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# Effects of bezafibrate in nonalcoholic steatohepatitis model mice with monosodium glutamate-induced metabolic syndrome

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ABSTRACT

Recently, we reported that monosodium glutamate-treated mice (MSG mice) developed severe hyperlipidemia and diabetes mellitus and several complications of obesity. MSG mice acquired fatty livers and subsequently underwent changes that are characteristic of nonalcoholic fatty liver disease (NAFLD)/nonalcoholic steatohepatitis (NASH). In the present study, the effects of bezafibrate on obesity, diabetes mellitus, and NAFLD/NASH were examined in MSG mice. A single dose of MSG (4 mg/g) was administered subcutaneously to neonatal male mice within 24 h of birth. Bezafibrate was mixed into the normal feed for 8 weeks. The weight and body mass index of MSG mice increased significantly despite the unchanged intake of food. Triglyceride and total cholesterol levels in blood, visceral adipose tissue, and interscapular adipose tissue rose significantly. In the livers of MSG mice, moderate centrilobular microvesicular steatosis, ballooning degeneration with Mallory bodies, and scattered infiltration of neutrophils and lymphocytes were observed. Centrilobular hepatocytes were 4-hydroxynonenal-positive in MSG mice. Bezafibrate ameliorated the severity of diabetes mellitus, hyperinsulinemia, and hyperlipidemia. Adiponectin and leptin concentrations in blood improved, and the accumulation of visceral fat was inhibited. The expression of acyl-CoA oxidase, a beta-oxidation gene, and carnitine palmitoyl transferase, which regulates lipid metabolism, increased markedly on administration of bezafibrate. The liver pathology in MSG mice also improved with bezafibrate; specifically, macro- and microvesicles in hepatocytes nearly disappeared, and NAFLD activity score improved. It is concluded that bezafibrate inhibits the accumulation of visceral fat, following amelioration of hyperlipidemia, in MSG-induced obese mice, due to improvements in diabetes mellitus, fatty liver, and NAFLD.



## 1. Introduction

Recently, the prevalence of lifestyle-related diseases, such as diabetes mellitus, hyperlipidemia, and hypertension, has increased due to rises in obesity and stress and the lack of exercise; the number of nonalcoholic fatty liver disease (NAFLD)/nonalcoholic steatohepatitis (Ludwig et al., 1980) (NASH) patients has also risen annually (Kim and Younossi, 2008). NASH is induced by infiltration of inflammatory cells caused by second hits such as oxidation stress, followed by NAFLD. NAFLD is a phenotype of the liver in metabolic syndrome (Grundy et al., 2005; Marchesini et al., 2003; Parekh and Anania, 2007).

Metabolic syndrome begins with the accumulation of visceral fat, and the accompanying insulin resistance depends primarily on quantitative changes in adipocytokine levels in enlarged adipocytes (Matsuzawa, 2006). Additionally, onset of NASH is closely related to the liver cirrhosis and a hepatic tumor, and adiponectin inhibits liver fibrosis (Kamada et al., 2003). NASH differs from fatty livers and is a progressive disease.

Improvements in obesity, insulin resistance, and fatty liver and the control of oxidative stress are critical steps to treating NASH (Koruk et al., 2004; Schreuder et al., 2008). In many animal models, diabetes mellitus, hyperlipidemia, and obesity can be induced by drugs or genetic manipulation (Li et al., 2003; Mathews and Leiter, 2005), and there are methionine and choline deficiency-induced NASH animal models (Fan and Qiao, 2009). Yet, no animal has been identified in which the many symptoms of human metabolic syndrome develop concomitantly and the effects of the medicine are evaluated with a part of the symptom.

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Therefore, a suitable animal model is needed in which the effects of an existing therapy on NASH can be examined.

Monosodium glutamate (MSG) is a widely used food seasoning. In a previous study, we observed that a single administration of MSG in newborn mice induces severe hyperlipidemia and diabetes mellitus and several complications of obesity in adulthood (Sasaki et al., 2009). Specifically, NASH develops, following obesity and fatty liver, and the ensuing histological changes in the liver are very similar to those in human metabolic syndrome (Nagata et al., 2006; Nakanishi et al., 2008; Sasaki et al., 2009).

The basal metabolism of MSG mice can decrease by hypofunction of autonomic nerves and brown adipose tissue (Bergen et al., 1998; Tsukahara et al., 1998; Yasuda et al., 2004). The cause of obesity in the MSG mouse mirrors the lack of physical activity and the development of metabolic disorder in humans, rendering it a useful model of the metabolic syndrome and NASH.

Bezafibrate, a PPARα agonist, is a lipid-lowering drug that can improve diabetes mellitus and fatty liver following hyperlipidemia; it also ameliorates NASH (Nagasawa et al., 2006; Nakano et al., 2007). It has been hypothesized that it does so via upregulation of mRNAs of acyl-CoA oxidase and carnitine palmitoyl transferase which activates fatty acid oxidation (Nagasawa et al., 2006). In this study we characterized the livers of MSG mice (a novel NASH animal model) and determined the effects of bezafibrate on several metabolic factors indicating obesity, diabetes mellitus and NAFLD.

## 2. Materials and methods

### 2.1. Chemicals

Bezafibrate, a dry powdered extract, was supplied by Kissei Pharmaceutical Co., Ltd. (Matsumoto, Japan). Bezafibrate 0.1% or 0.3% was mixed into the Powder Feed MF (Oriental Yeast Co., Ltd., Tokyo, Japan).

