

# Therapeutic effects of the dipeptidyl peptidase-IV inhibitor, sitagliptin, on non-alcoholic steatohepatitis in FLS-*ob/ob* male mice

TAKUMI ONOYAMA, MASAHIKO KODA, TOSHIAKI OKAMOTO, MANABU KISHINA, TOMOMITSU MATONO, TAKAAKI SUGIHARA and YOSHIKAZU MURAWAKI

Division of Medicine and Clinical Science, Department of Multidisciplinary Internal Medicine, Faculty of Medicine, Tottori University, Yonago 683-8504, Japan

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**Abstract.** Non-alcoholic steatohepatitis is characterized by hepatic fat accumulation, inflammation and varying degrees of fibrosis. The dipeptidyl peptidase-IV enzyme is important in glucose metabolism, as well as lipid accumulation, extracellular matrix metabolism and immune stimulation. Furthermore, the enzyme activity of dipeptidyl peptidase-IV is known to be increased in non-alcoholic steatohepatitis. Therefore, dipeptidyl peptidase-IV inhibitors are potential therapeutic agents for non-alcoholic steatohepatitis. The present study assessed the therapeutic effects of sitagliptin, a dipeptidyl peptidase-IV inhibitor, on non-alcoholic steatohepatitis using fatty liver Shionogi-*ob/ob* male mice. Sitagliptin (2 mg/kg/day; n=10) or placebo (control; n=10) was orally administered to fatty liver Shionogi-*ob/ob* mice for 12 weeks, and hepatic steatosis,

fibrosis, inflammation and oxidative stress were assessed in comparison with the controls. Sitagliptin administration reduced body weight and blood glucose levels, and improved hepatic fibrosis. It also inhibited the gene expression levels of fatty acid synthase, transforming growth factor- $\beta$ 1, tissue inhibitor of metalloproteinases-1, procollagen-type 1, tumor necrosis factor- $\alpha$ , monocyte chemoattractant protein-1 and enhanced peroxisome proliferator activated receptor- $\alpha$ . Furthermore, a marked attenuation of hepatic stellate cell activation and Kupffer cells was observed in the sitagliptin group. A decrease in oxidative stress and apoptosis was also observed. Sitagliptin attenuated the progression of hepatic fibrosis by improving lipid metabolism, inflammation and oxidative stress in non-alcoholic steatohepatitis.

## Introduction

Non-alcoholic steatohepatitis (NASH) is characterized by hepatic fat accumulation, inflammation and varying degrees of fibrosis. The central pathophysiological issue in patients afflicted with NASH is insulin resistance. Improvement of insulin resistance has therapeutic potential in preventing the progression of NASH (1). Previously, dipeptidyl peptidase (DPP)-IV inhibitors have been used as novel oral drugs for the treatment of type 2 diabetes. DPP-IV is an enzyme, which inactivates incretins, including glucagon-like peptide-1 and gastric inhibitory polypeptide, which regulates blood glucose primarily via stimulation of glucose-dependent insulin release. As a result, the activation of DPP-IV leads to the development of glucose intolerance and hepatic steatosis (2). DPP-IV enzyme has widespread organ distribution throughout the body and has pleiotropic biological functions (3-5). DPP-IV is involved in glucose metabolism and lipid accumulation, degradation of the extracellular matrix, appetite regulation and immune stimulation via its peptidase activities (6-10). The liver is one of the organs expressing DPP-IV to a high degree (11). The mRNA expression of DPP-IV is also increased in the liver of non-alcoholic fatty liver disease (NAFLD) (12). Serum DPP-IV activity and the expression of hepatic DPP-IV are correlated with hepatic steatosis and NAFLD severity (13). DPP-IV deficient rats exhibit a reduction of hepatic steatosis, hepatic

*Correspondence to:* Professor Masahiko Koda, Division of Medicine and Clinical Science, Department of Multidisciplinary Internal Medicine, Faculty of Medicine, Tottori University, Nishi-Machi 36-1, Yonago 683-8504, Japan  
E-mail: masakoda@grape.med.tottori-u.ac.jp

**Abbreviations:** NASH, non-alcoholic steatohepatitis; DPP, dipeptidyl peptidase; NAFLD, non-alcoholic fatty liver disease; FLS, fatty liver shionogi; MCD, methionine/choline-deficient; AST, aminotransferase; ALT, alanine aminotransferase; HSC, hepatic stellate cell, SMA,  $\alpha$ -smooth muscle actin; 8-OHdG, 8-hydroxy-2-deoxyguanosine; 4-HNE, 4-hydroxynonenal; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; PCR, polymerase chain reaction; TGF- $\beta$ 1, transforming growth factor- $\beta$ 1; CTGF, connective tissue growth factor; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ ; MCP-1, monocyte chemoattractant protein-1; TIMP-1, tissue inhibitor of metalloproteinases-1; PPAR- $\alpha$ , peroxisome proliferator activated receptor; SREBP1c, sterol regulatory element-binding protein 1c; FAS, fatty acid synthase; MTP, microsomal triglyceride transfer protein

**Key words:** non-alcoholic steatohepatitis, sitagliptin, fatty liver Shionogi-*ob/ob* mice, dipeptidyl peptidase-IV inhibitor, hepatic fibrosis, oxidative stress, hepatic stellate cell, apoptosis

inflammatory and pro-fibrogenic cytokines compared with the wild-type rats (14). Therefore, DPP-IV may be involved in not only insulin resistance, but also hepatic lipogenesis, inflammation and fibrosis during the progression of NASH. Therefore, DPP-IV inhibitors may have favorable effects for four pathways in the treatment of NASH, including insulin resistance, hepatic lipogenesis, inflammation and fibrogenesis.

