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Original

Increase in Matrix Metalloproteinase-2 and 9 in the Liver of Nonalcoholic Steatohepatitis (NASH) Model Rats

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Abstract: Nonalcoholic steatohepatitis (NASH) is regarded as a hepatic manifestation of the metabolic syndrome which can progress to hepatic cirrhosis and hepatocellular carcinoma. It is thought that matrix metalloproteinases (MMPs) play an important role in hepatic fibrosis and we previously reported a correlation between oxidative stress and MMP-9 expression. However, the expression of MMPs and tissue inhibitors of metalloproteinases (TIMPs) in the progression of NASH is unclear. In this study we used spontaneously hypertensive and hyperlipidemic rats (SHHR) fed a high-fat diet and 30% sucrose solution (HFDS) as a model for NASH, in order to clarify the relationships between oxidative stress, liver weight (LW), MMPs and TIMPs at various time-points in the progression of NASH. Male SHHR and Sprague-Dawley (SD) rats were divided into four groups: SHHR-normal diet (ND), SHHR-HFDS, SD-ND and SD-HFDS. Hepatic fibrosis was clearly increased at 13 months in SHHR-HFDS, resembling NASH. LW and oxidative stress markers in plasma were increased in SHHR-HFDS compared to the other groups. Oxidative stress was correlated with LW in all rats. Expression of MMP-2, MMP-9, TIMP-1 and TIMP-2 mRNA, measured by real-time polymerase chain reaction, was increased in the liver of SHHR-HFDS at 13 months. This study suggests that oxidative stress, MMPs and TIMPs may play an important role in the progression of NASH.

Key words: matrix metalloproteinase (MMP), nonalcoholic steatohepatitis (NASH), oxidative stress, tissue inhibitor of metalloproteinase (TIMP)

Introduction

Nonalcoholic fatty liver disease (NAFLD) is the most common form of chronic liver disease, paralleling the epidemic of obesity^{1, 2)}. NAFLD is defined as the accumulation of lipid, primarily triacylglycerols, in individuals who do not consume a large amount of alco-

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hol³). Nonalcoholic steatohepatitis (NASH) is a progressive form of NAFLD that is diagnosed by histopathological features⁴. NAFLD and NASH have been recently recognized as hepatic manifestations of lifestyle-related diseases^{2, 5, 6}.

Spontaneously hypertensive hyperlipidemic rats (SHHRs) were developed as a stable model of early vascular degeneration⁷, since there are few rat models for atherosclerosis. SHHRs have persistently high systolic blood pressure (above 150 mmHg) and a plasma total cholesterol concentration above 150 mg/dL. In addition, vascular intimal lesions and lipid deposits have been observed under the endothelial cells in the aorta of SHHRs, but not in spontaneously hyperlipidemic rats or controls⁸. Moreover, invasive changes occur in the subendothelium of SHHR when nitric oxide production is inactivated, followed by a high fat diet and sucrose water treatment (HFDS)⁹, and a previous study found visceral fat accumulation and increased oxidative stress in SHHR-HFDS^{10, 11}. We have found fibrosis and cell inflammation around the central vein, and fat accumulation in hepatocytes on liver histology in SHHR-HFDS; thus we use SHHR-HFDS as a rat model of NASH.

Systemic oxidative stress is associated with visceral fat accumulation and the metabolic syndrome¹². In addition, oxidative stress plays an important role in the progression of NASH¹³. As we have shown in a previous study¹¹, the diacron reactive oxidative metabolites (d-ROMs) test can be used as an indicator of oxidative stress.

Matrix metalloproteinases (MMPs) are zinc ion-dependent enzymes that help to regulate the extracellular matrix, cellular migration, and tissue remodeling¹⁴. MMPs are associated with liver fibrosis; in particular, MMP-2 and MMP-9 play important roles in the progression of liver fibrosis¹⁵, and MMP-9 activity and expression are associated with oxidative stress¹⁶. Our previous studies showed that mRNA expression of MMP-9 in white blood cells (WBCs) is significantly higher in SHHR-HFDS compared to other rats¹¹. Moreover, MMPs are tightly regulated by tissue inhibitors of metalloproteinases (TIMPs)¹⁷.

Therefore, in this study we examined SHHR-HFDS as a model of NASH to clarify the relationships between oxidative stress, liver weight (LW), MMPs and TIMPs in the progression of NASH.

Materials and Methods

Animals and samples

Four-month-old male SHHRs and SD rats were each divided into two groups: a control group fed a regular diet (ND; CE2; CLEA Japan Inc., Tokyo, Japan) and a HFDS-fed group. The regular diet comprised 8.9% water, 25.4% protein, 4.4% fat, 4.1% fiber, 6.9% carbohydrate and 50.3% nitrogen-free extracts, containing 342.2 kcal/100 g. The high-fat diet (HFD) consisted of 8.2% water, 23.4% protein, 11.0% fat, 3.8% fiber, 6.3% carbohydrate, and 46.3% nitrogen-free extracts, containing 378.0 kcal/100 g. Until the age of 4 months, the regular diet was available to all groups *ad libitum*. Thereafter, the two HFDS groups received NG-nitro-L-arginine methyl ester (Nacalai Tesque Co., Kyoto, Japan) in the drink-

ing water (100 mg/L) for 1 month, and then the high-fat diet with 30% sucrose solution ad libitum for 4, 6 and 8 months, thus we obtained rats of 9, 11 and 13 months of age. The present study used SHHRs with a systolic blood pressure over 150 mmHg, as determined by the tailcuff method (PS-100; Riken Kaihatsu, Tokyo, Japan). The rats were housed in a semi-barrier system under controlled room temperature (23 ± 1 °C), humidity ($55\pm 5\%$), and lighting (lights on from 6 AM to 6 PM). All studies were conducted according to the “Guiding Principles for the Care and Use of Laboratory Animals” of Showa University¹¹⁾.

