Original Article

Protective Effects of Ipragliflozin, a Sodium-glucose Cotransporter 2 Inhibitor, on a Non-alcoholic Steatohepatitis Mouse Model

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ABSTRACT

Background The options for the treatment of nonalcoholic steatohepatitis (NASH) are limited. We examined the effects of ipragliflozin, a sodium-glucose cotransporter 2 inhibitor, on the fatty liver Shionogi (FLS)-*ob/ob* mice, a non-alcoholic steatohepatitis mouse model.

Methods FLS-*ob/ob* male mice were treated with vehicle ($n = 10$) and ipragliflozin ($n = 8$). Serum metabolic markers, histopathology of the liver, hepatic cholesterol and triglyceride levels and hepatic mRNA levels related to fibrosis, lipid metabolism and endoplasmic reticulum (ER) stress were compared between the two groups.

Results The body weight and hepatic cholesterol and triglyceride levels were significantly decreased in the ipragliflozin group compared with the control group. Hepatic steatosis and fibrosis were significantly ameliorated by the treatment with ipragliflozin. Hepatic infiltration of macrophage, expression levels of 8-hydroxy-2-deoxyguanosine (8-OHdG) and hepatic mRNA levels of ER stress markers were not significantly modulated by the treatment with ipragliflozin.

Conclusion Ipragliflozin can be a therapeutic option for patients with NASH. The precise mechanisms of action need to be clarified in future studies.

Key words ipragliflozin; non-alcoholic steatohepatitis; sodium-glucose cotransporter 2 inhibitor

Nonalcoholic steatohepatitis (NASH) is a subtype of nonalcoholic fatty liver disease (NAFLD) which can progress to liver cirrhosis and cancer. Among the situation that the number of patients with NASH has been increasing worldwide, several novel drugs are under clinical trials including an inhibitor of apoptosis signal-regulating kinase-1 (ASK-1), a farnesoid X receptor (FXR) agonist and a fibroblast growth factor (FGF)-19 agonist.[1,2] Since there are no established treatments for NASH as of 2018, the application of novel drugs for NASH is long-awaited.

Sodium-glucose cotransporter 2 (SGLT2) inhibitors are a new class of oral anti-diabetic agents which inhibit SGLT2-mediated renal glucose reabsorption and induce glycosuria. In addition to the lowering effects on blood glucose levels and body weight, some SGLT2 inhibitors (empagliflozin and canagliflozin) have been shown to improve cardiovascular outcomes in high-risk individuals and to slow the progression of diabetic kidney disease.[3] Recently, SGLT2 inhibitors have also been demonstrated to benefit the liver in mouse models of NASH[4,5] and patients with NASH.[6,7] However, the precise mechanisms by which SGLT2 inhibitors ameliorate NASH are largely unknown.

We have previously reported that the fatty liver Shionogi (FLS)-*ob/ob* mice made by transferring the leptin[ob] gene develop steatohepatitis with increased oxidative stress and fibrosis.[8] By employing the FLS-*ob/ob* mice as a mouse model of NASH, we investigated the effects of ipragliflozin, a sodium-glucose cotransporter 2 inhibitor, on the liver and explored the mechanisms of the actions.

MATERIALS AND METHODS
Animals and treatment with ipragliflozin
A total of 18 male FLS-*ob/ob* mice obtained from Shionogi Research Laboratories (Shiga, Japan) were housed in a room-controlled environment (temperature of 24 ± 2 °C and a 12-h light/dark cycle). The mice were allowed access to water and standard pellet chow (CE-2, 4.6% fat; CLEA Japan, Tokyo, Japan) *ad libitum*. All experiments were performed in accordance with

the Animal Experimentation Guidelines of Tottori University (h26-Y-008).