### 2.2. Animals and experimental design

Male ICR-MSG and normal mice, aged 11 weeks (30 and 10 animals, respectively), were generated as follows. Pregnant Crlj:CD-1(ICR) mice (12 gestational days) were purchased from Charles River Laboratories Japan Inc. MSG (4 mg/g, Wako Pure Chemical Industries, Ltd., Tokyo, Japan) or physiological saline (Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan) was administered subcutaneously as a single dose to the resulting male neonates within 24 h of birth. MSG- and saline-treated mice (normal) were weaned at age 3 weeks. The animals were housed in a dedicated animal room under controlled conditions (temperature: 23 ± 2 °C, humidity: 50 ± 10%, lighting hours: 7:00–19:00). After 1 week of acclimation, they were given Powder Feed MF and water ad libitum. At age 12 weeks, bezafibrate groups were given MF based feed that contained 0.1% or 0.3% bezafibrate ad libitum for 8 weeks. This study was performed in accordance with the animal experimentation guidelines of Musashino University.

### 2.3. Growth curve and body mass index (BMI)

To generate the growth curve, the weight of the mice was measured once per week. Body weight and length (from the nose to anus) were measured at 8 weeks. BMI was calculated as body weight (g)/body length$^2$ (cm).

### 2.4. Food intake

Food intake for each cage was determined at 0 and 4 weeks after administration of bezafibrate for 3 days, and the mean daily food intake per mouse (g/day/mouse) was calculated after deducting the food that had spilled.

### 2.5. Urinary glucose

All mice were forced to pass urine at 0, 2, 4, 6, and 8 weeks after administration of bezafibrate, and the urine was examined for the presence of glucose using Ames Urinalysis Test Paper (Lab Sticks, Bayer Medical Ltd., Tokyo, Japan). The rate of the appearance of urinary glucose was calculated.

### 2.6. Glucose tolerance test

The glucose tolerance test was performed in all groups at 7 weeks after administration of bezafibrate (age 19 weeks). After the mice fasted overnight, 2 g/kg glucose was injected into the peritoneal cavity, and blood samples were collected from the orbital cavity venous plexus using a capillary tube at 0, 30, 60, and 120 min. Plasma glucose levels were measured, per the following section.

### 2.7. Blood biochemistry

At 8 weeks (age 20 weeks), blood samples were obtained from the orbital cavity venous plexus under nonfasting conditions and centrifuged to generate plasma. Plasma glucose, total cholesterol and triglyceride concentrations were determined using commercial kit (Wako Pure Chemical Industries, Ltd., Tokyo, Japan) (Sasaki et al., 2009). Plasma insulin concentrations were measured using commercial kit (Shibayagi Co., Ltd., Gunma, Japan) (Sasaki et al., 2009). Plasma adiponectin concentrations were measured using commercial kit (R&D Systems, Inc., USA) (Sasaki et al., 2009). Plasma leptin concentrations were determined using the Mouse Leptin ELISA kit (AKRLP-011, Shibayagi, Gunma, Japan). Plasma free fatty acid concentrations were determined with the NEFA C-test Wako (Wako Pure Chemical Industries, Ltd., Tokyo, Japan) kit. Plasma aspartate amino transferase (AST) and alanine amino transferase (ALT) concentrations were measured using the Transaminase CII-test Wako (Wako Pure Chemical Industries, Ltd., Tokyo, Japan) kit.

### 2.8. Weights of visceral fat, subcutaneous fat, and brown adipose tissue

At 6 weeks (age 18 weeks), each mouse was anesthetized with nembutal (50 mg/kg, i.p.), and abdominal and subcutaneous fat were measured by X-ray computerized tomography (CT) (La Theta, Aloka Co., Ltd., Tokyo, Japan). The amounts of visceral and subcutaneous fat were calculated at individual cross-sites by scanning from xiphisternum to sacrum at 1.5-mm intervals (nos. 1–10, Fig. 5A).

Mice were sacrificed at 8 weeks (age 20 weeks), and the mesenteric adipose tissue as visceral adipose tissue, interscapular adipose tissue, pancreas, and liver were removed. Peritesticular adipose tissues were excluded from calculations of visceral adipose tissue weight. For interscapular adipose tissue weight calculations, brown adipose tissue and surrounding white adipose tissue were included.

### 2.9. Triglyceride and cholesterol levels in liver

The fat content in the liver was extracted as described by Freedman et al. (Freedman et al., 2005). Briefly, mouse livers were homogenized in 1 M NaCl. Liver tissue homogenates were extracted with chloroform/methanol (2:1) and 1 M NaCl. The organic phase was collected, dried, and resuspended in Triton X-100/methanol (2:1). Triglyceride and cholesterol levels were measured using biochemical test kits (Wako Pure Chemical Industries, Ltd., Tokyo, Japan).

### 2.10. mRNA levels by real-time quantitative RT-PCR

Total liver RNA was isolated using the RNeasy Mini Kit (QIAGEN, Tokyo, Japan) according to the manufacturer's instructions. Total RNA