Preparation and biochemical determination of plasma samples

Blood samples were taken from the inferior vena cava under pentobarbital anesthesia (35 mg/kg, intraperitoneal administration) and mixed with 3.2% sodium citrate solution in a volume ratio of 9:1. After 15 minutes of centrifugation at 3,000 rpm, the supernatant, as citrated plasma, was used for analysis. The rats were killed by decapitation and the liver and visceral fat (VisF) were isolated and weighed.

Plasma levels of total cholesterol and total triglycerides were determined with commercially available kits (Cholesterol E-test and Triglyceride E-test, respectively; Wako Pure Chemical Industries Ltd., Tokyo, Japan). Plasma levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were determined using the Transaminase CII-test (Wako)¹⁰⁾. Oxidative stress was measured in rat plasma using the d-ROMs test (Free Radical Elective Evaluator; Wismerll Co., Ltd., Tokyo, Japan) by gently mixing 20 μ l of plasma sample and 1 mL of buffer solution in a cuvette, before adding 10 μ L of the chromogenic substrate. After mixing, the cuvette was immediately incubated in the thermostatic block of the analyzer for 5 minutes at 37°C and then the absorbance at 505 nm was recorded. Measurements are expressed as Carr units, with 1 Carr corresponding to 0.8 mg/L H₂O₂¹¹⁾.

Morphological study

The fresh left lobe of liver was harvested from all rats and stored in saline on ice. It was then dissected from the surrounding tissues and fixed in 10% neutral buffered formalin (pH 7.4; Wako). Sections of the liver were stained with hematoxylin-eosin (HE) and Masson trichrome (MT).

Preparation of total RNA

Total RNA was extracted from the median lobe of the rat liver. Liver samples (80 ~ 500 mg) and RNAiso Plus (2 mL; Takara Bio, Shiga, Japan) were mixed, before total RNA was isolated using the Takara FastPure RNA kit and quantified by optical density (260 nm). Each RNA sample was diluted with RNase-free water and mRNA concentrations were equalized. Total RNA was reverse transcribed using the ExScriptTM RT reagent kit (Takara).

Quantitative real-time polymerase chain reaction

Quantitative real-time polymerase chain reaction (Q-PCR) was used to quantify the mRNA levels of MMP-2, MMP-9, TIMP-1, TIMP-2, TIMP-3 and superoxide dismutase 1 (SOD1) in rat liver. The cDNAs were amplified using primers designed by ProbeFiber software (Roche Diagnostic K.K., Tokyo, Japan). Table 1 details the primers used, Roche Universal ProbeLibrary Probe numbers, and accession numbers. Amplification was performed with a LightCycler (Roche) using the LightCycler TaqMan Master mix (Roche). The PCR reaction parameters were as follows: 95°C for 10 minutes, 45 cycles (except MMP-9, only MMP-9; 40 cycles) of 10 seconds at 95°C, 30 seconds at 60°C, and 1 second at 72°C. Fluorescence data were analyzed with LightCycler software (Roche). The mRNA levels were compared to 18 s rRNA as a standard, and relative expression ratios were obtained¹¹⁾.

MMP-2 and MMP-9 immunostaining in the liver

Liver sections (5 μ m) were dewaxed in xylene and placed on slides in the glass slide chamber, which was filled with processing buffer (citric acid buffer, pH 6.0). Sections were blocked for 1 hour in phosphate-buffered saline (PBS) containing 5% normal goat serum and then exposed to primary antibody (rabbit polyclonal antibody, 1:200) against either rat MMP-2 (sc-10736; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or MMP-9 (AB19016; Millipore Co., Billerica, MA), overnight at 4°C. The slides were then washed in PBS and incubated with anti-rabbit IgG secondary antibody. After being rinsed in PBS, tissues were finally counterstained with HE. Immunostaining of sections was uniformly timed and assessed by microscopy¹¹⁾.

Statistical analysis

Data were analyzed using the Mann-Whitney U-test. Correlations were calculated by the Pearson's product moment correlation coefficient. All data are expressed as mean \pm S.E.M. $P < 0.05$ was considered significant.

Table 1. Nucleotide sequences of the primers used for polymerase chain reaction.