Eighteen FLS-*ob/ob* mice (12 weeks old) were divided into 2 groups; the control group ($n = 10$) and ipragliflozin group ($n = 8$). Mice in the ipragliflozin group were administered ipragliflozin orally at a dose of 1 mg/kg via a gastric tube every day for 12 weeks. The control group received vehicle, a standard pellet chow, instead of ipragliflozin via a gastric tube every for 12 weeks. After 12 weeks, the mice were sacrificed under pentobarbital anesthesia injection (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 tissues were collected, weighed, snap frozen in liquid nitrogen and stored at −80 °C. Liver specimens were fixed in 10% buffered formalin (Wako Pure Chemical Industries, Osaka, Japan) and embedded in paraffin (Wako Pure Chemical Industries) for histological analysis.

Biochemistry
The blood samples were immediately separated by centrifugation at 2000 g for 15 min at 4 °C and stored at −80 °C until further use. Plasma levels of alanine aminotransferase (ALT), cholesterol and triglyceride were measured using an autoanalyzer (7170; Hitachi, Tokyo, Japan).

Histopathology of the liver
Four-µm-thick sections of formalin-fixed and paraffin-embedded liver were stained with hematoxylin-eosin (HE) for evaluation of lipid drops and picrosirius red counterstained with fast green for evaluation of fibrosis, respectively. The area of lipid drops and fibrosis was measured per specimen in 10 randomly selected fields/section (magnification x200) by Win ROOF ver. 5.71 (Mitsutani, Tokyo, Japan) and Olympus BX51N-34 microscope (Olympus, Tokyo, Japan).

Hepatic cholesterol and triglyceride levels
Snap frozen liver samples (50 mg) were homogenized, mixed with chloroform-methanol mixture (2:1 v/v; Wako Pure Chemical Industries), organic phase was dried and resolubilized in 2-propanol containing 10% Triton X-100. Total cholesterol and triglyceride levels were determined using commercial enzymatic kits (Cholesterol E-test and Triglyceride E-test; Wako Pure Chemical Industries).

RNA extraction and reverse transcription polymerase chain reaction (PCR)
Tissue samples were homogenized and total RNA was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany). Concentrations of RNA were determined by measuring absorbance at 260 nm, and the quality of RNA was verified by electrophoresis on ethidium-bromide-stained 1% agarose gels. Total RNA (2 µg) was reverse transcribed in a final volume of 11.5 µL containing 4 µL of 5x standard buffer, 2 µL of 0.1M dithiothreitol, 1 µL of SuperScript II RNase H-reverse transcriptase (Invitrogen, Carlsbad, CA), 2 µL of 10M dNTPs (Promega, Madison, WI), 1 µL of 50 pmol/µL Random Primer (Promega), 0.5 µL of 100 pmol/µL Oligo(dt)15 Primer (Promega) and 1 µL of 40 U/µL ribonuclease inhibitor (Wako Pure Chemical Industries, Osaka, Japan). Samples were incubated at 37 °C for 60 min, 95 °C for 5 min and cooled to 4 °C for 5 min.

Quantitative real-time PCR assays (7900HT Fast Realtime PCR system; Applied Biosystems, Carlsbad, CA) were proceeded in a final volume of 10 µL of solution containing 250 nM Universal ProbeLibrary probe (Roche, Basel, Switzerland), 900 nM forward primer, 900 nM reverse primer, 5 µL EXPRESS qPCR Supermix with Premixed Rox (Invitrogen) and 2 µL cDNA. The mRNA levels of activating transcription factor 3 (ATF3) (GenBank; NM_007498.3), C/EBP-homologous protein (CHOP) (GenBank; NM_007837.3), C-JUN, nuclear protein 1 (NUPR1) (GenBank; NM_010591.2), peroxisome proliferator activated receptor-α (PPAR-α) (GenBank; NM_011144.6), procollagen-type I (GenBank; NM_007742), sterol regulatory element-binding protein 1c (SREBP1c) (GenBank; NM_011480.3), tissue inhibitor of metalloproteinases-1 (TIMP-1) (GenBank; NM_001044384), transforming growth factor-β1 (TGF-β1) (GenBank; NM_011577), and tumor necrosis factor-α (TNF-α) (GenBank; NM_013693.2) were assessed using the 7900HT Fast Real-Time PCR System with SDS2.3 software (Applied Biosystems) and β-actin as an internal standard. Thermal cycle conditions were as follows: hold at 95 °C for 20 s followed by 45 cycles of 95 °C for 1 s and 60 °C for 20 s. The relative mRNA expression levels were compared using the $2^{-\Delta\Delta CT}$ method.