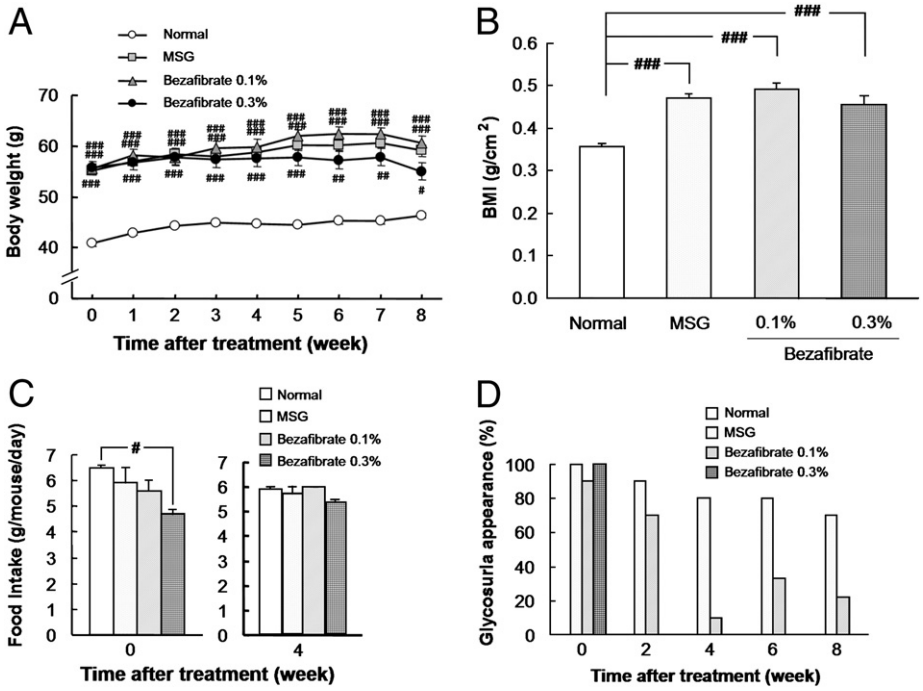


**Fig. 1.** Effects of bezafibrate on body weight, body mass index, food intake, and glycosuria in male MSG mice. Body weight (A) and body mass index (BMI) in MSG mice (B) were significantly higher than in normal mice. Food intake in the bezafibrate 0.3% group decreased compared with the normal group at 0 weeks (C). Glycosuria in the MSG group increased compared with the normal group at 0 weeks. Data are means ± S.E.M. (n = 9–10/group). #P<0.05, ##P<0.01, and ###P<0.001, vs. normal group.

(1.0 μg) was reverse-transcribed in a 20-μL reaction using the QuantiTect® Reverse Transcription kit (QIAGEN, Tokyo, Japan) according to the manufacturer's protocol.

Gene expression in livers were quantified by real-time PCR (Mini-opticon™ system, Bio-Rad laboratories, Inc, Tokyo, Japan). PCR reactions were prepared using the QuantiTect SYBR Green PCR kit (QIAGEN, Tokyo, Japan) in 25 μL. The cycling parameters were 15 min at 95 °C and 40 cycles of 95 °C (15 s), 60 °C (30 s), and 72 °C (30 s), followed by melting curve analysis.

The following primers were used:acyl-CoA oxidase (ACO), AF006688 forward 5′-TCTTCTTGAGACAGGGCCCAG-3′, reverse 5′-GTTCCGACTAGCCAGGCATG-3′; carnitine palmitoyl transferase 1(CPT1), NM_013495.2 forward 5′-CTTCCAAGGCAGAAGAGTGGG-3′, reverse 5′-GAACCTTGGCTGCGGTAAGAC-3′; phosphoenolpyruvate carboxykinase (PEPCK), NM_011044.2| forward 5′- GCTCCTGGCACCTCAGTGAA-3′, reverse 5′-ACGTTGGTGAAGATGGTGTTTT-3′; proliferator-activated receptor α (PPARα), NM_011144.5 forward 5′-ATTTGCTGTGGAGATCGGCC-3′, reverse 5′-TGGTTGCTCTGCAGGTGGAG-3′; multidrug resistance-associated protein 2 (MRP2), NM_013806.2| forward 5′-TGTTGGGCTTATGGTTCTCC-3′, reverse 5′-CGAATGCTGTTCACTTGCTC; β-actin, NM_007393.3 forward 5′-CCATCCTGCGTCTGGACCTG-3′, reverse 5′-TTCCCTCTCAGCTGTGGTGG-3′.

Gene expression by real-time PCR was calculated relative to β-actin.

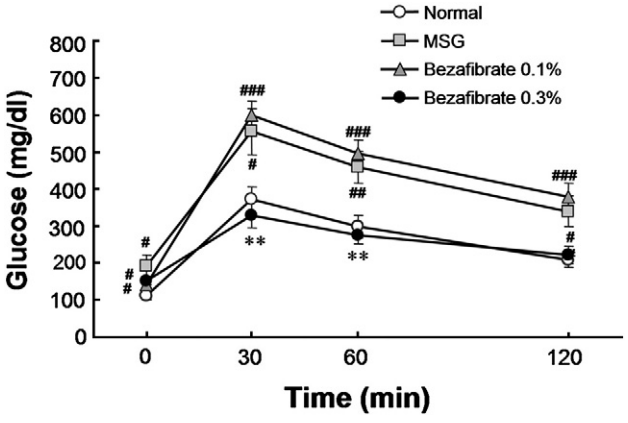


**Fig. 2.** Effects of bezafibrate on glucose tolerance test in male MSG mice at 7 weeks after treatment. Plasma glucose levels following intraperitoneal glucose injection (time 0) in fasted mice. Plasma glucose levels at 30 min after glucose loading (0 and 30 min, #: P<0.05) and at 120 min (60 min, ##: P<0.01, 120 min, #: P<0.05). The bezafibrate 0.3% group showed a significant decrease compared with the MSG group (30 and 60 min, **: P<0.01). Data are means ± S.E.M. (n = 9–10/group). #P<0.05, ##P<0.01, and ###P<0.001 vs. normal group. **P<0.01 vs. MSG group.

### 2.11. Histopathological examination

The visceral adipose tissue, interscapular adipose tissue, pancreas, and liver of all groups were fixed with 10% phosphate-buffered formalin solution and examined by light microscopy after HE staining. The degree of histopathology was scored 0 for normal, 1 for slight, 2 for moderate, and 3 for severe. Liver histology was scored using the system that was proposed by the NASH Clinical Research Network (Kleiner et al., 2005), based on 4 semiquantitative items—steatosis (0–2), lobular inflammation (0–1), hepatocellular ballooning (0–2), and fibrosis (0–1). Three representative areas were scored in each section, and average values were used for scoring. The sum of steatosis, lobular inflammation, and hepatocellular ballooning scores constituted the NAFLD activity score.

