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REVIEW

Metabolism of N-nitrosodimethylamine, methylation of macromolecules, and development of hepatic fibrosis in rodent models

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Abstract

Hepatic fibrosis and cirrhosis are chronic diseases affecting liver and a major health problem throughout the world. The hallmark of fibrosis and cirrhosis is inordinate synthesis and deposition of fibril forming collagens in the extracellular matrix of the liver leading to nodule formation and loss of normal architecture. Hepatic stellate cells play a crucial role in the pathogenesis and progression of liver fibrosis through secretion of several potent fibrogenic factors that trigger hepatocytes, portal fibrocytes, and bone marrow–derived fibroblasts to synthesize and deposit several connective tissue proteins, especially collagens between hepatocytes and space of Disse. Regulation of various events involved in the activation and transformation of hepatic stellate cells seems to be an appropriate strategy for the arrest of hepatic fibrosis and liver cirrhosis. In order to unravel the molecular mechanisms involved in the pathogenesis and progression of hepatic fibrosis, to determine proper and potent targets to arrest fibrosis, and to discover powerful therapeutic agents, a quick and reproducible animal model of hepatic fibrosis and liver cirrhosis that display all decompensating features of human condition is required. This review thoroughly evaluates the biochemical, histological, and pathological features of *N*-nitrosodimethylamine-induced model of liver injury, hepatic fibrosis, and early cirrhosis in rodents.

Keywords Hepatic fibrosis · Liver cirrhosis · Rodent model · *N*-Nitrosodimethylamine · NDMA · Dimethylnitrosamine

Introduction

Hepatic fibrosis is the result of a pathological response of most chronic liver injury and a serious health problem worldwide. Hepatic fibrogenesis is a physiological process that converts into a pathological situation due to contour stimulus from the causative agent. Various types of chronic liver injury could lead to fibrosis that gradually develops into liver cirrhosis and may result in liver cancer [1, 2]. The hallmark of hepatic fibrosis is an excessive synthesis and abnormal deposition of connective tissue components, especially interstitial collagens in the extracellular matrix of the liver [3–7]. It is the result of an abnormal and repeated wound healing response generated as a result of chronic liver injury from various factors, such as drugs, alcohol, non-alcoholic steatohepatitis (NASH), hepatitis B and C viral (HBV & HCV) infections, autoimmune hepatitis, and cholestatic liver diseases [8, 9]. The pathogenesis of hepatic fibrosis triggers from oxidative stress caused from elevated levels of reactive oxygen species (ROS). This is followed with cellular injury and production of a series of inflammatory cytokines mainly transforming growth factor-β1 (TGF-β1), tumor necrosis factor-α (TNF-α), and connective tissue growth factor (CTGF) [10–14]. The elevated levels of various cytokines and growth factors induce activation and transformation of resting round hepatic stellate cells (HSCs) into star-shaped myofibroblast-like cells with the expression of α-smooth muscle actin (α-SMA) filaments as a characteristic marker [15–18]. The transformed stellate cells lose their retinoid deposits (vitamin A), proliferate rapidly, and remarkably express a number of connective tissue proteins, especially collagens, fibronectins, laminin, and hyaluronic acid that accumulate in the extracellular space of hepatic parenchyma [19–21]. Stellate cells also contribute to liver regeneration and serve as a progenitor cell population with hepatobiliary characteristics [22, 23].

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The metabolism and detoxification of all drugs and molecules are mostly happening in the liver through the cytochrome P450 family of enzymes, which is one of the reasons of chronic liver injury during the persistent detoxification process [24–26]. The chronic liver injury and hepatic necrosis activate several cytokines and growth factors that trigger regenerative response and initiates wound healing [1, 27, 28]. The repeated wound healing process leads to scarring of hepatic parenchyma along with blockage of hepatic sinusoids, which results in portal hypertension [29–31]. Extensive scarring of the hepatic mass causes nodular formation and distortion of normal architecture that prevents liver's ability to regenerate by itself. Hepatic fibrosis or the process of fibrogenesis is reversible up to a particular stage, which is hard to define or explain precisely. Once the process of hepatic fibrosis transforms into cirrhosis with extensive distortion of lobular architecture and nodular formation, then it is unlikely to reverse even if the injurious stimulus is removed permanently. Therefore, it is important to stop or terminate the causative agent well in advance to prevent transformation of hepatic fibrosis to advanced cirrhosis [32, 33]. Portal hypertension and extensive hepatocyte impairment lead to deregulation of normal functioning of the liver resulting in liver failure [34, 35]. The repeated wound healing process involving cellular regenerative events could cause genomic aberrations of normal mitotic process or mutations in tumor-suppressor genes leading to the development of hepatocellular carcinoma [36–41].