Fatty liver Shionogi (FLS)-*ob/ob* mice are generated by transferring the *Lep<sup>ob</sup>* gene into the genome of FLS mice without obesity, which then spontaneously develop chronic hepatic steatosis. The features of FLS-*ob/ob* mice are hyperphagia, obesity, hyperlipidemia and diabetes mellitus (15). They exhibit histologically severe steatosis, hepatocellular ballooning and advanced hepatic fibrosis, increased oxidative stress, elevated inflammatory, as well as pro-fibrotic cytokine production, increased apoptosis of hepatocytes and the mice also develop cirrhosis and liver tumors (16,17). FLS-*ob/ob* mice are the closest animal model to metabolic syndrome-associated NASH in humans and possess targets of DPP-IV inhibitors. Previously, sitagliptin, a DPP-IV inhibitor, was reported to attenuate methionine/choline-deficient (MCD) diet-induced steatohepatitis (18). However, this model is markedly different compared with the features of human NASH. The present study aimed to confirm whether sitagliptin improved steatohepatitis in the FLS-*ob/ob* mice by modifying insulin resistance, hepatic lipogenesis, inflammation, fibrosis and oxidative stress.

## Materials and methods

**Animals.** Twenty male FLS-*ob/ob* mice (age, 8 weeks; body weight, 40.14±3.39 g) were obtained from Shionogi Research Laboratories (Shiga, Japan) and housed under a controlled temperature of 24±2°C and a 12-h light/dark cycle. The mice were provided with water and standard powder chow (CE-2, 4.6% fat; CLEA Japan, Inc., Tokyo, Japan) *ad libitum*. Food consumption and body weight were monitored throughout the observation period to equalize the dietary intake between the two groups. All experiments were performed in accordance with the Animal Experimentation Guidelines of Tottori University (Yonago, Japan). The study was approved by the ethics committee of Tottori University, Yonago, Japan (approval no. 11-Y-54).

**Administration of sitagliptin.** Male FLS-*ob/ob* 12-week-old mice were randomly assigned to the control or sitagliptin group (n=10/group). Sitagliptin (2 mg/kg/day; Merck & Co., Inc, Kenilworth, NJ, USA) was administered as a bolus every afternoon *per os* for 12 weeks through a gastric tube. The control group was administered water. Blood was drawn from the tail vein after 4 h fasting and the blood glucose level was measured every 4 weeks. After 12 weeks, the animals were sacrificed under pentobarbital anesthesia (Dainippon Sumitomo Pharma, Osaka, Japan) and blood was collected from the right ventricle. The plasma samples were frozen and stored at -80°C. The liver and visceral fat were weighed, snap frozen in liquid nitrogen and stored at -80°C. Liver specimens were also fixed in 10% buffered formalin (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and embedded in paraffin (Wako Pure Chemical Industries, Ltd.) for histological analysis.

**Analysis of hepatic cholesterol and triglyceride contents.** Snap frozen liver samples (50 mg) were homogenized and extracted with chloroform-methanol (2:1, v/v; Wako Pure Chemical Industries, Ltd.), and subsequently the organic phase was dried and resuspended in 2-propanol, containing 10% Triton X-100. The total cholesterol and triglyceride levels were measured using a Cholesterol E-test (Wako Pure Chemical Industries, Ltd.) and a Triglyceride E-test (Wako Pure Chemical Industries, Ltd.), respectively.

**Biochemical analyses.** The blood samples were immediately separated via centrifugation at 2,000 x g for 15 min at 4°C, and were stored at -80°C until further use. The serum samples were analyzed to determine the levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT).

**Measurement of areas of hepatic steatosis.** Neutral lipids in frozen-fixed, cryostat-embedded liver sections (4-μm thick) were stained with Oil Red O (Sigma-Aldrich, St. Louis, MO, USA). The areas of hepatic steatosis were subsequently measured in 10 randomly selected fields (magnification, x400; Olympus BX51N-34; Olympus Corporation, Tokyo, Japan) in each specimen using Win ROOF version 5.71 software (Mitani Corporation, Tokyo, Japan).

**Measurement of hepatic fibrosis area with sirius red staining.** Formalin-fixed, paraffin-embedded liver sections (4 μm thick) were stained with Picrosirius red (Chroma-Gesellschaft Schmid GmbH & Co., Münster, Germany) and counterstained with fast green (Chroma-Gesellschaft Schmid GmbH & Co.). The areas of hepatic fibrosis were subsequently measured in 10 randomly selected fields in each specimen (magnification, x400) using Win ROOF version 5.71 software and the Olympus BX51N-34 microscope.