Inflammatory cells in the liver
Inflammatory cells in the liver was evaluated by immunohistochemical staining with an anti-F4/80 monoclonal antibody (Abcam, Tokyo, Japan) in accordance with the manufacturer's instructions. Immuno-positive cells were counted in 10 ocular fields (magnification x400) per specimen.

Oxidative stress in the liver
Oxidative stress in the liver was evaluated by immu-

Table 1. The impact of ipragliflozin treatment on FLS-*ob/ob* mice

	Control (n = 10)	Ipragliflozin (n = 8)	P value
Body weight (g)	58.0 ± 4.6	52.6 ± 4.5	0.024*
Liver weight (g)	7.36 ± 2.46	7.05 ± 2.80	0.110
Liver/body weight ratio	0.13 ± 0.03	0.13 ± 0.04	0.213
Visceral fat weight (g)	2.345 ± 0.395	2.385 ± 0.505	0.182
Serum ALT (U/L)	338.5 ± 187.5	263.5 ± 174.5	0.301
Serum cholesterol (mg/dL)	283.5 ± 60.5	321.5 ± 84.5	0.657
Serum triglyceride (mg/dL)	250 ± 126	313 ± 105	0.018*
Hepatic cholesterol (mg/dL)	69.0 ± 20.0	51.4 ± 15.6	0.003*
Hepatic triglyceride (mg/dL)	2479 ± 690	2182 ± 819	0.021*

*$P < 0.05$ compared with the control group.
FLS, fatty liver Shionogi.

Table 2. Hepatic mRNA levels in FLS-*ob/ob* mice treated with vehicle (control) and a SGLT2 inhibitor (ipragliflozin)

	Control (n = 10)	Ipragliflozin (n = 8)	P value
Procollagen I	1.35 ± 0.74	0.94 ± 0.71	0.062
TGF-β1	1.04 ± 0.44	0.90 ± 0.24	0.534
TIMP-1	0.98 ± 0.63	0.77 ± 0.48	0.131
TNF-α	0.76 ± 0.24	1.24 ± 0.69	0.016*
PPAR-α	1.42 ± 0.42	0.60 ± 0.28	0.0004***
SREBP1c	2.53 ± 1.53	1.75 ± 1.18	0.183
ATF3	0.74 ± 0.26	0.82 ± 0.56	0.214
CHOP	0.70 ± 0.30	0.80 ± 0.37	0.790
C-JUN	0.78 ± 0.26	0.77 ± 0.40	0.131
NUPR1	0.97 ± 0.41	0.81 ± 0.27	0.183

* $P < 0.05$, *** $P < 0.001$ compared with the control group.
ATF3, activating transcription factor 3; CHOP, C/EBP-homologous protein; C-JUN, jun proto-oncogene; FLS, fatty liver Shionogi; NUPR1, nuclear protein 1; PPAR-α, peroxisome proliferator activated receptor-α; SREBP1c, sterol regulatory element-binding protein 1c; TGF-β1, transforming growth factor-β1; TIMP-1, tissue inhibitor of metalloproteinases-1; TNF-α, tumor necrotic factor-α.

nohistochemical staining with an anti-8-hydroxy-2-deoxyguanosine (8-OHdG) monoclonal antibody (Nikken Seil, Shizuoka, Japan) according to the instructions from the manufacturer. Immuno-positive cells in 10 ocular fields (magnification x400) per specimen were counted and the values were expressed as ratios (%) of fields. Analysis of immune-positive cells was carried out by using Win ROOF ver. 5.71 software.

Statistical analysis
The significance of differences between the groups was analyzed by the Mann-Whitney test. All statistical tests were performed using StatFlex ver. 6.0 for Windows software (Artech, Osaka, Japan). All values are expressed as means ± standard deviation. $P < 0.05$ was considered significant.