N-Nitrosodimethylamine or dimethylnitrosamine

N-Nitrosodimethylamine, (NDMA) or dimethylnitrosamine (DMN) [$(CH_3)_2N_2O$, Mol. Wt. 74.08] is a yellow, semi-volatile oily liquid with a characteristic odor. Table 1 depicts most of the physical and chemical properties of NDMA. It forms as a byproduct or waste product during several industrial processes and present in trace amounts in tobacco smoke condensate and cured or smoked meat products [42–45]. NDMA could be formed from dimethylamine during chlorination of water [46] and can be degraded into dimethylamine and nitrite by fermenting bacteria [47]. Chlorination or chloramination of organic nitrogen-containing wastewater treatment could produce NDMA at potentially harmful levels [48]. The hepatotoxicity of NDMA was first reported by Barnes and Magee in 1954 following two cases of liver cirrhosis in an industry in UK [49]. Later NDMA has been extensively studied and characterized as a potent hepatotoxin, mutagen, and carcinogen [5, 50–53]. The highly reactive metabolic intermediates are responsible for the toxicities produced by NDMA and related nitrosamines and not by the parent compound [52, 54]. The biologic half-life of NDMA is < 10 min in rodents and about 20 min in non-human primates [55, 56]. As per US Environmental Protection Agency (EPA), the maximal admissible concentration of NDMA in drinking water is 7 ng/L [57].

Mechanism of DNA and protein methylation by *N*-nitrosodimethylamine

Since NDMA is primarily metabolized and degrades in the liver, its toxicity mainly affects the liver, especially in the centrilobular area due to diffusion of the compound from the hepatic central veins [58]. Metabolic degradation of NDMA in the liver is largely through the microsomal membrane-bound enzyme, cytochrome P-4502E1 [59–61]. The metabolic activation and degradation of NDMA by CYP2E1 is depicted as structural flowchart in Fig. 1. The activation of NDMA involves α-C-hydroxylation by CYP2E1 to form hydroxymethylnitrosamine and subsequent dealkylation of reactive intermediates to form methyldiazohydroxides and finally alkylating agents [62]. The metabolism of NDMA in the liver produces methylamine, formaldehyde, and methanol and a highly reactive alkylating intermediate "methylcarbocation" that reacts with nucleic acids and proteins to form methylated macromolecules (Fig. 1). NDMA methylates proteins [63, 64] and DNA [65] and forms specific DNA adducts [66, 67]. It was demonstrated that in vitro preparations of human liver slices can also metabolize NDMA and methylates its DNA in the same order as rat liver slices [68]. While the basic principles of the metabolism of NDMA and related nitrosamine compounds are well established, only limited information is available about the biochemical and molecular biological mechanisms of organotropy in nitrosamine-related carcinogenesis [69–72].

N-Nitrosodimethylamine induced model of hepatic fibrosis

Madden et al. [73] first developed the new canine model of hepatic fibrosis employing NDMA. Later, Jenkins et al. [74] had shown that serial administrations of NDMA in rats could produce a reproducible model of hepatic fibrosis, cirrhosis, and portal hypertension, as seen in human beings. Jezequel and co-workers studied various aspects of the pathophysiological and biochemical events associated with the development of NDMA-induced hepatic fibrosis and demonstrated that it is a good and reproducible animal model to investigate the early events involved in the pathogenesis of human liver fibrosis [75–82]. Recently, the model has been widely used to study the molecular mechanisms involved in the pathogenesis of hepatic fibrosis [83–86]. Furthermore, NDMA-induced model of chronic liver injury has been employed to investigate the

Table 1 Physical and chemical properties of *N*-nitrosodimethylamine

Property	Value/description
Chemical formula	$C_2H_6N_2O$
Molecular weight	74.083 g/mol
Solubility in water	290 g/L at 20 °C
Specific gravity/density at 20 °C/4 °C	1.0048 g/mL
Melting point	< 25 °C (estimated)
Boiling point	153.1 °C (307.5 °F)
Vapor pressure at 20 °C	2.7 mmHg
Flash point	61.0 °C (141.8 °F)
LD_{50} (rat)	37.0 mg/kg (oral)
Physical description (physical state at RT)	Yellow liquid with no distinct odor
Biohazards	Carcinogen, hepatotoxic