Gene		Sequence	Accession number	Probe number
MMP-2	Sense primer	5'-ctccactacgcttttctcgaat-3'	NM_031054.2	#60
	Antisense primer	5'-tgggtatccatctccatgct-3'		
MMP-9	Sense primer	5'-cctctgcatgaagacgacataa-3'	NM_031055.1	#42
	Antisense primer	5'-ggtcagggttagagccacga-3'		
TIMP-1	Sense primer	5'-cagcaaaaaggcctctgtaaa-3'	NM_053819.1	#76
	Antisense primer	5'-tggctgaacagggaaacact-3'		
TIMP-2	Sense primer	5'-ctggacgttgaggaaagaa-3'	L31884.1	#12
	Antisense primer	5'-acagagggtaatgtcatcttg-3'		
TIMP-3	Sense primer	5'-tgcaactttgtggagagggtg-3'	U27201.1	#123
	Antisense primer	5'-aggcaagtagtagcaggacttgat-3'		
SOD1	Sense primer	5'-ggtccagcggatgaagag-3'	NM_017050.1	#5
	Antisense primer	5'-ggacacattggccacacc-3'		

Results

Table 2 shows the level of body weight, VisF, hemoglobin A_{1C}, total cholesterol, total triglycerides, AST, ALT, and d-ROMs in the SD-ND, SD-HFDS, SHHR-ND and SHHR-HFDS groups. There was no significant change in body weight between the two SHHR groups. In both the SD and SHHR groups, VisF and LW (Fig. 1A) were either significantly elevated or tended to be elevated after ingestion of HFDS. LW in SHHR-HFDS was significantly increased at 11 and 13 months compared with other rats. Plasma hemoglobin A_{1C}, AST and ALT levels were unchanged among the SD and SHHR groups. The level of oxidative stress (d-ROMs) in SHHR-HFDS was higher than in other rats at 9 and 13 months. A significant positive correlation (Pearson $r = 0.712$, $P < 0.0001$) between LW and d-ROMs in all rats is shown in Fig. 1B.

Photographs of the livers from 9-month-old rats are shown in Fig. 2. The images of both the SD and SHHR groups show fatty liver caused by the HFDS feeding. Fig. 3 shows the results of the morphological study with a representative sample of liver from each group of SHHRs and SD rats at 13 months, stained by HE and MT. Hepatocyte ballooning and steatosis are well recognized with HFDS feeding. Severe fibrosis and cell inflammation around the central vein were observed in SHHR-HFDS but only slightly detected in SD-HFDS. Figs. 4 and 5 are photomicrographs of the liver showing the immunostaining of MMP-2 and MMP-9, respectively. In SHHR-HFDS, staining of MMP-2 was detected mainly in extracellular spaces, while staining of MMP-9 was observed in the WBCs, hepatocytes, bile ducts and biliary canaliculi. Staining of MMP-2 and MMP-9 was barely evident in the livers from the three other groups of rats.

SOD1 mRNA expression in the liver of rats, measured by Q-PCR, is shown in Fig. 6. SOD1 mRNA expression in SHHR-HFDS decreased to 42% of SD-ND and 20% of SHHR-ND at 13 months. MMP-2 and MMP-9 mRNA expression in rat liver, measured by Q-PCR, are shown in Figs. 7A and 7B, respectively. In SHHR-HFDS, MMP-2 mRNA expression was increased 3.18, 2.76 and 16.6 fold at 9, 11 and 13 months, respectively, compared to SD-ND at the same months. SHHR-HFDS showed a time-dependent 1.65-, 8.83- and 49.47- fold increase in MMP-9 mRNA at 9, 11 and 13 months, respectively, compared to SD-ND. TIMP-1, TIMP-2 and TIMP-3 mRNA expression in rat liver, measured by Q-PCR, are shown in Figs. 8A, 8B and 8C, respectively. In SHHR-HFDS at 13 months, TIMP-1 and TIMP-2 mRNA expression were increased 8.67 and 28.96 fold, respectively, compared to SD-ND. There was little variation in TIMP-3 mRNA expression in all groups and no significant differences were seen between SD-ND and SHHR-HFDS at 9 or 13 months.

Discussion

The present study demonstrates that the increased LW associated with oxidative stress markers progressed to liver fibrosis. In addition, MMP-2 and MMP-9 expression in SHHR-

Table 2. BW, VisF, HbA1c, TC, TG, AST, ALT and d-ROMs in SD-ND, SD-HFDS, SHHR-ND and SHHR-HFDS at 9, 11 and 13 months of age