**Immunostaining for α-smooth muscle actin (SMA).** The present study immunohistochemically detected α-SMA by staining with mouse monoclonal anti-α-SMA antibody (cat. no. MS-113-R7; Thermo Fisher Scientific, Fremont, CA, USA) without dilution. Goat anti-mouse Ig, from the Histofine® Mouse Stain kit (cat. no. 414322; Nichirei Biosciences, Inc., Tokyo, Japan), was used as the secondary antibody without dilution. The activation of hepatic stellate cells (HSC) was assessed by measuring the areas of α-SMA staining using Win ROOF version 5.71 software in 10 randomly selected fields (magnification, x400; Olympus BX51N-34 microscope) in each specimen.

**Analysis of inflammatory cell infiltration of liver tissue.** F4/80, which is a mature mouse cell surface glycoprotein expressed at high levels on Kupffer cells (19), was immunohistochemically stained using a rat monoclonal anti-F4/80 mouse antibody (cat. no. ab6640; Abcam, Tokyo, Japan) diluted at 1:100 with 0.01 M/l phosphate-buffered saline (PBS), according to the manufacturer's instructions. Goat anti-rat secondary antibody, from the Histofine® Simple Stain™ Mouse MAX-PO (Rat) kit (cat. no. 414311; Nichirei Biosciences, Inc.) was used without dilution. The immunopositive cells were analyzed in 10 intralobular ocular fields (magnification, x400; Olympus BX51N-34 microscope) in each specimen.

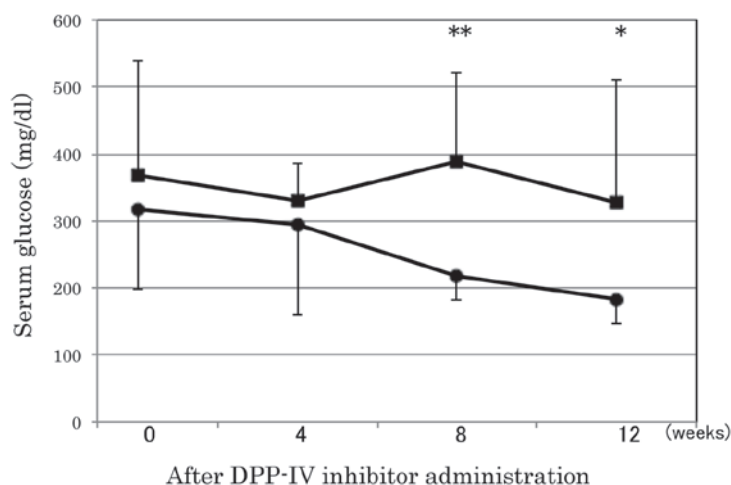


Figure 1. Effect of sitagliptin administration on serum glucose levels in mice. The levels of serum glucose were significantly lower in the sitagliptin group (●) compared with the control group (■) at 8 and 12 weeks. \* $P<0.05$  and \*\* $P<0.01$ , compared with the control group.

Table I. Effects of sitagliptin administration on various characteristics of the mice.

Characteristic	Control group (n=10)	Sitagliptin group (n=10)
Body weight (g)	56±8	48±9 <sup>a</sup>
Liver weight (g)	7.8±2.5	6.0±2.7
Liver/body weight ratio	0.14±0.03	0.12±0.04
Visceral fat weight (g)	2.3±0.4	2.2±0.6
Weekly dietary intake (g)	37±12	36±10
Serum AST (U/l)	183±73	158±61
Serum ALT (U/l)	226±107	232±195

<sup>a</sup> $P<0.05$  and <sup>b</sup> $P<0.01$ , compared with the control group. AST, aspartate aminotransferase; ALT, alanine aminotransferase.

**Analysis of oxidative stress.** Oxidative stress was assessed by immunohistochemical staining to detect 8-hydroxy-2-deoxyguanosine (8-OHdG), a marker of oxidative DNA damage (16), using a monoclonal mouse anti-8-OHdG antibody (cat. no. MOG-020P; Nikken SEIL, Shizuoka, Japan), diluted with 200  $\mu$ l distilled water, according to the manufacturer's instructions. Goat anti-mouse Ig, from the Histofine® Mouse Stain kit, served as the secondary antibody without dilution. The immunopositive cells were analyzed using Win ROOF version 5.71 software in 10 intralobular ocular fields (magnification, x400; Olympus BX51N-34 microscope) in each specimen, and the values were expressed as the ratios (%) of fields. Furthermore, the present study semi-quantified 4-hydroxynonenal (4-HNE), which was immunohistochemically stained using a monoclonal mouse anti-4-HNE antibody (cat. no. MHN-020P; Nikken SEIL), diluted with 200  $\mu$ l distilled water, according to the manufacturer's instructions. Goat anti-mouse Ig, from the Histofine® Mouse Stain kit, was used as the secondary antibody without dilution. A total of 10 randomly selected fields in each specimen, which were stained with 4-HNE (magnification, x400) were classified into immunopositive grades 1, 2, 3 or 4 (0-10%, 11-20%, 21-30%

and >30%, respectively) and the mean values of the 10 fields were calculated.

**Analysis of apoptotic cells in liver tissue.** The apoptotic cells in liver tissue were detected *in situ* by specific labeling of nuclear DNA fragmentation using terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL). The sections were deparaffinized in xylene, rehydrated, washed with PBS and digested with 20  $\mu$ g/ml proteinase K (Wako Pure Chemical Industries, Ltd.) for 10 min at room temperature. The fragmented DNA was detected via the TUNEL method using the Apop Tag Plus Peroxidase *In Situ* Apoptosis Detection kit (EMD Millipore, Billerica, MA, USA), according to the manufacturer's instructions. The numbers of stained and unstained cells were counted using Win ROOF version 5.71 software in 10 intralobular ocular fields (magnification, x400) in each specimen.

**RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis.** Hepatic tissue samples were homogenized and the total RNA was extracted using the RNeasy Lipid Tissue Mini kit (Qiagen, Hilden, Germany). The RNA concentrations were determined by measuring the absorbance at 260 nm using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific), and the RNA quality was confirmed by electrophoresis on ethidium bromide stained 1% agarose gels. The total RNA (2  $\mu$ g) was reverse transcribed in a final volume of 11.5  $\mu$ l, containing 4  $\mu$ l 5X standard buffer, 2  $\mu$ l 0.1 M dithiothreitol, 1  $\mu$ l SuperScript II RNase H reverse transcriptase (Invitrogen Life Technologies, Carlsbad, CA, USA), 2  $\mu$ l 10 mM dNTP (Promega, Madison, WI, USA), 1  $\mu$ l 50 pmol/ $\mu$ l random primer (Promega), 0.5  $\mu$ l 100 pmol/ $\mu$ l oligo (dt) 15 Primer (Promega) and 1  $\mu$ l 40 U/ $\mu$ l ribonuclease inhibitor (Wako Pure Chemical Industries, Ltd.). The mixtures were incubated at 37°C for 60 min, 95°C for 5 min and subsequently cooled to 4°C for 5 min using a MyCycler™ Thermal Cycler (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