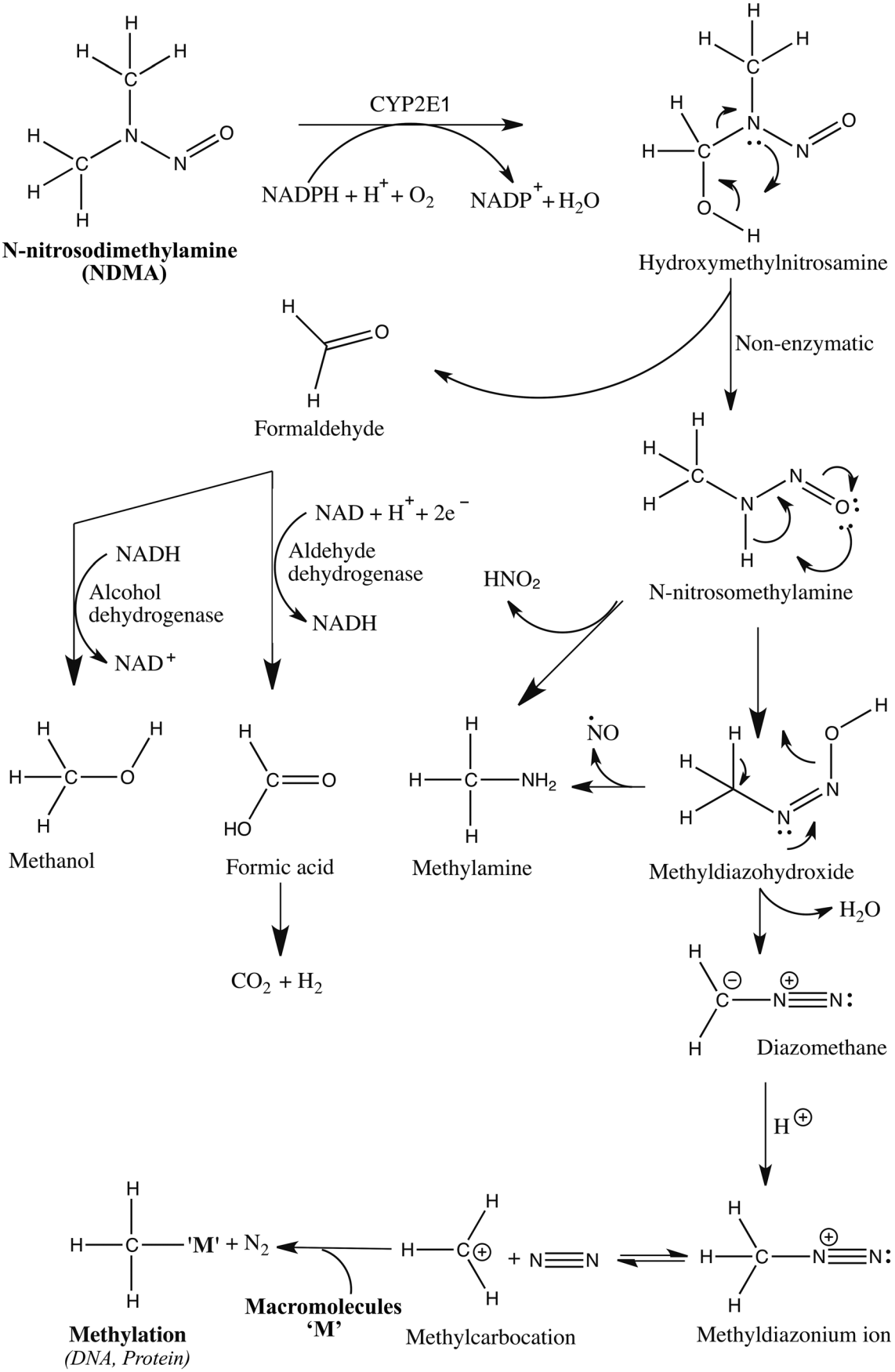
g/mol grams per mole, *g/L* grams per liter, *°C* degrees Celsius, *g/mL* grams per milliliter, *°F* degrees Fahrenheit, *mm Hg* millimeters of mercury, LD_{50} median lethal dose (lethal dose 50%), *mg/kg* milligram per kilogram, *RT* room temperature