	N	BW (g)	Vis Fat (g)	HbA1c	T-Chol (mg/dl)	TG (mg/dl)	AST	ALT	d-ROMS
	9 m	665.8 ± 25.7	23.3 ± 4.1 ^{*1}	2.9	108.7 ± 8.0 ^{*5}	120.6 ± 6.6	23.0 ± 3.7	21.0 ± 2.4	3171 ± 6.3
SD-ND	10	613.1 ± 30.0	18.2 ± 2.4	2.4	60.0 ± 4.2	168 ± 32.3	25.3 ± 4.2	32.0 ± 6.0	2679 ± 15.8
	13 m	724.3 ± 87.2	24 ± 7.1	2.9	155.8 ± 70.1	325.8 ± 134.6	28.0 ± 8.0	20.1 ± 0.5	256 ± 1.4
	9 m	656 ± 16.8	21 ± 2.0 ^{*2}	3	84.7 ± 11.5	84.4 ± 8.0	25.8 ± 3.7	27.4 ± 5.0	593 ± 13.1
SD-HFDS	11 m	691.8 ± 25.2	32.5 ± 2.8	2.5	108.0 ± 13.2	103.4 ± 14.2	18.0 ± 2.8	23.8 ± 4.4	384.5 ± 11.4
	13 m	819.8 ± 35.4	46.4 ± 78 ^{*1*2}	3.1	254.7 ± 32.8 ^{*5}	972 ± 22.5	171 ± 2.8	16.9 ± 1.5	313.5 ± 4.0
	9 m	565.8 ± 19.4	12.1 ± 0.6 ^{*4}	3	204.2 ± 8.5	172.8 ± 23.5	20.7 ± 2.0	26.9 ± 4.5	355.4 ± 11.7 ^{*7}
SHHR-ND	11 m	592 ± 9.8	18.1 ± 0.85	2.4	183.1 ± 176 ^{*6}	233.1 ± 31.3	14.8 ± 1.4	21.2 ± 2.9	3073 ± 75 ^{*8}
	13 m	657 ± 15.7	24.3 ± 1.9	3	279.3 ± 3.6	251.1 ± 15.5	21.4 ± 10.5	19.3 ± 1.9	244 ± 5.2
	9 m	631.8 ± 19.7	42.5 ± 3.8 ^{*3}	3	492.3 ± 41.9	94.1 ± 11.2	18.8 ± 1.0	22.2 ± 2.7	699.5 ± 71 ^{*7*9}
SHHR-HFDS	11 m	662.2 ± 15.8	51.3 ± 3.7 ^{*3}	2.4	612.3 ± 98.1	166 ± 43.0	24.7 ± 3.3	271 ± 5.8	578.3 ± 15.2 ^{*8}
	13 m	685.4 ± 20.3	65.9 ± 4.5 ^{*4}	2.8	635.5 ± 76.1 ^{*6}	95.0 ± 11.1	176 ± 1.3	16.4 ± 0.4	586.7 ± 1.1 ^{*9}

Body weight (BW), visceral fat (VisF), hemoglobin A_{1c} (HbA_{1c}), total cholesterol (TC), total triglycerides (TG), aspartate aminotransferase (AST), alanine aminotransferase (ALT) and diacylglycerol reactive metabolites (d-ROMs) in Sprague-Dawley rats on normal diet (SD-ND), SD-high-fat diet with sucrose (SD-HFDS), spontaneously hypertensive hyperlipidemic rats-ND (SHHR-ND) and SHHR-HFDS at 9, 11 and 13 months of age.

The results are presented as mean ± S.E.M.

P < 0.05 for ^{*1}, and ^{*3}; *P* < 0.001 for ^{*2}, ^{*4}, ^{*5}, ^{*6}, ^{*7}, ^{*8}, and ^{*9}.

^{*1}, SD-ND at 9 months vs SD-HFDS at 13 months; ^{*2}, SD-HFDS at 9 months vs SD-HFDS at 13 months; ^{*3}, SHHR-HFDS at 9 months vs SHHR-HFDS at 13 months; ^{*4}, SHHR-ND at 9 months vs SHHR-HFDS at 13 months; ^{*5}, SD-ND at 9 months vs SD-HFDS at 13 months; ^{*6}, SHHR-ND at 11 months vs SHHR-HFDS at 13 months; ^{*7}, SHHR-ND at 9 months vs SHHR-HFDS at 9 months; ^{*8}, SHHR-ND at 11 months vs SHHR-HFDS at 11 months; ^{*9}, SHHR-HFDS at 9 months vs SHHR-HFDS at 11 months.

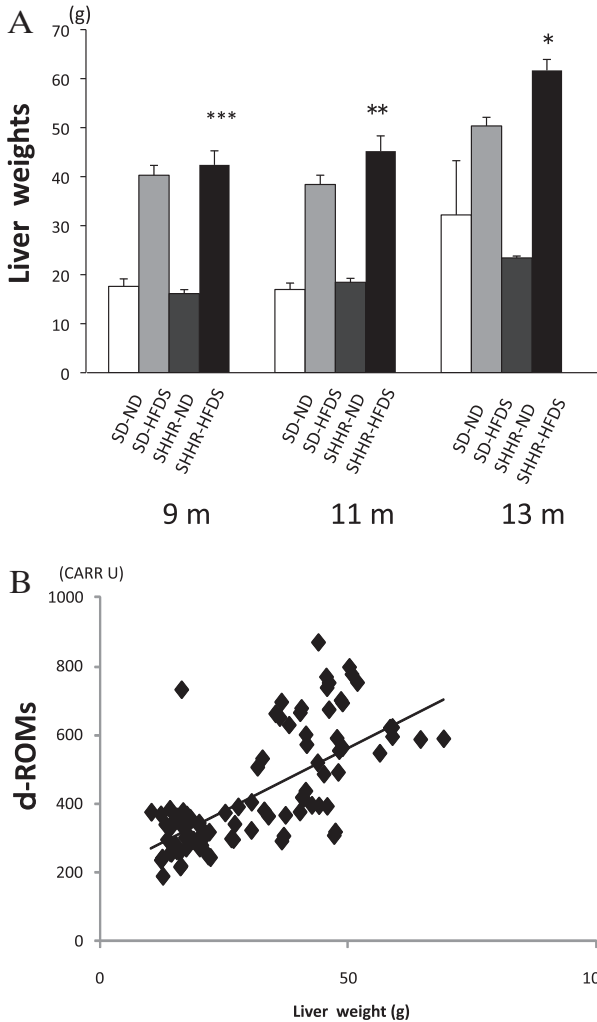


Fig. 1. (A) Liver weight (LW) in Sprague-Dawley rats on a normal diet (SD-ND), SD-high fat diet with sucrose (SD-HFDS), spontaneously hypertensive hyperlipidemic rats-ND (SHHR-ND) and SHHR-HFDS. Data are expressed as mean \pm S.E.M. * $P < 0.05$, SHHR-HFDS vs SD-HFDS, SD-ND, and SHHR-ND at 13 months. ** $P < 0.05$, SHHR-HFDS vs SHHR-ND; SD-ND vs SD-HFDS at 11 months. *** $P < 0.05$, SHHR-HFDS vs SHHR-ND; SD-ND vs SD-HFDS at 9 months.