RESULTS
The impact of ipragliflozin treatment on FLS-*ob/ob* mice
After FLS-*ob/ob* mice were treated with vehicle (control group) and a SGLT2 inhibitor (ipragliflozin group) for 12 weeks, several metabolic parameters were compared between the two groups (Table 1). The body weight and hepatic cholesterol and triglyceride levels were significantly decreased in the ipragliflozin group compared with the control group. Serum triglyceride level was significantly higher in the ipragliflozin group than the control group. There was no significant difference between the two groups in the liver weight, liver to body weight ratio, visceral fat weight, serum ALT and cholesterol levels. These results suggest that the treatment with ipragliflozin ameliorated fatty liver change in FLS-*ob/ob* mice.

Effects of ipragliflozin on hepatic steatosis
To confirm the above findings, hepatic steatosis levels in FLS-*ob/ob* mice were examined in the control and ipragliflozin groups. As expected, the area of hepatic steatosis was significantly decreased in the ipragliflozin group compared with the control group ($P < 0.05$, Figs. 1A–C).

The molecular mechanisms of the inhibitory effects of hepatic steatosis by ipragliflozin were investigated by examination of the expression levels of PPAR-α and SREBP1c mRNA, which are relevant to hepatic steatosis, in the two groups of FLS-*ob/ob* mice (Table 2). Expression levels of serum PPAR-α mRNA were significantly decreased in the ipragliflozin group compared with the control group ($P < 0.001$) and those of SREBP1c were not different between the two groups.

Effects of ipragliflozin on liver fibrosis
The impact of ipragliflozin treatment on liver fibrosis in FLS-*ob/ob* mice was examined in the control and ipragliflozin groups. Sirius red staining revealed that area of liver fibrosis was significantly decreased in the ipragliflozin group compared with the control group ($P < 0.001$; Figs. 2A–C). The anti-fibrotic effect of ipragliflozin on the liver was substantiated by the result that hepatic mRNA levels of procollagen-1 tended to be

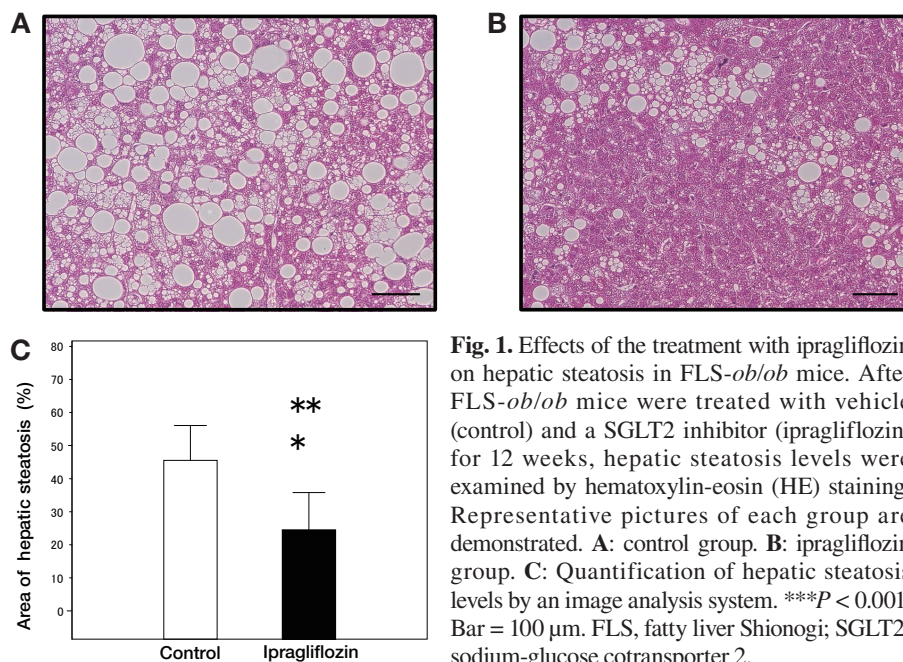suppressed in the ipragliflozin group compared with the control group (Table 2).

The molecular mechanisms involved in the anti-fibrotic effects of ipragliflozin were investigated at the mRNA levels of TGF-β1 and TIMP-1. Hepatic expression levels of these molecules were not significantly modified by the treatment of ipragliflozin (Table 2).