Source: Technical Fact Sheet—United States Environmental Protection Agency

arrest of activation of hepatic stellate cells and to study various therapeutic approaches to prevent progression of fibrosis to liver cirrhosis [87–92]. We have extensively studied various pathological and biochemical events involved during the pathogenesis of NDMA-induced hepatic fibrosis in rodents. Our studies covered glycoprotein metabolism [21], collagen biosynthesis and metabolism [5–7], LDH isoenzymes [93], biochemical abnormalities [94], oxidative stress and osteopontin [14, 95–99], hyaluronic acid and hyaluronidase [100, 101], mineral and trace element metabolism [11, 102, 103], antioxidants [14, 104, 105] and gene therapy [17, 106], lysosomal fragility [107, 108], role of metalloproteinases [18, 109, 110], and various therapeutic approaches [111, 112]. Furthermore, we recently reviewed the molecular mechanisms involved in the pathogenesis of NDMA-induced hepatic fibrosis [1]. These studies clearly demonstrated that NDMA-induced rodent model of hepatic fibrosis and early cirrhosis is a quick and reproducible animal model to study various events involved in the pathogenesis of human hepatic fibrosis and also to screen antifibrotic agents that could reverse fibrosis and to arrest the progression liver fibrosis to cirrhosis.

NDMA-induced hepatic fibrosis and early cirrhosis in rats

NDMA-induced model of hepatic fibrosis and early cirrhosis in albino rats is a well-established model and has been extensively studied [5, 7, 11]. It is a quick and reproducible animal model of hepatic fibrosis and early cirrhosis of human beings depicting portal hypertension and other decompensating features [74]. The best course of administration of NDMA (Sigma-Aldrich #PHR2407, density 1.01 g/mL) to induce hepatic fibrosis in rats is serial intraperitoneal injections in doses of 1 mg/100 g body weight (10 μl diluted to 1 ml with 0.15 mol/L sterile NaCl) on three consecutive days of each week over a period of 21 days [5]. The schematic presentation of the timeline for NDMA-induced hepatic fibrosis is delineated in Fig. 2 (Protocol A). Around 3-month-old albino rats of the Wistar strain are ideal for induction of hepatic fibrosis using NDMA. Young adult rats that are free from diseases could produce uniform fibrosis in 3 weeks without much mortality. The animals should be provided with commercial rat feed pellets and water available ad libitum and maintained in a 12-h light/12-h dark cycle in an air-conditioned, humidity controlled animal house. Treated animals could be sacrificed either on days 7, 14, and 21 from the beginning of exposure or at the end of the treatment period along with the vehicle-treated control animals. Blood could be collected from the right jugular vein after a deep cut with a scalpel blade. Surgically remove the liver tissue rapidly, cut the median lobe into pieces of 3-mm thick, and instantly fix in 10% phosphate-buffered formalin for histopathological and immunohistochemical studies. Sacrifice of the animals on 7th, 14th, and 21st days following NDMA treatment would be ideal for the study of pathobiochemical alterations and mechanisms involved in the pathogenesis of hepatic fibrosis. Sacrifice of the animals at the endpoint would be suitable for the study of early events involved in liver cirrhosis. Figure 3 depicts Azan trichrome staining for collagen on 7th, 14th, and 21st days following NDMA treatment. Staining for collagen was absent in the livers of control animals except in central veins (Fig. 3a). Fibrosis was initiated on day 7 accompanied with the deposition of collagen fibers between central vein and portal tracts (Fig. 3b). On day 14 following NDMA treatment, there was intermittent deposition of thick collagen fibers in


N-nitrosodimethylamine
(NDMA)
CYP2E1
NADPH + H+ + O2
NADP+ + H2O
Hydroxymethylnitrosamine
Non-enzymatic
Formaldehyde
NAD + H+ + 2e−
NADH
Aldehyde
dehydrogenase
NADH
Alcohol
dehydrogenase
NAD+
HNO2
N-nitrosomethylamine
NO
Methanol
Formic acid
Methylamine
Methyldiazohydroxide
H2O
CO2 + H2
Diazomethane
H+
'M' + N2
Macromolecules
'M'
Methylcarbocation
Methyldiazonium ion
Methylation
(DNA, Protein)

◀ **Fig. 1** Schematic representation of metabolic degradation of *N*-nitrosodimethylamine (NDMA) depicting formation of free radical and methylation of macromolecules in liver. The metabolic activation and degradation of NDMA by cytochrome P4502E1 produces formaldehyde and methanol that would degrade further. The highly reactive methyl cation covalently binds with nucleic acids and proteins to form methylated macromolecules that could lead to carcinogenicity

hepatic parenchyma (arrows). There was bridging necrosis and fibrosis between portal tract and central veins (Fig. 3c). All the liver sections from the 21st day animals demonstrated extensive fibrosis and deposition of thick collagen fibers between central vein and portal tracts along with early cirrhosis (Fig. 3d).