(B) Correlation between oxidative stress and liver weight (LW). A significant positive correlation (Pearson $r = 0.71$, $P < 0.0001$) between LW and diacron reactive oxidative metabolites (d-ROMs) was observed in all rats.

HFDS increased dramatically at 13 months with the progression of NASH.

Several previous studies have established a relationship between obesity and lifestyle-related diseases. In particular, visceral obesity is directly correlated with the clustering of lifestyle-related diseases, leading to various ailments¹⁸⁻²⁰. Our previous report showed that visceral fat accumulation is closely associated with increased oxidative stress¹¹. In the present study, there was also a significantly positive relationship between VisF and LW in all groups, with the underlying pathological conditions of hypertension and hyperlipidemia having a weak yet direct effect on this relationship. Moreover, NASH model rats developed intense fibrosis in the liver by 13 months of age. The most accepted theory to explain the progression from simple NAFLD to NASH is the “two-hit hypothesis” wherein fat accumulation is sufficient to induce the progression to steatohepatitis, but renders the liver

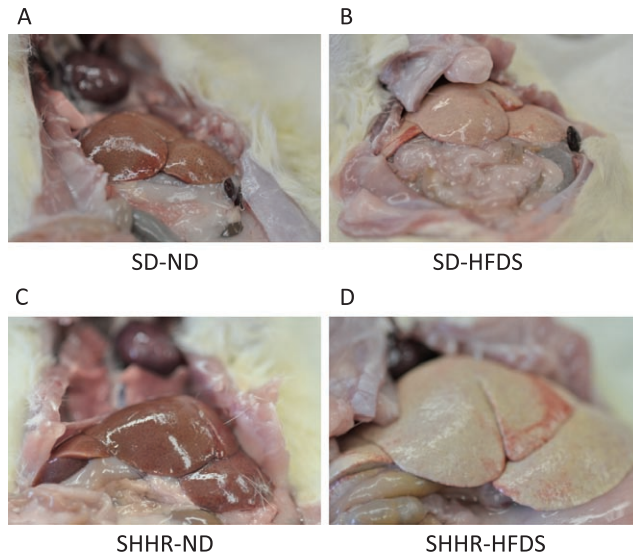


Fig. 2. Liver photographs after abdominal dissection in (A) Sprague-Dawley rats on a normal diet (SD-ND), (B) SD-high-fat diet with sucrose (SD-HFDS), (C) spontaneously hypertensive hyperlipidemic rats-ND (SHHR-ND) and (D) SHHR-HFDS at 9 months of age.

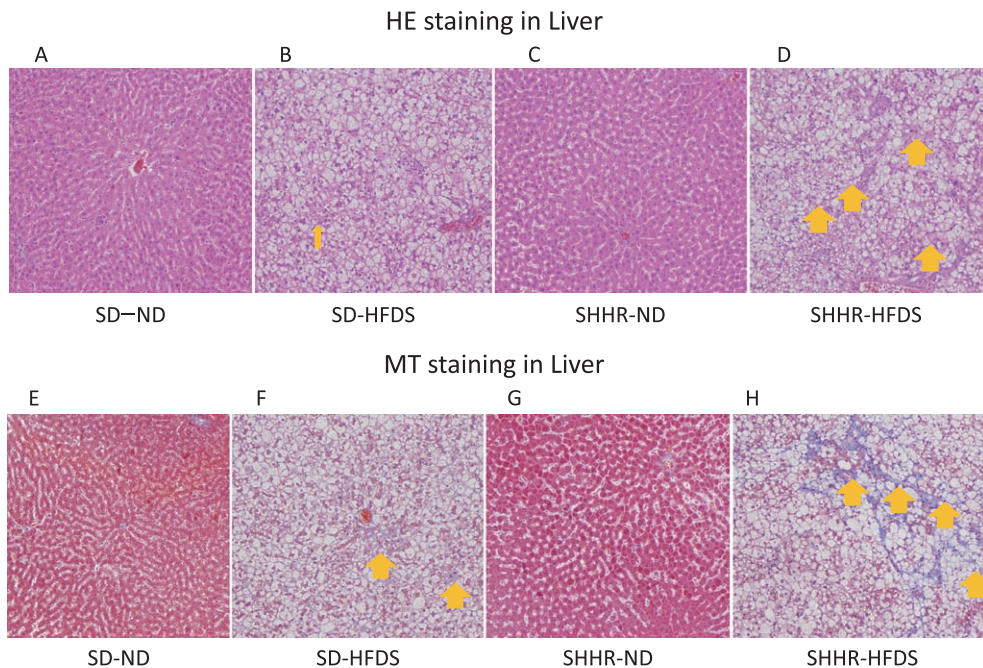


Fig. 3. Histological changes in the liver of rats at 13 months of age. Panels (A)-(D) shows hematoxylin-eosin staining and (E)-(H) shows Masson trichrome (MT) staining in: (A) and (E), Sprague-Dawley rats on a normal diet (SD-ND); (B) and (F), SD-high-fat diet with sucrose (SD-HFDS); (C) and (G), spontaneously hypertensive hyperlipidemic rats-ND (SHHR-ND); and (D) and (H), SHHR-HFDS. Hepatocyte ballooning and cell inflammation are indicated by the yellow arrows. Fibrosis is indicated by the yellow arrows following MT staining in (E)-(H). Original magnification $\times 100$.