For immunohistochemical analysis, deparaffinized liver specimens were incubated with rabbit polyclonal antibodies against SQSTM1/A170/p62 (Wako Chemicals USA, Inc., Richmond, USA) and 4-hydroxynonenal (4-HNE, OXIS International, Foster City, CA) to detect Mallory bodies and oxidative stress, respectively. Control normal rabbit serum was used as a negative control. Envision-peroxidase was used as the second antibody (DAKO, Glostrup, Denmark), and 3,3′diaminobenzidine (DAB) was used as the substrate.

**Table 1**
Effects of bezafibrate treatment on blood chemistry, hepatic lipid contents and liver mRNA levels in male MSG.

| Group | Normal (N = 10) | MSG (N = 10) | Bezafibrate 0.1% (N = 9) | Bezafibrate 0.3% (N = 10) |
|---|---|---|---|---|
| Glucose (mg/dl) | 176.6 ± 2.6 | 418.0 ± 64.1[a] | 251.1 ± 21.5[b,c] | 173.9 ± 7.7[d] |
| Insulin (ng/ml) | 3.9 ± 1.0 | 103.8 ± 29.0[a] | 113.1 ± 37.3[a] | 14.9 ± 5.3 |
| Adiponectin (μg/ml) | 3.70 ± 0.11 | 3.25 ± 0.23[e] | 3.45 ± 0.18 | 3.95 ± 0.17[c] |
| Leptin (ng/dl) | 1.23 ± 0.43 | 9.33 ± 1.53[b] | 13.47 ± 1.99[b] | 6.04 ± 1.49 |
| Triglyceride (mg/dl) | 131.0 ± 16.2 | 203.0 ± 28.5[e] | 176.1 ± 25.2 | 120.9 ± 10.9[c] |
| Total cholesterol (mg/dl) | 105.3 ± 3.9 | 151.2 ± 6.0[a] | 161.7 ± 6.5[b,c] | 107.2 ± 5.1[c] |
| Free fat acid (mEq/l) | 1.24 ± 0.16 | 1.52 ± 0.13 | 1.50 ± 0.15 | 1.12 ± 0.09 |
| AST (Karmen U/dl) | 59.8 ± 7.7 | 87.8 ± 7.9[e] | 90.8 ± 13.2 | 153.6 ± 35.6[b] |
| ALT (Karmen U/dl) | 18.8 ± 2.8 | 52.1 ± 7.5[b] | 63.1 ± 12.8 | 87.8 ± 18.8[a] |
| Relative liver weight (g/kg body weight) | 52.6 ± 1.1 | 70.7 ± 4.0[b] | 90.4 ± 1.9[b,f] | 119.6 ± 5.0 [e,d] |
| Cholesterol content (mg/g liver) | 3.11 ± 0.73 | 5.24 ± 0.89 | 5.90 ± 0.93[e] | 2.91 ± 0.66 |
| Triglyceride content (mg/g liver) | 15.1 ± 5.3 | 39.9 ± 3.9[a] | 44.6 ± 7.3[a] | 22.1 ± 5.1 |
| Liver mRNA level | | | | |
| CPT1 | 96 ± 12 | 100 ± 6 | 152 ± 23 | 241 ± 55[c,e] |
| ACO | 81 ± 10 | 100 ± 10 | 352 ± 46[b,f] | 545 ± 71[b,d] |
| PEPCK | 116 ± 14 | 100 ± 13 | 54 ± 6[a,c] | 52 ± 9[b,f] |
| PPARα | 78 ± 6 | 100 ± 7[e] | 109 ± 12[b] | 87 ± 8 |
| MRP2 | 102 ± 10 | 100 ± 14 | 134 ± 22 | 185 ± 38[c,e] |

AST: aspartate amino transferase, ALT: alanine amino transferase, CPT1: carnitine palmitoyl transferase 1, ACO: acyl-CoA oxidase, PEPCK: phosphoenolpyruvate carboxykinase, PPARα: peroxisome proliferator-activated receptor α, MRP2: multidrug resistance-associated protein 2. Data are presented as means ± S.E.M.
[a] $P<0.01$ vs. normal.
[b] $P<0.001$ vs. normal.
[c] $P<0.05$ vs. MSG.
[d] $P<0.001$ vs. MSG.
[e] $P<0.05$ vs. normal.
[f] $P<0.01$ vs. MSG.

### 2.12. Statistical analysis

All the values, except those of histopathological findings and urinary glucose are expressed as mean ± S.E.M. The statistical significance was evaluated by student's t-test between the groups of normal and MSG mice. For multiple comparisons, post-hoc analysis was performed with Tukey's test between the normal or MSG mice and each bezafibrate group. For the histological findings, Mann–Whitney test was performed between the normal and MSG mice. Steel–Dwass test was performed between the normal or MSG mice and each bezafibrate group. The significance level for each statistical analysis was accepted at $P<0.05$.

## 3. Results

### 3.1. Growth parameters and food intake

The weight of MSG mice was significantly higher compared with normal mice ($P<0.001$). No significant difference was observed between the MSG and bezafibrate groups (Fig. 1A). The BMI of the MSG mice was significantly greater than that of normal mice ($P<0.001$); there was no significant difference between the MSG and bezafibrate groups (Fig. 1B). Food intake decreased in the 0.3% bezafibrate group compared with normal group at 0 weeks ($P<0.05$) (Fig. 1C).

### 3.2. Glycosuria

Glycosuria increased in the MSG group compared with the normal group on day 1 of the experiment (0 weeks). Bezafibrate (0.1 and 0.3%) inhibited MSG-induced glycosuria in a dose-dependent manner. Specifically, glycosuria disappeared in the bezafibrate 0.3% group at age 2 weeks (Fig. 1D).

### 3.3. Glucose tolerance test

Plasma glucose levels were significantly higher in MSG mice than in normal mice at 30 min after glucose loading (0 and 30 min: $P<0.05$) and remained elevated at 120 min (60 min: $P<0.01$, 120 min: $P<0.05$). The bezafibrate 0.3% group experienced a significant decrease in plasma glucose compared with the MSG group (30 and 60 min: $P<0.01$); thus, plasma glucose levels of bezafibrate 0.3% group after glucose loading were comparable to those of normal group (Fig. 2).