Another course of NDMA administration to induce hepatic fibrosis and early cirrhosis in rats is serial intraperitoneal injections in doses of 1 mg (prepared in 0.15 M sterile NaCl)/100 g body weight for 7 consecutive days [100]. The schematic representation of this course is depicted in Fig. 2 (Protocol B). The animals could be injected without anesthesia by holding them in left hand and inject with right hand. Frequent anesthesia could affect the health of the animals and may interfere with uniform development of liver fibrosis. A set of animals could be sacrificed on days 7, 14, and 21st following NDMA administrations along with sham-treated control animals. Alternatively, the animals could be sacrificed on day 21 or could keep up to day 28 or beyond. Keeping the treated animals beyond the day 21 may result in regression of fibrosis in certain animals. Besides, a good percentage of animals could die. However, a few animals could progress to cirrhosis and may produce ascites with portal hypertension. Jenkins et al. [74] maintained NDMA-treated rats up to 24–48 weeks after the cessation of NDMA treatment. At 24 weeks after the treatment with NDMA, they reported cirrhosis with diffuse nodularity and fibrosis, marked portal hypertension, and accumulation of ascites as seen in human beings. Figure 4 depicts Masson's trichrome staining for collagen and histochemical staining for hyaluronic acid (HA) in paraffin liver sections of rats injected with NDMA at a concentration of 1 mg/100 g body weight for 7 consecutive days and sacrificed on day 21 after the start of NDMA treatment. The sham-treated control animal livers did not show staining for collagen or hyaluronic acid. On day 21 following serial administrations of NDMA, the animal livers depicted extensive deposition of mature collagen fibers (Fig. 4b, arrows). There was marked bridging fibrosis with early nodular cirrhosis. Staining for HA depicted marked accumulation of HA in the fibrotic areas indicating extensive synthesis and deposition (Fig. 4d). The activated stellate cells are mainly responsible for synthesis of HA during pathogenesis of hepatic fibrosis [100]. In the 7 day course of serial administrations of NDMA, massive hepatic necrosis and collapse of liver parenchyma may occur in certain cases [100]. NDMA-induced model of liver fibrosis would not produce hepatic tumors or carcinogenesis, which generally occurs after treatment with N-nitrosodiethylamine (NDEA).