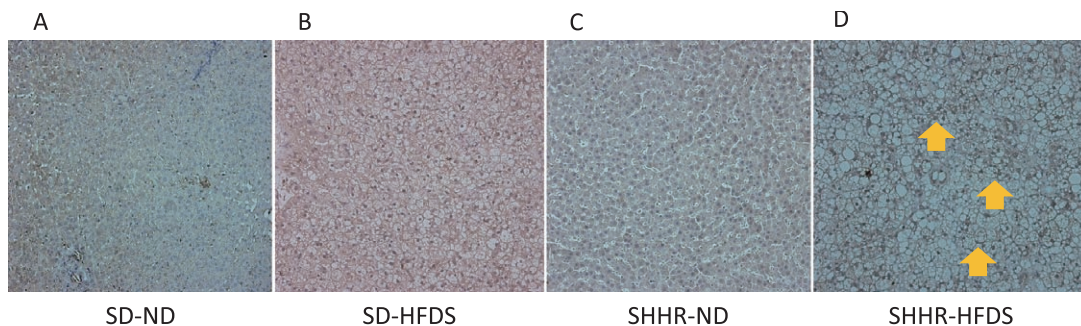


Fig. 4. Immunostaining of MMP-2 in the liver.

MMP-2 stained brown with yellow arrows indicating the staining of MMP-2. (A) Sprague-Dawley rats on a normal diet (SD-ND), (B) SD-high-fat diet with sucrose (SD-HFDS), (C) spontaneously hypertensive hyperlipidemic rats-ND (SHHR-ND) and (D) SHHR-HFDS. Original magnification $\times 100$.

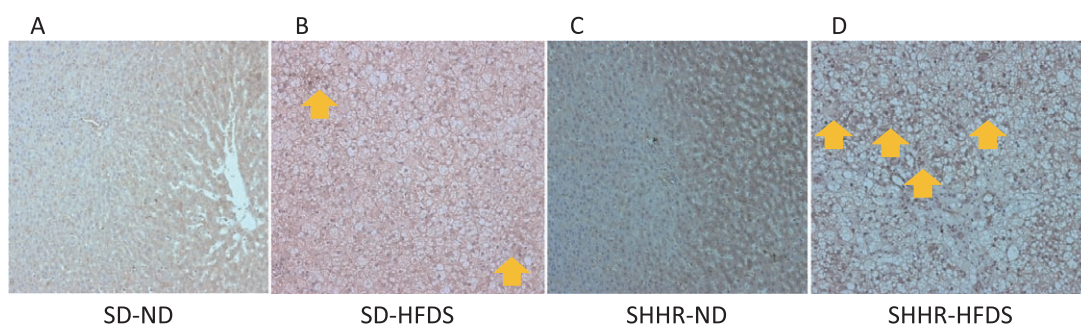


Fig. 5. Immunostaining of MMP-9 in the liver.

MMP-9 stained brown with yellow arrows indicating the staining of MMP-9. (A) Sprague-Dawley rats on a normal diet (SD-ND), (B) SD-high-fat diet with sucrose (SD-HFDS), (C) spontaneously hypertensive hyperlipidemic rats-ND (SHHR-ND) and (D) SHHR-HFDS. Original magnification $\times 100$.

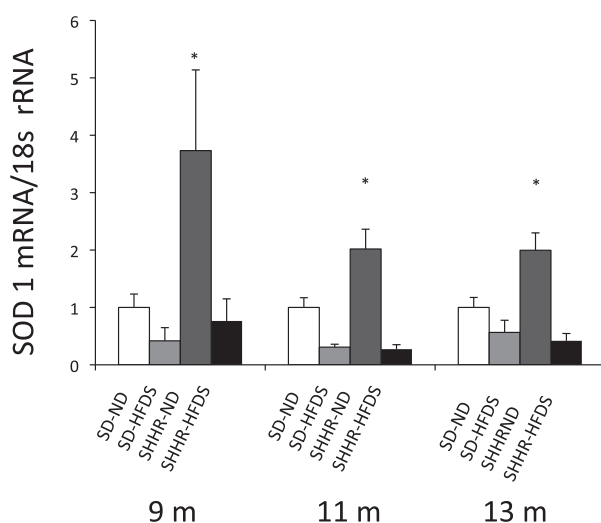


Fig. 6. SOD1 mRNA expression in the liver of Sprague-Dawley rats on a normal diet (SD-ND), SD-high-fat diet with sucrose (SD-HFDS), spontaneously hypertensive hyperlipidemic rats-ND (SHHR-ND) and SHHR-HFDS, relative to SD-ND, as determined by quantitative real-time polymerase chain reaction. Data are shown as mean \pm S.E.M. * $P < 0.05$, SHHR-ND vs SD-ND, SD-HFDS and SHHR-HFDS at 9, 11 and 13 months.

