCC in response to fat high sugar Western Diet [104,115]. In a time period of 16–22 weeks, the majority of mice develops robust NASH and fibrosis, hepatocellular carcinoma may developed in most mice at week 32–52 [104].

2.7.5. Is there a good NASH model for portal hypertension?

However, albeit no perfect NASH models exists up to date for an optimal hemodynamic evaluation of the portal hypertensive syndrome, several features of NASH can be modeled. Some of the promising novel pharmacological treatments of NASH have been developed in animal models [116–118] and continue to show efficacy in phase 3 trials [119]. Importantly, all differences between NASH phenotypes dependent on animal species, strains and even gender should be kept in consideration [93,109,120].

3. Animal models in comparison

Each model has specific features, advantages and disadvantages and thus, the animal model should be chosen according to the research questions that want to be answered. Species, strain and sex should be chosen in regard to the main endpoint parameter of the study while comprising significant influence on the phenotype of the model. The length of timeline of each model should be chosen by the stage of disease which is in the focus of interest. For the inflammatory phase of cirrhosis and early fibrogenesis a shorter timeline is recommended, whereas a longer timeline is better suited for advanced fibrosis or portal hypertension. In this regard, consider treatment settings: prevention treatment vs. therapeutic/regressive treatment. In addition, the different application routes, administration schedules, environmental or housing condition may influence the severity of disease as most evident from the CCl_4 and TAA intoxication models where some heterogeneity is observed in the fibrogenic and inflammatory response. Even the experience of the experimenter, different manufacturers, or the storing of the diets have an impact on the overall performance. Therefore, the establishment of an animal model at a laboratory needs time and effort. At last, the potential for disease reversibility of an animal model is essential to answer questions concerning the mechanisms involved in resolution of hepatic inflammation, steatosis or liver fibrosis regression (Table 1).

For decision support in question of which model fits best to certain scientific issues, Table 1 summarizes the models by advantages/disadvantages, timelines, species, strain and sex and Table 2 provides a summary of utilities of each animal model presented.

Table 2
Utility in each animal model.

	PPVL	gOIVC	NCPH	BDL	CCl_4	TAA	NASH
Disturbed intra-hepatic microcirculation	−	−	+	+ +	+ + +	+ + +	+ +
Hyperdynamic circulation	+ + +	n/a	n/a	+ +	+ +	+ +	n/a
Formation of portosystemic collaterals	+ + +	n/a	n/a	+ +	+ +	+ +	n/a
Development of ascites	+ +	+ + +	n/a	+ +	+ + +	+ + +	n/a
Bleeding model	+ + +	n/a	n/a	+ +	n/a	n/a	n/a

Table 3
Anesthesia regimes for rats (Sprague Dawley).

		Advantages	Disadvantages
MMF-K	MMF-K: Medetomidine [0.3 mg/kg] & midazolam [1 mg/kg] & fentanyl [0.015 mg/kg] subcutaneous in combination with ketamine [10 mg/kg] intramuscular. Antagonization of medetomidine by atipamezole [1 mg/kg] and midazolam by flumazenil [0.1 mg/kg] subcutaneous for a quick recovery time period from anesthesia (recommended) is possible after 20–30 min.	(+) Broad range of efficacy (+) Safer application routes (+) Complete antagonization at any time	(−) Availability (−) Costs
KX	KX: 80–100 mg/kg ketamine with 5–10 mg/kg xylazine in physiological saline solution; intraperitoneal. It is necessary to re-dosed ketamine (reduced dose 20-30 mg/kg, intramuscular) after 30 to 45 min to induce continuous surgical-plane anesthesia. Antagonization is possible by atipamezole after 30 min.	(+) Availability (+) Costs	(−) High rate of wrongly administered injection (−) Antagonization at earliest after 30 min possible

4. Anesthesia regimes recommended for surgical interventions in rodents

Surgeries used for induction of the models are recommended to be performed in aseptic conditions in an operation room by experienced researchers. There are several anesthesia regimes available. Table 3 summarized the specific advantages and disadvantages of two different anesthesia regimens for rats (Sprague Dawley). In order to avoid potential cardiovascular and respiratory adverse events of all anesthetics, we recommend intubation and ventilation of the animals during the surgery. This will improve safety reasons and reduce morbidity due to complications of anesthesia. However, special equipment is needed for ventilation or even inhalation anesthesia.

The usage of a heating pad to maintain physiological body temperature and controlling it through a probe is essential during surgery. Importantly, this will also support a physiological metabolism rate and a balanced hemostasis that reduce potential bleedings.

Nomenclature

BDL	bile duct ligation
CCl_4	carbon tetrachloride
CDHFD	choline-deficient high fat diet
gOIVC	gradual occlusion of inferior vena cava
HCC	hepatocellular carcinoma
HCS	hyperdynamic circulatory syndrome
HFD	high fat diet
IHVR	intrahepatic vascular resistance
IVC	inferior vena cava
MCD	methionine choline deficient diet
NAFLD	non-alcoholic fatty liver disease
$NaNO_2$	sodium nitrite
NASH	non-alcoholic steatohepatitis
NCPH	non-cirrhotic idiopathic portal hypertension
PBC	primary biliary cholangitis
PHT	portal hypertension
PPVL	partial portal vein ligation
TAA	thioacetamide
s.c.	subcutaneous
i.p.	intraperitoneal
p.o.	per os (oral administration)

Transparency document

The Transparency document associated this article can be found, in online version.

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