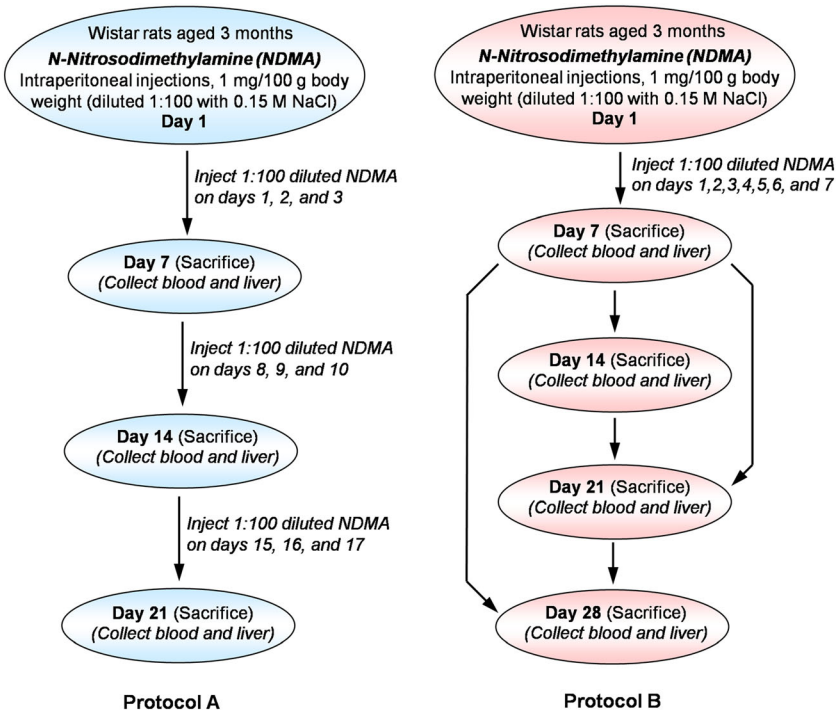


Fig. 2 Schematic presentation of two different protocol timeline for *N*-nitrosodimethylamine-induced hepatic fibrosis in Wistar rats. **a** Protocol for hepatic fibrosis. The animals could be injected with NDMA in doses of 1 mg/100 g body weight (density 1.01 g/mL, diluted 1:100 with 0.15 M sterile NaCl) on three consecutive days of a week over a period of 21 days. **b** Protocol for hepatic fibrosis and early cirrhosis. The animals could be injected with 1:100 diluted NDMA in doses of 1 mg/100 g body weight for 7 consecutive days. The injected animals may be maintained up to 21 or 28 days for the development of hepatic fibrosis or early cirrhosis, respectively

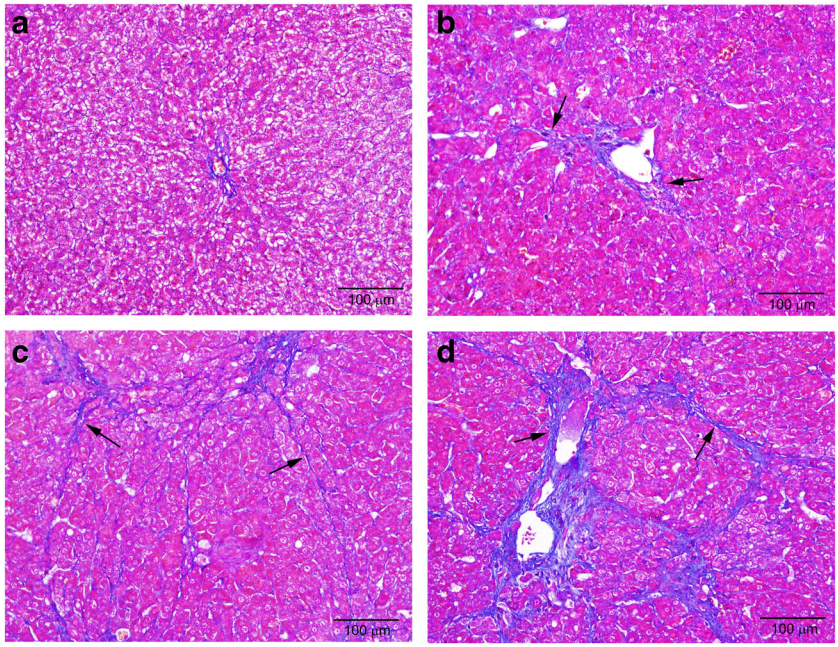


Fig. 3 Azan trichrome staining for collagen during the pathogenesis of NDMA-induced hepatic fibrosis in rats. The animals were administered with NDMA as serial intraperitoneal injections in doses of 1 mg/100 g body weight on three consecutive days of each week over a period of 21 days. Treated animals were sacrificed on days 7, 14, and 21 from the beginning of exposure. Paraffin-embedded liver sections were stained for collagen along with untreated control samples (× 100). **a** Untreated control liver. Staining for collagen was absent in hepatic parenchyma. **b** Day 7. Initiation of fibrosis and deposition of collagen fibers between central vein and portal tracts (arrows). **c** Day 14. Intermittent deposition of thick collagen fibers in hepatic parenchyma (arrows). There was bridging necrosis and fibrosis between portal tract and central veins. Focal fatty changes were present. **d** Day 21. Extensive fibrosis and deposition of thick collagen fibers between central vein and portal tracts (arrows). Early nodular cirrhosis was present