### 3.4. Blood biochemistry

Plasma glucose, insulin, leptin, triglyceride, total cholesterol, AST, and ALT concentrations were significantly higher in the MSG group compared with the normal group ($P<0.01$, $P<0.01$, $P<0.001$, $P<0.05$, $P<0.01$, $P<0.05$, and $P<0.001$, respectively) (Table 1), and adiponectin concentrations were lower than in the normal group ($P<0.05$). Bezafibrate (0.3%) ameliorated the MSG-induced increase in plasma glucose ($P<0.001$), adiponectin ($P<0.05$), triglyceride ($P<0.05$), and total cholesterol ($P<0.05$). In the bezafibrate 0.3% group, AST and ALT concentrations increased significantly compared with the normal group ($P<0.05$ and $P<0.01$).

### 3.5. Fat accumulation

Visceral adipose tissue and interscapular adipose tissue weight increased significantly in the MSG group ($P<0.001$, $P<0.001$) compared with the normal group (Fig. 3A). Bezafibrate failed to improve these measures.

By CT, severe visceral fat accumulation was observed in MSG mice, which was inhibited by bezafibrate (0.3%). Specifically, the amounts of visceral fat in CT scanning site nos. 3, 4, and 5 decreased significantly compared with the MSG group ($P<0.001$, $P<0.01$, and $P<0.05$, respectively) (Fig. 3C).

Severe subcutaneous fat accumulation was observed in the neck and abdomen in MSG animals. In the bezafibrate group, subcutaneous fat accumulated less markedly in a dose-dependent manner. In the bezafibrate (0.3%), the amount of the fat (CT scanning site nos. 4, 5, and 6) decreased significantly compared with the MSG group ($P<0.05$) (Fig. 3D).

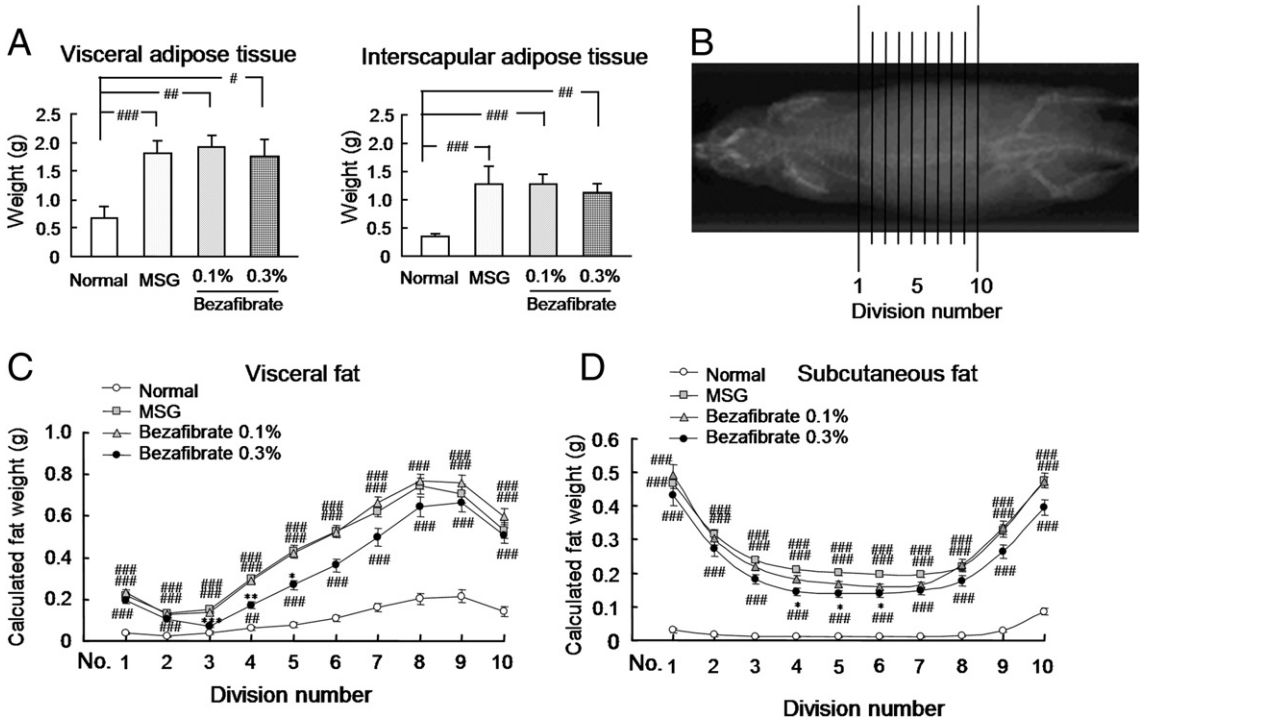


**Fig. 3.** Effects of bezafibrate on weight of adipose tissue, abdominal scanning site, and amount of adipose tissue by X-ray computerized tomography in male MSG mice 6 weeks after treatment. The weights of visceral adipose tissue and interscapular adipose tissue (A). By X-ray computerized tomography (CT) (B), severe accumulation of visceral fat was observed in MSG mice, and the accumulation of fat was inhibited by bezafibrate (C). Severe accumulation of subcutaneous fat was observed around the neck and abdomen in the MSG group. In the bezafibrate group, the accumulation of the subcutaneous fat decreased dose-dependently (D). Data are means ± S.E.M. (n = 9–10/group). ##P<0.01 and ###P<0.001 vs. normal group, *P<0.05, **P<0.01, and ***P<0.001 vs. MSG group.

### 3.6. Hepatic lipid content and liver weight

The relative liver weight in the MSG group was significantly higher than in the normal group (P<0.001), and the weights in bezafibrate (0.1% and 0.3%) significantly increased to a greater extent than MSG group (P<0.01, P<0.001) (Table 1). The hepatic triglyceride (P<0.01) content in the MSG group was higher compared with the normal group, but hepatic cholesterol content did not differ significantly between groups (Table 1).