**Real-time PCR.** Quantitative real-time PCR assays (7900HT Fast Realtime PCR system; Applied Biosystems



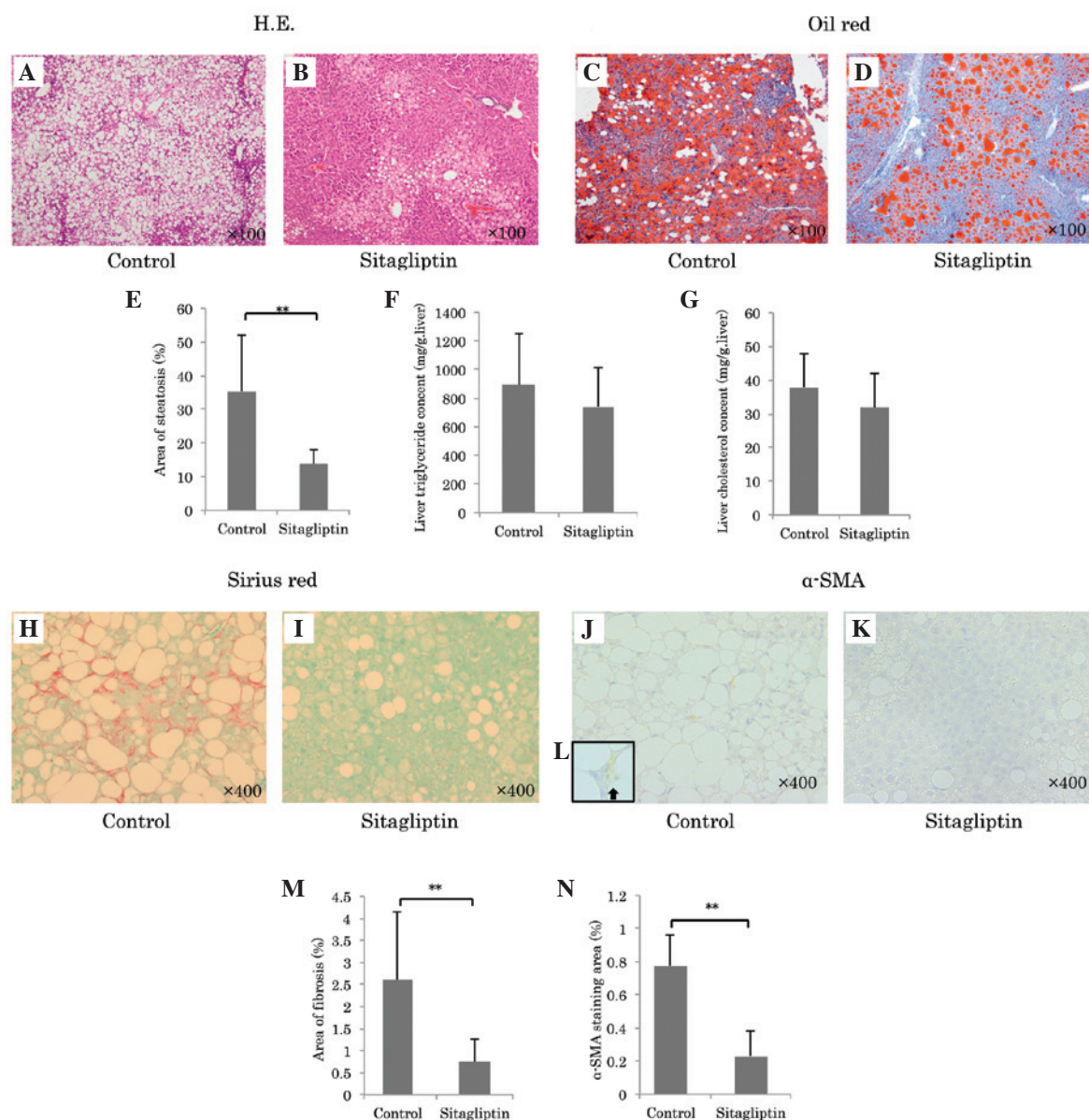


Figure 2. Histological analyses of liver tissues. Representative images of the H.E. staining (magnification, x100) of the (A) control group and (B) sitagliptin groups. Representative images of Oil Red O staining (magnification, x100) in the (C) control and (D) sitagliptin groups. (E) The proportion (%) of hepatic steatosis area stained with Oil Red O was measured using image analysis (\*\* $P < 0.01$ ). A comparison of (F) hepatic triglyceride content and (G) hepatic cholesterol content between the groups. Hepatic fibrosis was determined by Sirius red staining. Representative images of sirius red staining of the (H) control and (I) sitagliptin groups. Representative images of  $\alpha$ -SMA immunostaining in the (J) control and (K) sitagliptin groups (magnification, x400). (L) demonstrates a higher magnification (x1,000) of an  $\alpha$ -SMA positive cell. (M) The proportion (%) of hepatic fibrosis area stained with Sirius red (magnification, x400) was measured using image analysis (\*\* $P < 0.01$ ). The area of fibrosis was significantly decreased in the sitagliptin group compared with the control group. (N) Quantitation of an area of  $\alpha$ -SMA immunostaining measured by image analysis (\*\* $P < 0.01$ ). The areas of  $\alpha$ -SMA immunostaining were significantly reduced in the sitagliptin group compared with the control. H.E., hematoxylin and eosin;  $\alpha$ -SMA,  $\alpha$ -smooth muscle actin.

Life Technologies, Foster City, CA, USA) were performed in a final volume of 10 ml, containing 250 nM Universal ProbeLibrary probe (Roche, Basel, Switzerland), 900 nM forward primer, 900 nM reverse primer, 5 ml EXPRESS qPCR Supermix with Premixed Rox (Invitrogen Life Technologies) and 2 ml cDNA. The mRNA expression levels of transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1; GenBank, NM\_011577), procollagen-type I (GenBank, U08020), connective tissue growth factor (CTGF; GenBank, NM\_010217), tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ; GenBank, NM\_013693), monocyte chemoattractant protein-1

(MCP-1; GenBank, NM\_100127112), tissue inhibitor of metalloproteinases-1 (TIMP-1; GenBank, NM\_011593), peroxisome proliferator activated receptor (PPAR- $\alpha$ ; GenBank, NM\_007988.3), sterol regulatory element-binding protein 1c (SREBP1c; GenBank, NM\_011480), fatty acid synthase (FAS; GenBank, AF127033) and microsomal triglyceride transfer protein (MTP; GenBank, NM\_008642) were assessed using the 7900HT Fast Real-Time PCR system with SDS2.3 software (Applied Biosystems Life Technologies) and  $\beta$ -actin (GenBank, NM\_007393) was used as an internal standard. The thermal cycle conditions were