## Effects of ipragliflozin on hepatic infiltration of macrophage

Because anti-inflammatory actions of SGLT2 inhibitors at the kidney level have been reported,[9] we evaluated whether ipragliflozin has impact on the infiltration of macrophage in the liver. The number of positive cells with F4/80, a representative marker of macrophage (Kupffer cells), was examined in FLS-*ob*/*ob* mice (Figs. 3A–C). The number of F4/80-positive cells did not show a significant difference between the control and ipragliflozin groups, although hepatic mRNA level of TNF-α, a pro-inflammatory cytokine, was significantly increased in the ipragliflozin group compared with the control group (Table 2).

Fig. 1. Effects of the treatment with ipragliflozin on hepatic steatosis in FLS-*ob*/*ob* mice. After FLS-*ob*/*ob* mice were treated with vehicle (control) and a SGLT2 inhibitor (ipragliflozin) for 12 weeks, hepatic steatosis levels were examined by hematoxylin-eosin (HE) staining. Representative pictures of each group are demonstrated. **A**: control group. **B**: ipragliflozin group. **C**: Quantification of hepatic steatosis levels by an image analysis system. ***$P < 0.001$. Bar = 100 μm. FLS, fatty liver Shionogi; SGLT2, sodium-glucose cotransporter 2.
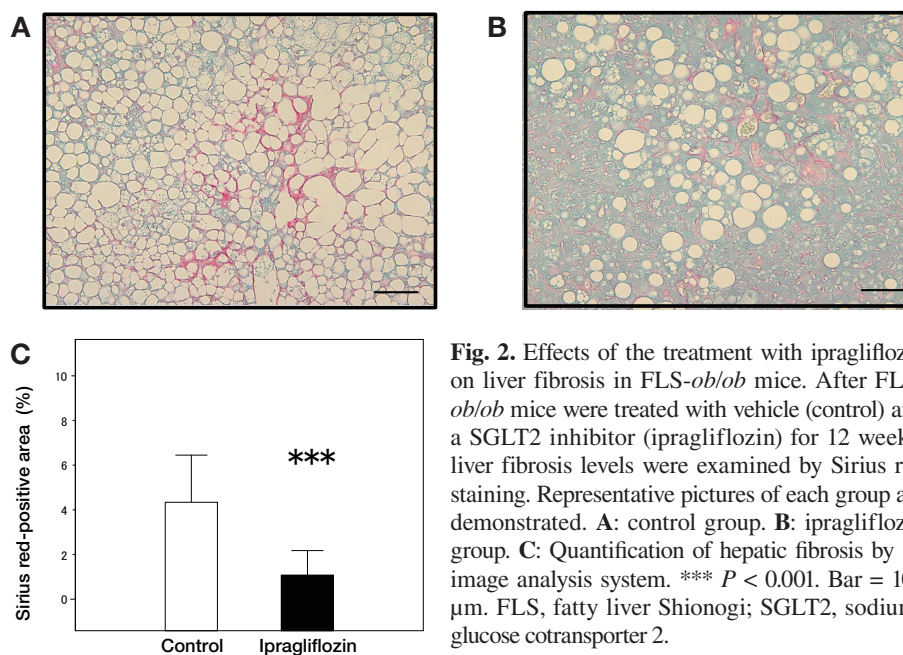
Fig. 2. Effects of the treatment with ipragliflozin on liver fibrosis in FLS-*ob*/*ob* mice. After FLS-*ob*/*ob* mice were treated with vehicle (control) and a SGLT2 inhibitor (ipragliflozin) for 12 weeks, liver fibrosis levels were examined by Sirius red staining. Representative pictures of each group are demonstrated. **A**: control group. **B**: ipragliflozin group. **C**: Quantification of hepatic fibrosis by an image analysis system. *** $P < 0.001$. Bar = 100 μm. FLS, fatty liver Shionogi; SGLT2, sodium-glucose cotransporter 2.