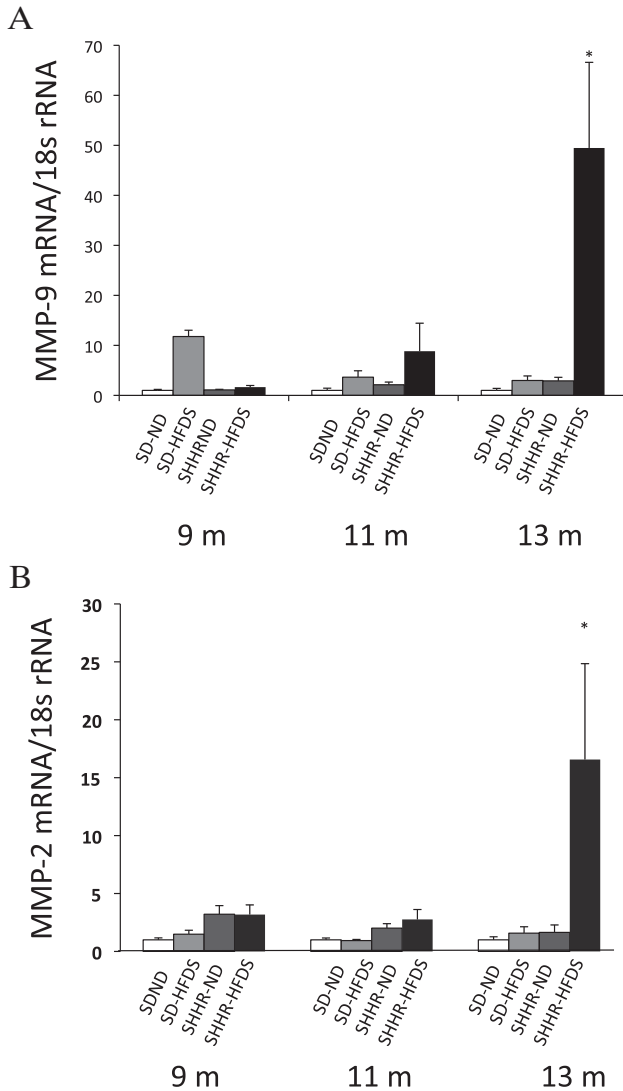


Fig. 7 (A) MMP-9 and (B) MMP-2 mRNA expression in the liver of Sprague-Dawley rats on a normal diet (SD-ND), SD-high-fat diet with sucrose (SD-HFDS), spontaneously hypertensive hyperlipidemic rats-ND (SHHR-ND) and SHHR-HFDS, relative to SD-ND, as determined by quantitative real-time polymerase chain reaction. Data are shown as mean \pm S.E.M. * $P < 0.05$, SHHR-HFDS vs SD-ND, SD-HFDS and SHHR-ND at 13 months.

more susceptible to a “second hit” that, once imposed upon the steatotic liver, causes further aberrations that culminate in the development of NASH^{13, 21)}. One of the key factors in this “second hit” is oxidative stress¹³⁾. Indeed, our NASH model rats had increased d-ROMs levels but not increased SOD1 expression. However, the livers of the SD-HFDS rats did not progress from simple NAFLD to NASH, because the oxidative stress markers in these rats were not increased, as they were in the NASH model rats. Thus, this study suggests that the progression from simple NAFLD to NASH requires oxidative stress in this model.

The remodeling process of damaged liver tissue includes the activation of quiescent