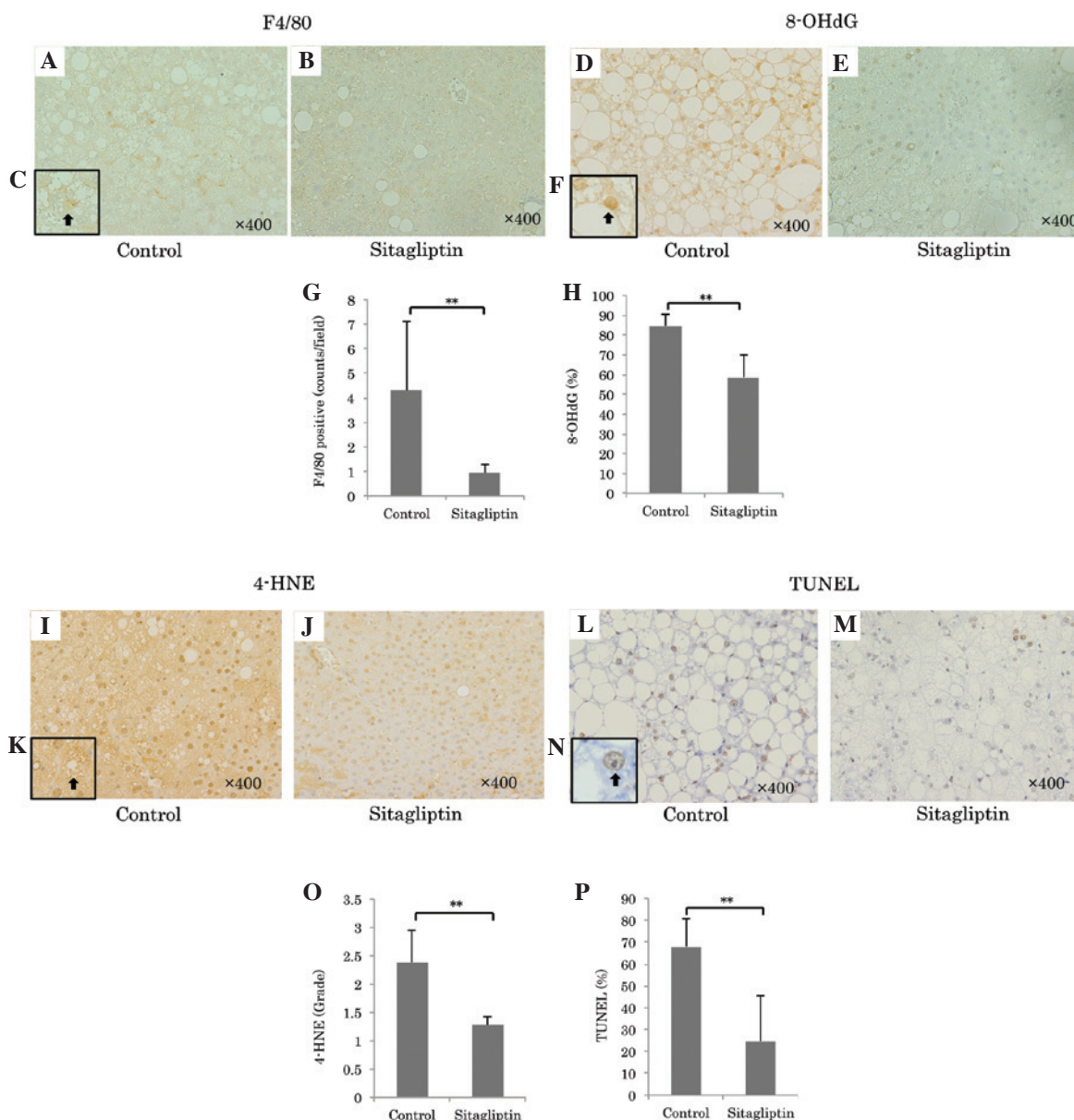


Figure 3. Representative images of F4/80 immunostaining for Kupffer cells (magnification, x400) in the (A) control and (B) sitagliptin groups. (C) Increased magnification (x1,000) of immunopositive F4/80 positive cells. Representative immunostaining for 8-OHdG (magnification, x400) of the (D) control and (E) sitagliptin groups. (F) A higher magnification of an 8-OHdG positive nucleus (x1,000, arrow). (G) Quantitation of F4/80 immunopositive cells in each group indicated that F4/80 immunopositive cells were significantly decreased in the sitagliptin group compared with the control (\*\* $P < 0.01$ ). Oxidative stress was determined by 8-OHdG and 4-HNE immunostaining. (H) A comparison of 8-OHdG immunopositive cells between the groups (\*\* $P < 0.01$ ). Immunostaining for 8-OHdG was significantly decreased in the sitagliptin group compared with the control. Immunostaining for 4-HNE (magnification, x400) in the (I) control and (J) sitagliptin groups. (K) 4-HNE immunopositive cell at a higher magnification (x1,000, arrow). Apoptotic cells were determined by TUNEL immunostaining (magnification, x400) in the (L) control and (M) sitagliptin groups. (N) A higher magnification (x1,000) of a TUNEL positive nucleus (arrow). (O) A comparison of 4-HNE immunopositive cells between the groups. Immunostaining of 4-HNE was significantly reduced in the sitagliptin group compared with the control (\*\* $P < 0.01$ ). (P) A comparison of the numbers of TUNEL positive cells between groups. The number of TUNEL-positive cells was significantly decreased in the sitagliptin group compared with the control (\*\* $P < 0.01$ ). 8-OHdG, 8-hydroxy-2-deoxyguanosine; 4-HNE, 4-hydroxynonenal; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling.

as follows: 95°C for 20 sec, followed by 45 cycles of 1 sec at 95°C and 20 sec at 60°C. The relative mRNA expression levels were calculated using the  $2^{-\Delta\Delta CT}$  method (20).

**Statistical analysis.** The significance of the differences between the groups was statistically analyzed using an unpaired Student's t-test. The data were statistically analyzed using StatFlex version 6.0 for Windows software (Artech Co, Ltd., Osaka, Japan). All data are expressed as the mean  $\pm$  stan-

dard deviation.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**Characteristics of the mice.** As shown in Table I, the body weight of the mice was significantly lower in the sitagliptin group compared with the control group. The liver weight and liver-to-body weight ratio in the sitagliptin group also

Table II. mRNA expression levels of various genes in the control and sitagliptin groups.

mRNA	Control group (n=10)	Sitagliptin group (n=10)
FAS	6.21±3.79	1.65±0.43 <sup>b</sup>
SREBP1c	1.98±0.62	1.59±0.42
PPAR- $\alpha$	2.07±0.63	2.83±0.59 <sup>b</sup>
MTP	1.63±0.37	1.44±0.28
Procollagen-type I	24.89±17.47	8.06±6.40 <sup>b</sup>
TGF- $\beta$ 1	2.40±0.46	1.65±0.47 <sup>b</sup>
CTGF	7.32±10.93	3.36±0.91
TIMP-1	11.43±6.53	4.05±2.69 <sup>b</sup>
TNF- $\alpha$	4.81±2.19	2.64±0.91 <sup>b</sup>
MCP-1	8.10±5.10	4.30±2.45 <sup>a</sup>

The data are presented as the relative mRNA levels compared with  $\beta$ -actin. <sup>a</sup>P<0.05 and <sup>b</sup>P<0.01, compared with the control group. FAS, fatty acid synthase; SREBP1c, sterol regulatory element-binding protein 1c; PPAR- $\alpha$ , peroxisome proliferator activated receptor- $\alpha$ ; MTP, microsomal triglyceride transfer protein; TGF- $\beta$ 1, transforming growth factor  $\beta$ 1; CTGF, connective tissue growth factor; TIMP-1, tissue inhibitor of metalloproteinases 1; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ . MCP-1, monocyte chemoattractant protein-1.

demonstrated a decreased tendency compared with the control group. Food consumption and visceral fat weight revealed no significant difference between the two groups. The serum levels of AST and ALT also revealed no significant difference between the two groups. The serum glucose was significantly lower in the sitagliptin group compared with the control group at 8 and 12 weeks after the administration of the DPP-IV inhibitor (Fig. 1).