Effects of ipragliflozin on oxidative stress and endoplasmic reticulum stress

Finally, we investigated the involvement of oxidative stress and endoplasmic reticulum (ER) stress in the inhibitory effect by ipragliflozin on liver steatosis and fibrosis in FLS-*ob*/*ob* mice. Since 8-OHdG has been reported to be a hallmark of oxidative stress,[10] expression levels of 8-OHdG in the liver was compared between the control and ipragliflozin groups (Figs. 4A–C). The number of 8-OHdG-positive cells was not significantly changed by the treatment with ipragliflozin. As representative ER stress markers, we measured hepatic mRNA levels of ATF3, CHOP, C-JUN and NUPR1 (Table 2). Any of these ER stress markers were not changed by the treatment with ipragliflozin.

DISCUSSION

We examined the effects of ipragliflozin, a SGLT2 inhibitor, on the metabolic factors and the liver in FLS-*ob*/*ob* mice, a mouse model of NASH. We found that

the body weight of FLS-*ob/ob* mice was significantly decreased by the treatment with ipragliflozin. This is consistent with the data in a human study.[11] We also observed that hepatic cholesterol and triglyceride levels were significantly decreased and serum cholesterol and triglyceride levels were increased after the treatment with ipragliflozin, which is in concordance with a previous report.[12] We speculate that uptake of serum cholesterol was decreased by the liver by ipragliflozin, because empagliflozin, another SGLT2 inhibitor, has been demonstrated to reduce hepatic expression levels of low-density lipoprotein cholesterol (LDL)-receptor in hamsters.[13]

It is contrary to previous studies in mice and human[5–7, 11] that serum ALT levels did not decrease after the treatment with ipragliflozin. This discrepancy may derive from the differences in the experimental conditions.

We have also demonstrated that the treatment with ipragliflozin ameliorated liver fibrosis in FLS-*ob/ob* mice. Although we were not able to identify the molecular mechanisms involved in the anti-fibrotic effects of ipragliflozin, the action appears to be mediated in a manner independent of TGF-β1 and TIMP-1.