### 3.7. Hepatic mRNA expression levels

Hepatic mRNA expression levels of CPT1, ACO, PEPCK, and MRP2 were equal in the MSG and normal groups. PPARα expression levels significantly increased in the MSG group (P<0.05) compared with the normal group. In the bezafibrate groups, such levels increased significantly compared with MSG group (CPT1; bezafibrate 0.3% group, P<0.05, ACO; bezafibrate 0.1% group, P<0.01, bezafibrate 0.3% group, P<0.001, and MRP2; bezafibrate 0.3% group, P<0.05) (Table 1). In the bezafibrate groups, PEPCK mRNA levels fell compared with MSG group, respectively (bezafibrate 0.1% group, P<0.05 and bezafibrate 0.3% group P<0.01) (Table 1).

### 3.8. Histopathological examination

In the livers of MSG mice, moderate centrilobular microvesicular steatosis (P<0.01) (Table 2, Fig. 4B, and E), scattered infiltration of neutrophils and lymphocytes, and ballooning degeneration were observed compared with the normal group (P<0.05 and P<0.001) (Table 2, Fig. 4A and D). Fibrotic changes were not prominent. The centrilobular microvesicular steatosis improved dose-dependently on bezafibrate treatment (Table 2, Fig. 4C and F), but the inflammatory

**Table 2**
Score assessment of steatosis, lobular inflammation, hapatocellular ballooning, NAFLD activity score and fibrosis in the liver.

| Score | | Steatosis(0–2) | | Lobular inflammation (0–1) | | Hepatocellular ballooning (0–2) | | NAFLD activity score (0–5) | | | Fibrosis (0-1A) | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 0–1 | 2 | 0 | 1 | 0 | 1–2 | 0–2 | 3–4 | 5 | 0 | 1A |
| Normal | N | 10 | 0 | 10 | 0 | 9 | 1 | 10 | 0 | 0 | 10 | 0 |
| (N = 10) | (%) | (100) | (0) | (100) | (0) | (90) | (10) | (100) | (0) | (0) | (100) | (0) |
| MSG | N | 3 | 7[a] | 6 | 4[b] | 0 | 10[c] | 1 | 7 | 2[c] | 8 | 2 |
| (N = 10) | (%) | (30) | (70) | (60) | (40) | (0) | (100) | (10) | (70) | (20) | (80) | (20) |
| Bezafibrate 0.1% | N | 3 | 6[a] | 7 | 2 | 2 | 7[b] | 3 | 6 | 0[a] | 9 | 0 |
| (N = 9) | (%) | (33.3) | (66.7) | (81.8) | (22.2) | (22.2) | (81.8) | (33.3) | (66.7) | (0) | (100) | (0) |
| Bezafibrate 0.3% | N | 7 | 3 | 6 | 4 | 7 | 3[d] | 7 | 2 | 1[e] | 10 | 0 |
| (N = 10) | (%) | (70) | (30) | (60) | (40) | (70) | (30) | (70) | (20) | (10) | (100) | (0) |

[a] P<0.01 vs. normal.
[b] P<0.05 vs. normal.
[c] P<0.001 vs. normal.
[d] P<0.01 vs. MSG.
[e] P<0.05 vs. MSG.

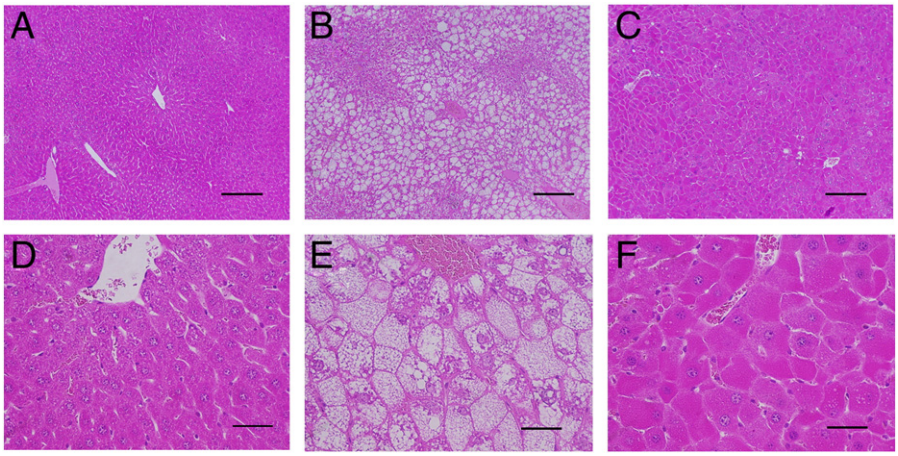


**Fig. 4.** Histopathological features of livers in male MSG- and bezafibrate-treated mice at 8 weeks. In the MSG group, moderate centrilobular microvesicular steatosis (B and E), ballooning degeneration and scattered infiltration of neutrophils and lymphocytes were observed compared with the normal group (A and D). The centrilobular microvesicular steatosis was improved dose-dependently by bezafibrate (C and F). Eosinophilic granular changes in hepatocytes (F) in bezafibrate-treated mouse—not observed in all MSG mice (A). A and D: normal, B and E: MSG, C and F: bezafibrate (0.3%). H&E stain. Bar = 200 (A–C), 50 μm (D–F).

and degenerative changes were unaffected (Table 2). In the bezafibrate 0.3% group, hepatocellular ballooning and NAFLD activity score were improved compared with MSG group ($P<0.01$ and $P<0.05$). Eosinophilic granular changes in hepatocytes (Fig. 4C and F) were observed in bezafibrate-treated mice but not in all MSG mice (Table 3).