**Effects of sitagliptin on hepatic steatosis.** To assess the effects of sitagliptin on lipid metabolism, the present study determined the hepatic steatosis area, hepatic lipid contents and gene expression of hepatic lipogenesis, lipolysis and lipid transporter. Oil Red O staining revealed that sitagliptin significantly reduced the area of hepatic steatosis (sitagliptin group, vs. control group; 13.8±4.3, vs. 35.4±16.5%; P<0.001; Fig. 2A-E). Hepatic total cholesterol and triglyceride contents in the sitagliptin group were lower compared with the control group (Hepatic cholesterol: Sitagliptin, 31.8±10.3, vs. control, 37.9±9.9 mg/g liver; Triglyceride: Sitagliptin, 741±276, vs. control: 894±359 mg/g liver), however, not significantly so (Fig. 2F and G). The mRNA expression levels of genes associated with hepatic steatosis were listed in Table II. The mRNA expression of PPAR- $\alpha$  was significantly increased in the sitagliptin group (sitagliptin, vs. control group; 2.83±0.59, vs. 2.07±0.63; P=0.0095). The mRNA expression of FAS was significantly decreased in the sitagliptin group (sitagliptin, vs. control group; 1.65±0.43, vs. 6.21±3.78; P=0.0013). The mRNA expression levels of SREBP1c and MTP were reduced in the sitagliptin group compared with the control, however, not significantly so. Taken together, these findings suggested that sitagliptin reduced lipid synthesis and the accumulation in the liver of FLS-*ob/ob* mice.

**Effects of sitagliptin on hepatic fibrosis.** To assess the possibility that sitagliptin reduced hepatic fibrosis, the present study determined the antifibrotic effects of sitagliptin in the FLS-*ob/ob* mice using Sirius red staining,  $\alpha$ -SMA staining and pro-fibrogenic cytokine gene expression. Sirius red staining revealed that sitagliptin reduced the area of fibrosis compared with the control (sitagliptin, vs. control; 0.74±0.54, vs. 2.61±1.53%; P=0.0016; Fig. 2H, I and M). Since activated HSCs are a major contributor to hepatic fibrogenesis, the present study measured the protein expression of  $\alpha$ -SMA, which is expressed by HSCs in response to liver injury. Notably, sitagliptin caused a profound decrease in the area of positive  $\alpha$ -SMA immunostaining compared with the control (sitagliptin, vs. control; 0.23±0.15, vs. 0.78±0.19%; P<0.001; Fig. 2J-L and N), which suggested that this treatment inhibited the activation of HSCs. The mRNA expression of parameters associated with extracellular matrix metabolism in the liver are listed in Table II. Sitagliptin significantly reduced the mRNA expression levels of procollagen I, TGF- $\beta$ 1 and TIMP-1 compared with the controls. The mRNA expression of CTGF tended to be lower in the sitagliptin group compared with the control group.

**Effects of sitagliptin on inflammatory reactions in the liver.** The process of hepatic fibrosis is driven primarily by inflammation in response to liver damage. Sitagliptin markedly reduced the number of F4/80 positive cells, representing liver macrophage Kupffer cells, compared with the control group (sitagliptin, vs. control; 0.95±0.33, vs. 4.31±2.78; P=0.0013; Fig. 3A-C and G) and reduced the quantity of mRNA for MCP-1 by 53% and TNF- $\alpha$  by 55% (Table II).

**Effects of sitagliptin on oxidative stress.** Oxidative stress is involved in the development of NASH. The present study determined oxidative stress by two methods: 8-OHdG as an index of DNA damage and 4-HNE as an index of a lipid peroxidation. Sitagliptin markedly reduced the ratio of 8-OHdG positive cells in the liver samples compared with the control (sitagliptin, vs. control; 58.6±11.6, vs. 84.5±5.8%; P<0.001; Fig. 3D-F and H) and significantly reduced the immunostaining grade for liver 4-HNE (sitagliptin, vs. control; 1.27±0.16, vs. 2.38±0.56; P<0.001; Fig. 3I-G and N).

**Effect of sitagliptin on hepatic apoptosis.** Hepatocytes damaged by oxidative stress undergo apoptosis. Sitagliptin significantly reduced the ratio of TUNEL positive cells in the liver samples compared with the control (sitagliptin, vs. control; 68.0±12.9, vs. 24.8±20.6%; P<0.001; Fig. 3K-M and O).