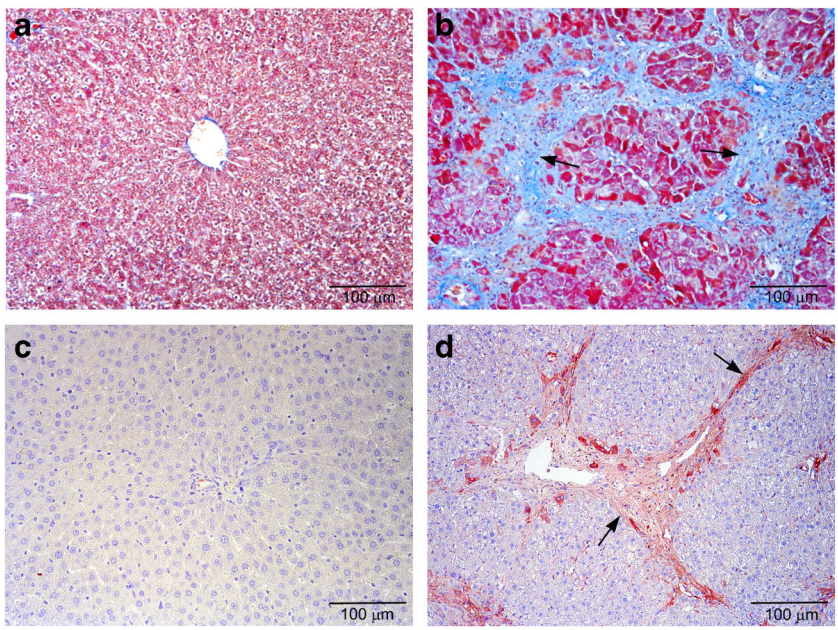


Fig. 4 Masson's trichrome staining for collagen and histochemical staining for hyaluronic acid (HA) in NDMA-induced hepatic fibrosis in rats. The animals were treated with NDMA as intraperitoneal injections in doses of 1 mg/100 g body weight for 7 consecutive days and sacrificed on day 21 from the beginning of treatment. Paraffin-embedded liver sections were stained for collagen and HA (× 100). **a**, **c** Sham-treated control animal livers did not show staining for collagen or HA, respectively. **b** Day 21. NDMA-treated animal livers depicted extensive deposition of mature collagen fibers (arrows). Bridging fibrosis and early nodular cirrhosis were present. **d** Marked accumulation of HA in fibrotic areas in conjunction with collagen indicating extensive synthesis of HA (arrows)

Murine model of NDMA-induced liver injury and fibrosis

Our studies demonstrated that C57BL/6 mice could not withstand NDMA in doses of 1 mg/100 g body weight on three consecutive days of each week over a period of 21 days (unpublished data). Almost all mice died within 7 days after the first three injections due to unknown reasons. A lower dose of 0.8 mg/100 g body weight would keep the animals alive. However, it was noticed that such a lower dose is not enough to induce moderate amount of fibrosis in the liver. We observed that 129/Sv mice could withstand NDMA doses much better compared with C57BL/6 mice. However, still a good percentage of mice may die during a 21-day course of NDMA administration as in rats. Besides, the surviving mice would not produce liver fibrosis as in the case of rats with a similar course of NDMA treatment. Figure 5 depicts hematoxylin and eosin (a, b) and Masson's trichrome (c, d) staining in paraffin liver sections of 129/Sv mice treated with NDMA in doses of 10 μg/g body weight (appropriately diluted with sterile saline) on three consecutive days of each week over a period of 21 days. There was moderate to severe centrilobular hepatocyte necrosis and dilatation of central veins on day 7 after the start of NDMA administration (Fig. 5a). On day 21 of NDMA administration, massive and intense centrilobular hemorrhagic necrosis was present (Fig. 5b). Masson's trichrome staining did not show collagen fibers in untreated control mice livers (Fig. 5c). However, on the 21st day of NDMA administration, there was deposition of thin collagen fibers in the hepatic parenchyma indicating centrilobular fibrosis (arrows) (Fig. 5d). Massive and intense centrilobular hemorrhagic necrosis was present (Fig. 5d). The data demonstrated that NDMA-induced liver injury in 129/Sv mice is not an appropriate model to investigate the biochemical and pathological changes during hepatic fibrosis. In other studies, MMP13$^{\pm}$ mice on a 129/Sv genetic background that were generated on C57BL/6J mice and MMP-1 transgenic mice generated on C57BL/6J X CBA/J were treated with similar course of NDMA as above and maintained them up to 28 days [18, 105, 109]. Those mice produced better fibrosis with less necrosis. However, about 50% of the NDMA-treated animals died during the course of the study. We have observed that intraperitoneal injections of CCl_4 at a dose of 5 μl/10 g body weight (diluted 1:19 with mineral oil) weekly twice for a period of 1 month (9 injections) is the best course of treatment to induce hepatic fibrosis in mice with zero death rate [96, 99]. This has been further demonstrated by other investigators also [113, 114].