We found that hepatic steatosis levels in FLS-*ob/ob* mice were significantly ameliorated by the treatment with ipragliflozin in concordance with previous reports.[5, 6, 11] Since PPAR-α regulates the catabolism of fatty acids in the liver by inducing several proteins including fatty acid transport protein (FATP) and long-chain fatty acid acetyl-CoA synthase (ACS)[14] and then benefits hepatic steatosis, it was unexpected that expression levels of PPAR-α mRNA were significantly inhibited by the
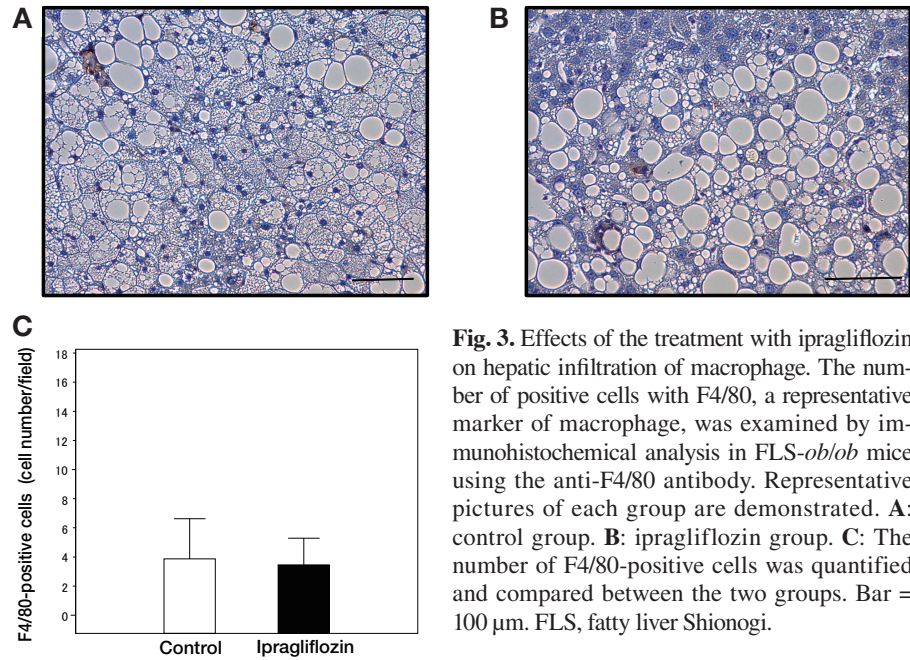
Fig. 3. Effects of the treatment with ipragliflozin on hepatic infiltration of macrophage. The number of positive cells with F4/80, a representative marker of macrophage, was examined by immunohistochemical analysis in FLS-*ob/ob* mice using the anti-F4/80 antibody. Representative pictures of each group are demonstrated. **A**: control group. **B**: ipragliflozin group. **C**: The number of F4/80-positive cells was quantified and compared between the two groups. Bar = 100 μm. FLS, fatty liver Shionogi.
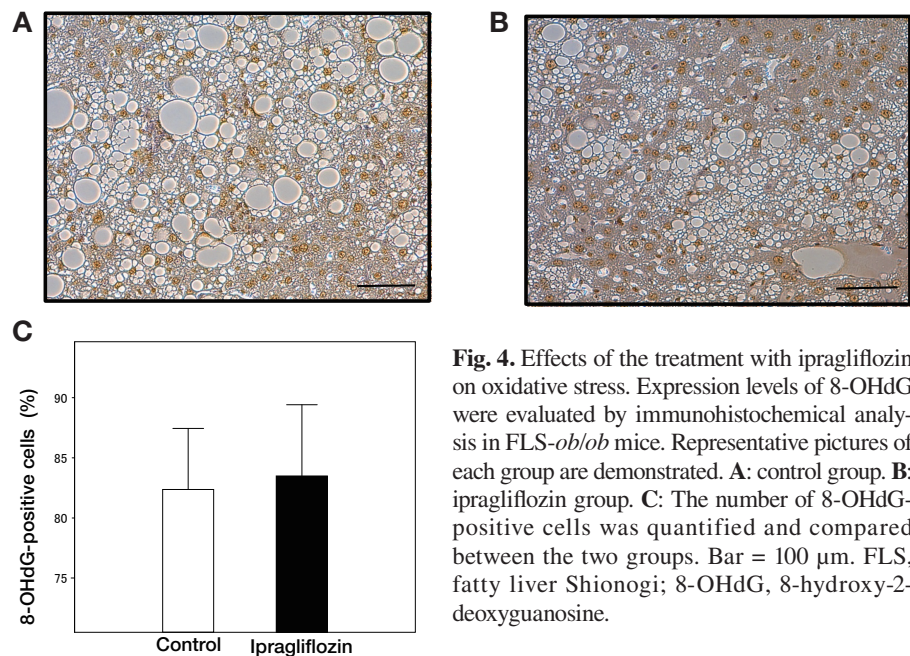
Fig. 4. Effects of the treatment with ipragliflozin on oxidative stress. Expression levels of 8-OHdG were evaluated by immunohistochemical analysis in FLS-*ob/ob* mice. Representative pictures of each group are demonstrated. **A**: control group. **B**: ipragliflozin group. **C**: The number of 8-OHdG-positive cells was quantified and compared between the two groups. Bar = 100 μm. FLS, fatty liver Shionogi; 8-OHdG, 8-hydroxy-2-deoxyguanosine.

treatment with ipragliflozin. This result may be because that fatty acids and fatty acid derivatives which serve as PPAR-α ligands[15] were suppressed by ipragliflozin.

This study includes some limitations. One is that we were not able to clarify the mechanisms by which ipragliflozin improved hepatic fatty change and fibrosis. The other limitation is that we were not able to explore whether ipragliflozin could inhibit liver carcinogenesis in this NASH mouse model.

Finally, we explored the mechanisms of the benefitial

effects of ipragliflozin on the hepatic steatosis in FLS-*ob/ob* mice. Our data suggest that inflammation, oxidative stress and ER stress are not involved in the action. Although we were not able to clarify the precise mechanisms, our work shows that ipragliflozin could be a novel drug for the treatment of hepatic steatosis and fibrosis in patients with NASH.

The authors declare no conflict of interest.

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