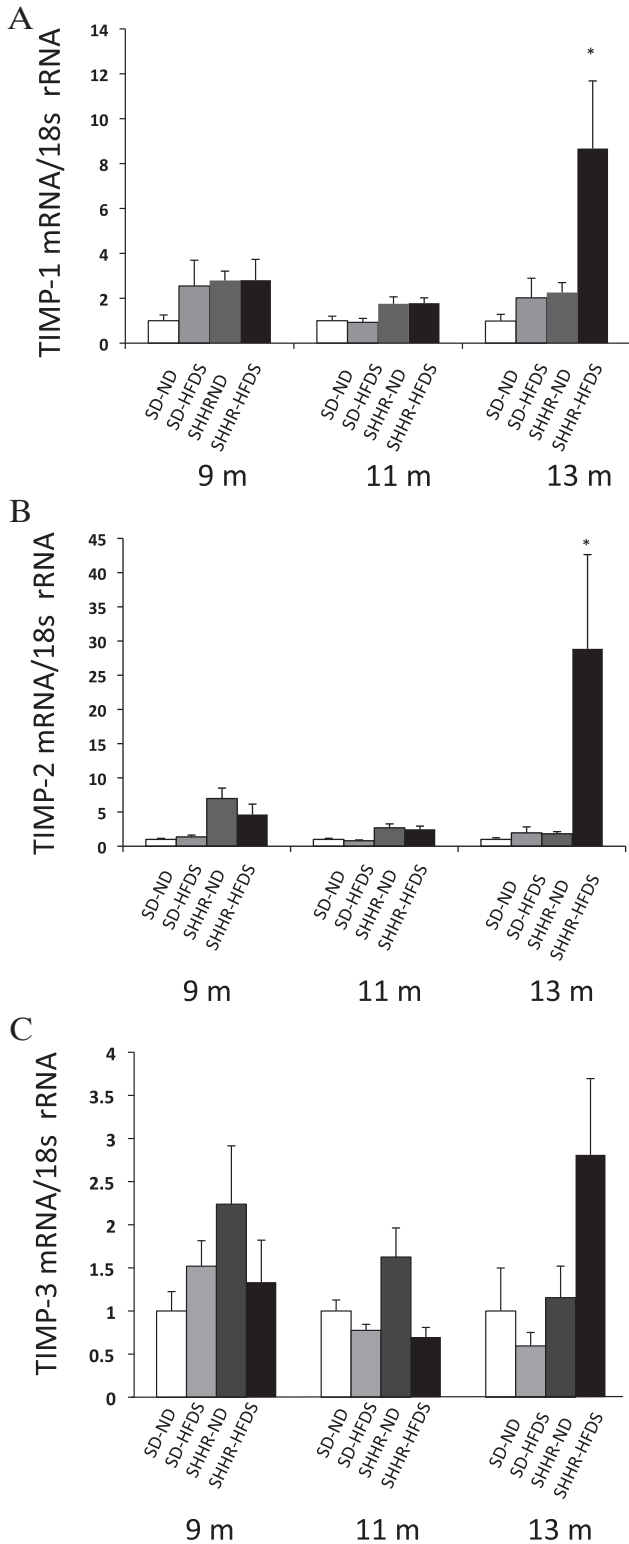


Fig. 8. (A) TIMP-1, (B) TIMP-2 and (C) TIMP-3 mRNA expression in the liver of Sprague-Dawley rats on a normal diet (SD-ND), SD-high-fat diet with sucrose (SD-HFDS), spontaneously hypertensive hyperlipidemic rats-ND (SHHR-ND) and SHHR-HFDS, relative to SD-ND, as determined by quantitative real-time polymerase chain reaction. Data are shown as mean \pm S.E.M. (A): * $P < 0.05$, SHHR-HFDS vs SD-HFDS and SHHR-ND at 13 months. (B): * $P < 0.05$, SHHR-HFDS vs SD-ND, SD-HFDS and SHHR-ND at 13 months.

hepatic stellate cells (HSCs) into fibroblast-like cells, which then produce matrix degradation proteins, collectively known as the MMP family^{22, 23}. From the known MMPs only a few are expressed in liver tissue¹⁵, and it is known that MMP-9 is produced at very low levels in normal liver²⁴. In the present study, MMP-9 mRNA expression increased dramatically in 13-month-old SHHR-HFDS, as it was produced at very low levels in the control liver. MMP-9 is produced by Kupffer cells in primary culture, by IL-1 β - and TNF- α -stimulated hepatocytes, IL-1 α -stimulated HSCs, and in carbon tetrachloride-induced liver injury¹⁵. In liver disease, HSCs and inflammatory mononuclear cells (e.g. lymphocytes and neutrophils) are also a source of MMP-9^{15, 24, 25}. In our previous study, SHHR-HFDS exhibited a higher MMP-9 expression in WBCs than other rat groups¹¹, and MMP-9 mRNA expression and activity is high in monocytes²⁶. In the present study, MMP-9 immunolabeled WBCs appeared in the SHHR-HFDS liver at 13 months, but did not appear in the other rat groups. Moreover, the immunolocalization of MMP-9 in hepatocytes, bile ducts and biliary canaliculi reflected the fact that these NASH model rats were severely affected and almost at a stage of hepatic cirrhosis in a part of NASH liver. TIMP-1 is a natural inhibitor of MMP, especially pro-MMP-9, acting to maintain the structural integrity of basement membranes and protects them from degeneration²⁷. The relative balance of MMP-9 and TIMP-1 is thus important in approximating the value of absolute MMP-9 activity¹⁷. In the present study, TIMP-1 expression in the liver was increased in SHHR-HFDS at 13 months, but the increase in MMP-9 was bigger than the increase in TIMP-1, thus MMP-9 activity would be increased in SHHR-HFDS at 13 months.

MMP-2 is constitutively expressed by many cell types, and its promoter is TATA-less, like many of the so-called housekeeping genes²⁸. In the present study, there were only small changes in MMP-2 mRNA expression up until 11 months, however expression increased markedly in SHHR-HFDS at 13 months compared with the other groups. Oxidative stress is thought to stimulate MMP-2 upregulation via signal-regulated kinase 1/2 and phosphatidylinositol 3-kinase on HSCs²⁹. Interestingly, TIMP-2 also inhibits all known members of the MMP family, but associates with membrane type 1-MMP and MMP-2 at the cell surface and regulates MMP-2 activation¹⁷. In the present study, TIMP-2 expression in liver was increased markedly in SHHR-HFDS at 13 months, enough to activate MMP-2 pro-type, leading to the immunolocalization of MMP-2 in extracellular spaces in SHHR-HFDS liver at 13 months. Hemmann and colleagues have suggested that continuous MMP-2 overexpression in HSCs with its proliferation and migration sequelae may foster the progression of fibrosis¹⁵. TIMP-3 is a natural inhibitor of MMPs and a disintegrin and metalloproteinase-17³⁰, but it did not change greatly in our present study.

In conclusion, the present study suggests that increased oxidative stress induces upregulation of MMP-2, MMP-9 and TIMP-2, followed by progression to fibrosis in the liver of NASH model rats. Regulation of the MMP family may lead to suppression of the progression of hepatic fibrosis. The SHHR model may help in the elucidation and treatment of NASH.

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