Summary

Compared with other models of hepatic fibrosis in rats, the NDMA-induced model is a quick, easy, and reproducible animal model for understanding the biochemical, pathophysiological,

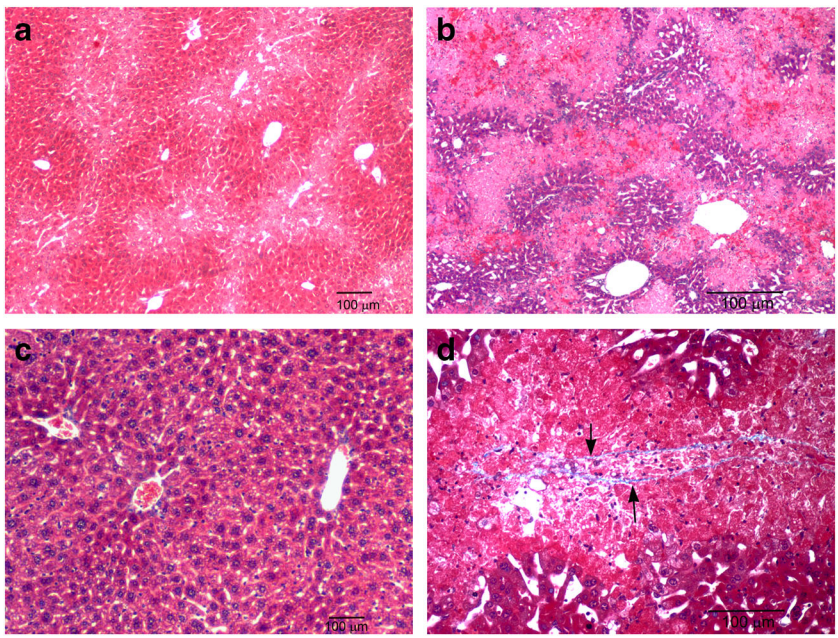


Fig. 5 Hematoxylin and eosin (**a**, **b**) and Masson's trichrome (**c**, **d**) staining in NDMA-treated mice liver. The animals were treated with NDMA in doses of 10 μg/g body weight on three consecutive days of each week over a period of 21 days. **a** NDMA 7 days. Moderate to severe centrilobular hepatocyte necrosis and dilatation of central veins (× 40). **b** NDMA 21 days (*animal died before sacrifice*). Massive and intense centrilobular hemorrhagic necrosis (× 40). **c** Untreated control mice liver. Absence of staining for collagen (× 100). **d** NDMA day 21. Deposition of thin collagen fibers in the hepatic parenchyma indicating centrilobular fibrosis (arrows). There was massive and intense hemorrhagic necrosis (× 100)

and molecular events associated with the development of hepatic fibrosis and liver cirrhosis. NDMA-induced liver injury in rats reflects changes that occur in human hepatic fibrosis, and illustrates many of the features, such as portal hypertension and ascites, as well as a number of other histopathological changes and biochemical abnormalities. One of the major differences noticed compared with other models is the early onset of collagen deposition, the major protein involved in hepatic fibrosis, and the key event towards the progression to liver cirrhosis. Another advantage of NDMA-induced model is that the liver damage is consistent and irreversible with the dosing regimen described. Overall, other animal models of fibrosis and cirrhosis of the liver are rarely accompanied by the decompensating features of the human condition, such as portal hypertension, ascites, hepatic encephalopathy, and esophageal varices. Finally, NDMA-induced model is an appropriate animal model to study the molecular mechanisms involved in the pathogenesis of hepatic fibrosis, and definitely a good model for rapid screening of antifibrotic agents.

Author contributions J. George carried out the major experiments, collected the data, analyzed and interpreted the data, and wrote the manuscript. M. Tsuchishima was involved in the conception and design of the study, provided materials, and evaluated the work. M. Tsutsumi obtained funding and critically evaluated the contents of the manuscript.

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Compliance with ethical standards

All the experimental works stated in the manuscript have been carried out in compliance with ethical standards. All the animals received food and water available ad libitum. All authors have read and approved the final version of the manuscript and tacitly or explicitly share responsibility.

Ethical approval All the animal experiments stated in this manuscript have been carried out with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 86-23, revised 1996). The animals received humane care as per the criteria outlined in the manual. The protocol was also approved by the Animal Care and Research Committee of Kanazawa Medical University on the Ethics of Animal Experiments.

Conflicts of interest The authors declare that they do not have no any conflicts of interest. to declare in connection with this manuscript.

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