## Discussion

The present study revealed that sitagliptin decreased blood glucose level, hepatic lipogenesis, pro-inflammatory cytokines, pro-fibrogenic cytokines and oxidative stress, consequently improving hepatic steatosis, inflammation and fibrosis. The 'two-hit' theory and 'multiple parallel hits' hypothesis have been proposed for the pathophysiology of NAFLD and NASH (21). The first hit is insulin resistance and leads to NAFLD. The following hits, oxidative stress, cytokine production and inflammation increase, result in the development of NASH (22-24).



Sitagliptin significantly improved hyperglycemia and reduced body weight compared with the controls under the identical dietary intake. The inhibition of DPP-IV by sitagliptin led to the attenuation of incretin degradation, consequently increasing insulin and reducing blood glucose levels. TNF- $\alpha$  leads to insulin resistance by impairing insulin signaling and reducing glucose transporters (22). The reduction of the gene expression of TNF- $\alpha$  in the sitagliptin group also improved insulin resistance. The loss of body weight by DPP-IV inhibitors is suggested to be due to two mechanisms, one of which is the loss of appetite by the inactivation of peptide YY (25). The other is the inhibitory effect on fat absorption from the gut. Notably, another DPP-IV inhibitor, vildagliptin, is reported to reduce the prandial triglyceride response to fat-rich meal intake by 85% (26). Furthermore, sitagliptin in combination with metformin reduce cholesterol and triglyceride levels (27). In the present study, since food consumption was unaltered between the two mouse groups, the weight loss in the sitagliptin group was likely due to the inhibition of fat absorption from the gut. Trials of weight loss improve liver function and liver histology in NAFLD (28). The improvement of liver histology by sitagliptin may originate from both weight-independent and weight-dependent effects (29,30).

Treatment with sitagliptin activated PPAR- $\alpha$  and attenuated SREBP1c and FAS. Therefore, the present study supported that DPP-IV affected lipid accumulation by the inactivation of PPAR- $\alpha$ , which is involved in  $\beta$ -oxidation of fatty acids, and the activation of SREBP1c and FAS, which are involved in hepatic lipogenesis (31). In the liver of high-fat-fed mice lacking DPP-IV, the expression of PPAR- $\alpha$  is upregulated, whereas the expression of SREBP1c is downregulated (10). SREBP1c stimulates several lipogenic enzymes, including FAS (32). In the present study, sitagliptin inhibited FAS more markedly compared with SREBP1c. This may be explained by the dose-dependency of sitagliptin (2 mg/kg/day). Sitagliptin inhibits plasma DPP-IV activity in a dose-dependent manner, from 0.1 to 3 mg/kg, in mice (33). Administration of a higher dose of sitagliptin may reduce the expression of SREBP1c. MTP transports triglycerides to very low-density lipoprotein. Enhanced expression of this gene (34) promotes the release of excess lipid from NAFLD livers. However, in the present study, sitagliptin revealed no affect on MTP gene expression. From these results, decreased steatosis by sitagliptin was caused by the attenuation of lipogenesis and the stimulation of lipolysis.

The present study demonstrated that sitagliptin reduced hepatic macrophages and Kupffer cells, which were measured by the F4/80 positive cell index. Furthermore, the expression levels of TNF- $\alpha$  and MCP-1 were reduced in the sitagliptin group. Several chemokines are known to be target peptides of DPP-IV (11). MCP-1 [chemokine (C-C motif) ligand 2] is produced by Kupffer cells and HSCs, and promotes hepatic inflammation by recruiting and activating macrophages (35-37). The plasma concentration of MCP-1 is reported to be reduced by treatment with DPP-IV inhibitors in patients with diabetes (38). DPP-IV inhibitors may reduce MCP-1 mediated C-C chemokine receptor type 2 macrophages. From these results, sitagliptin induced a marked reduction in intrahepatic inflammation.

In addition to the significant reduction of  $\alpha$ -SMA positive cells, activated myofibroblasts/HSCs, reduced hepatic fibrosis area and decreased gene expression of procollagen I, TGF- $\beta$ 1 and TIMP-1 were observed in the sitagliptin group compared with the control group. Similarly, sitagliptin reduced the activation of HSC in MCD fed mice (18). The mechanisms by which sitagliptin attenuated fibrosis are most likely mediated through inhibition of HSC activation. HSCs are activated by TGF- $\beta$ 1 or oxidative stress. The present study immunohistochemically analyzed 8-OHdG and 4-HNE to determine the effects of sitagliptin on oxidative stress. Oxidative stress is pivotal as a factor in liver disease, which progresses from steatosis to steatohepatitis. Fatty acid oxidation represents an important source of reactive oxygen species, which induce lipid peroxidation and initiate DNA damage, which are assessed as 4-HNE and 8-OHdG production, respectively. It was demonstrated that sitagliptin ameliorated 8-OHdG and 4-HNE immunostaining in the liver tissues and attenuated reactive oxygen species production. The present study revealed that sitagliptin suppressed the activation of HSC via the attenuation of oxidative stress and the inhibition of pro-fibrogenic and pro-inflammatory cytokines (TGF- $\beta$ 1, TNF- $\alpha$  and MCP-1). Additionally, sitagliptin was reported to directly suppress the proliferation of HSCs in rats (39). Therefore, sitagliptin may directly and indirectly suppress the activation of HSCs.

In conclusion, sitagliptin attenuated the progression of hepatic fibrosis by improving fatty deposition and inhibiting inflammation. This treatment also decreased oxidative stress, and pro-inflammatory and fibrogenic cytokines.

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