Mallory bodies were observed in the MSG (Fig. 5B) and bezafibrate groups (Fig. 5C). These changes in the normal group (Fig. 5A) were not observed. The degree of these changes in MSG and bezafibrate groups was the same. We noted diffuse 4-HNE-positive signals in centrilobular hepatocytes in MSG mice (Fig. 5E) compared with normal animals (Fig. 5D). In contrast, granular positive signals in the bezafibrate group were detected only in the cell membrane in hepatocytes near the bile capillaries (Fig. 5F).

In the pancreas of MSG mice, we observed slight or moderate islet hypertrophy compared with normal animals (Table 3); this pathology was not improved by bezafibrate treatment. In the interscapular brown adipose tissue of MSG mice, atrophy and replacement by white adipocytes were observed versus the normal group (Table 3). In the bezafibrate 0.3% group, the changes in brown adipose tissue improved slightly ($P<0.05$) (Table 3).

**Table 3**
Histopathological findings in liver, pancreas and brown adipose tissue.

| Findings | Groups | No. of animals | Severity[a] | | | |
|---|---|---|---|---|---|---|
| | | | − | + | + + | + + + |
| Centrilobular fatty change in liver | Normal | 10 | 10 | 0 | 0 | 0 |
| | MSG | 10 | 1 | 2 | 7 | 0[b] |
| | Bezafibrate 0.1% | 9 | 2 | 3 | 4 | 0[c] |
| | Bezafibrate 0.3% | 10 | 6 | 3 | 1 | 0[d] |
| Eosinophilic granular change of hepatocytes | Normal | 10 | 10 | 0 | 0 | 0 |
| | MSG | 10 | 10 | 0 | 0 | 0 |
| | Bezafibrate 0.1% | 9 | 6 | 2 | 1 | 0 |
| | Bezafibrate 0.3% | 10 | 1 | 3 | 6 | 0[c,d] |
| Islets hypertrophy in pancreas | Normal | 10 | 10 | 0 | 0 | 0 |
| | MSG | 10 | 4 | 4 | 2 | 0[c] |
| | Bezafibrate 0.1% | 9 | 3 | 4 | 2 | 0[c] |
| | Bezafibrate 0.3% | 10 | 4 | 5 | 1 | 0[e] |
| Atrophy of brown adipose tissue | Normal | 10 | 10 | 0 | 0 | 0 |
| | MSG | 10 | 0 | 1 | 3 | 6[b] |
| | Bezafibrate 0.1% | 9 | 1 | 0 | 2 | 6[b] |
| | Bezafibrate 0.3% | 10 | 4 | 2 | 3 | 1[e,f] |

[a] Each abnormality was divided into three grades of severity from + to + + +, mild, moderate and severe, qualitatively.
[b] $P<0.001$ vs. normal.
[c] $P<0.01$ vs. normal.
[d] $P<0.05$ vs. normal.
[e] $P<0.01$ vs. MSG.
[f] $P<0.05$ vs. MSG.

## 4. Discussion

In a previous study, we found that MSG mice developed obesity at age 2 months and their weight increased by approximately 60 g at age 5 months (Nagata et al., 2006; Sasaki et al., 2009). Glycosuria appeared at age approximately 3 months of age, and insulin resistance developed, as demonstrated by glucose tolerance test. Severe edema was observed in the arcuate nucleus of the hypothalamus within 1 day after administration of MSG in newborn mice; at age 12 months, the edema and degeneration of nerve cells in the hypothalamus reversed completely.

In the present study, the weight and BMI of MSG mice increased significantly compared with normal mice, but food intake was equal. Therefore, the main cause of obesity was not thought to be a disturbance of appetite, suggesting that obesity is caused by a decrease in energy metabolism, secondary to brown adipose tissue (Tsukahara et al., 1998) and autonomic nerve dysfunction (Yasuda et al., 2004). Body weight, BMI, and food intake did not differ significantly between the bezafibrate and MSG groups, and abdominal obesity clearly improved and the amounts of visceral adipose tissue in CT scanning site nos. 3, 4, and 5 decreased. These sites mainly indicate the mesenteric adipose tissue. The rate of appearance of urinary glucose decreased on treatment with bezafibrate dose-dependently, and glucose tolerance improved in the bezafibrate 0.3% group. Mesenteric adipose tissue regulates the secretion of adipocytokines (Kanaya et al., 2004), and the inhibition of fat accumulation by bezafibrate has been suggested to improve insulin resistance and declines in adiponectin (Hiuge et al., 2007). In addition, these scanning sites corresponded to human's abdomen (Tokunaga et al., 1983) and the increase at this site resembles that observed in metabolic syndrome.

Additionally, by histopathology, the atrophy of brown adipose tissue was inhibited, suggesting that bezafibrate ameliorates basal energy expenditures and insulin sensitivity by upregulating $\beta_3$-adrenoreceptor

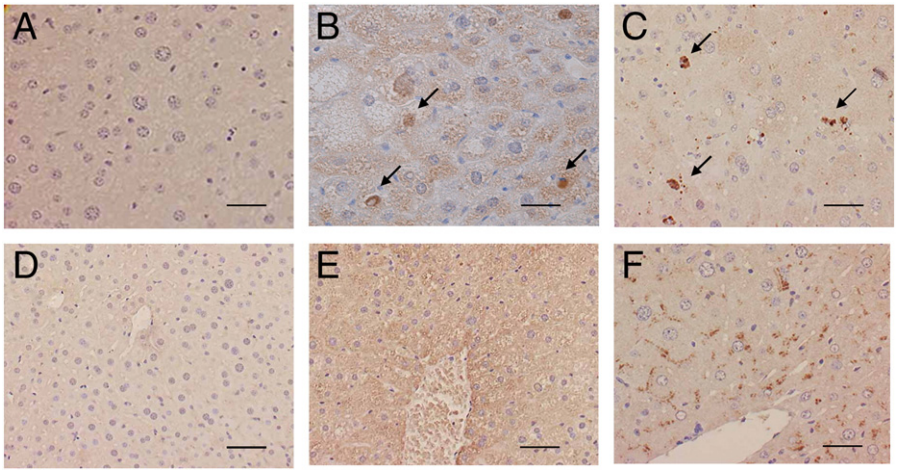


**Fig. 5.** Immunohistological features of livers in male MSG- and bezafibrate-treated mice at 8 weeks. Mallory bodies in the MSG (arrows in B) and bezafibrate groups (arrows in C), compared with the normal group (A). 4-HNE signals in centrilobular hepatocytes in MSG mice (E), compared with normal animals (D). In the bezafibrate group, positive granular signals were detected on the cell membrane of hepatocytes around the bile capillaries (F). A and D: normal, B and E: MSG, C and F: bezafibrate (0.3%). Immunohistochemical reaction of SQSTM1/A170/p62 to detect Mallory bodies (A–C). 4-HNE to detect oxidative stress (D–F). Bar = 50 μm.

(Inokuma et al., 2006). Therefore, we suspect that bezafibrate impedes the accumulation of visceral fat and, as a result, improves MSG-induced insulin resistance.

Cholesterol, triglyceride, and free fat acid levels increased in the blood and liver of MSG mice. Bezafibrate inhibits very low-density lipoprotein, following activation of PPARα, inhibition of free fatty acid composition, and promotion of beta-oxidation (Goldenberg et al., 2008). In this study, bezafibrate decreased PEPCK mRNA, cholesterol, triglycerides, and free fatty acid in blood and liver. ACO, a beta-oxidation gene, and CPT1, which regulates lipid metabolism, were up-regulated by bezafibrate, suggesting that energy metabolism by adipose tissue increased (Pickavance et al., 2005). However, in this condition bezafibrate did not significantly increase PPARα mRNA.

This cause is uncertain, but the swelling of hepatocytes that related to the increase of peroxisome was observed, suggesting that PPARα played a role in the decrease of triglyceride in blood. In liver, increases in relative weight, AST and ALT levels in blood, and PPARα mRNA expression were observed in MSG mice. All livers in the MSG group were yellow and severely swollen. In these mice, centrilobular microvesicular steatosis and Mallory bodies were observed, based on assessment of the livers. Cholesterol and triglyceride levels in the liver increased compared with the normal group. In addition, we noted infiltration of neutrophils, single-cell necrosis and increase of mitosis. Therefore, we equated the condition of 20-week-old MSG mice to the early stages of NASH (Diehl, 2005).

Cholesterol and triglyceride levels in the liver in the bezafibrate 0.3% group decreased compared with the MSG group, and the indices of pathological changes without inflammation also improved. Specifically, macrovesicles in the hepatocytes nearly vanished. Therefore, we reasoned that the ameliorative effect of bezafibrate on the fatty liver was robust. In contrast, in the bezafibrate group, AST and ALT levels increased and eosinophilic granular change with swelling of hepatocytes deteriorated. Because the peroxisome increased by administration of bezafibrate, these findings were thought to be a specific change in rodent (Gray and de la Iglesia, 1984; Nakajima et al., 2009). Therefore, liver swelling in the bezafibrate 0.3% group is a different pathology from NASH and metabolic syndrome; thus, this parameter cannot be applied to humans directly. However, the rise of the AST and ALT is rarely being reported as a side effect of bezafibrate in human and attention might be needed for use in the liver disease patients (Goldenberg et al., 2008).

In our pathological examination, bezafibrate did not ameliorate the infiltration of neutrophils, suggesting that the development of liver cirrhosis and tumors, following fatty liver, is not inhibited by bezafibrate. Severe fatty liver with Mallory bodies is a main cause of cirrhosis and tumors, as are chronic inflammation and hepatocyte necrosis (Kleiner et al., 2005; Ludwig et al., 1980). These changes are induced by diabetes mellitus and oxidative stress and are known as the NASH-inducing mechanisms (Fan and Qiao, 2009; Kim and Younossi, 2008; Marchesini et al., 2003; Parekh and Anania, 2007; Schreuder et al., 2008).

4-HNE is a specific index of lipid hyperoxidation and a good marker of oxidative stress (Malone and Hernandez, 2007). In this study, diffuse 4-NHE signals increased in centrilobular hepatocytes in MSG mice (Fig. 5E). In the bezafibrate group, however, such signals were detected only in the cell membrane of hepatocytes around bile capillaries (Fig. 5F). 4-HNE is conjugated by glutathione, and this complex is excreted in bile by multidrug resistance-associated protein 2 (MRP2) (Ji et al., 2002, 2004; Reichard et al., 2003). Further, bezafibrate enhances bile secretion by activating MRP2 (Nishioka et al., 2005; Yamazaki et al., 2005).

In this study, although MRP2 mRNA levels increased in the bezafibrate group, the 4-HNE that was detected might be glutathione conjugates of 4-HNE; thus, we reasoned that the increased 4-HNE signals in the bezafibrate group reflected the secretory processes (Cheng et al., 2005) of oxidative stress. Further, we hypothesized that bezafibrate decreases oxidative stress and heals injured hepatocytes. In addition, by histopathology, the fatty liver and NAFLD activity score improved markedly on bezafibrate treatment, suggesting that bezafibrate inhibits the progression of NAFLD. However, bezafibrate did not clearly ameliorate the inflammation in the liver, suggesting that the development of NASH, liver cirrhosis and tumors, following fatty liver, might not be inhibited by bezafibrate.

## 5. Conclusion

Bezafibrate inhibits the accumulation of visceral fat, following amelioration of hyperlipidemia, in MSG-induced obese mice, due to improvements in diabetes mellitus and NAFLD. However, bezafibrate did not ameliorate the inflammation in the liver, suggesting that the development of NASH, liver cirrhosis and tumors, following fatty liver, might not be inhibited by bezafibrate.

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