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Original Article

Benefits of combination low-dose pioglitazone plus fish oil on aged type 2 diabetes mice

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ABSTRACT

The elderly patients with type 2 diabetes suffer more adverse drug events than young adults due to pharmacokinetic and pharmacodynamic changes associated with aging. Reducing the risks of these medication-related problems are equally important for the clinical care of older type 2 diabetes patients. Pioglitazone is used for treating type 2 diabetes as an oral antidiabetic drug. Despite pioglitazone is used helpful insulin sensitizers, the accumulation of subcutaneous fat is considered a major adverse effect of pioglitazone therapy. We investigated to reduce the adverse effect of pioglitazone by combination with fish oil rich in eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in aged diabetic KK mice. The accumulation of subcutaneous fat associated with high-dose pioglitazone is reduced by fish oil, suppressing lipogenesis and stimulating fatty acid β-oxidation in the liver. Our data suggest that adding fish oil to low-dose pioglitazone results in antidiabetic efficacy similar to that of the high-dose without concomitant body weight gain.

1. Introduction

Multiple factors associated with aging promote glucose intolerance and insulin resistance (IR), such as accumulation of visceral and peripheral fat tissues, reduction of skeletal muscle that plays a role in glucose uptake and fatty acid β-oxidation, alteration of insulin secretion, and dysregulation of insulin signaling pathways [1–4]. Type 2 diabetes in older adults is also strongly associated with debilitating and fatal complications like retinopathy, nephropathy, neuropathy, coronary heart disease, and cerebrovascular disease [5]. Improving IR in elderly patients with type 2 diabetes is vital for preventing these complications, thereby maintaining quality of life and reducing the public healthcare burden.

In addition to higher type 2 diabetes and associated morbidity risks, older people suffer more adverse drug events than young adults due to pharmacokinetic and pharmacodynamic changes associated with aging [6]. Elderly type 2 diabetes patients are at greater risk not only of acute adverse

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events from oral antidiabetic drugs such as hypoglycemia, but also chronic impairments like dementia [7]. Therefore, strategies for reducing the risks of these medication-related problems are equally important for the clinical care of older type 2 diabetes patients.

Thiazolidinediones (TZDs) are selective peroxisome proliferator-activated receptor (PPAR)γ agonists used for treating type 2 diabetes. TZDs reduce IR in peripheral tissues without stimulating insulin secretion, thereby protecting against hypoglycemia and preserving pancreatic β-cell function [8,9]. Although TZDs are efficacious for reducing IR in various rodent models of diabetes and in type 2 diabetes patients [10–14], use by older adults may increase risks of body weight gain, heart failure, bone fracture, and bladder cancer [15]. Furthermore, risks of adverse events from TZDs increase with dose [16–18], suggesting that adjunct treatments allowing for reduced TZD doses could help minimize adverse effects in older patients.

We have also researched into ways of the combination treatment for obesity and lifestyle-related disease, such as type 2 diabetes and dyslipidemia. KK mice have been used in our studies, because of the merit to examine on lipid metabolism in moderate obesity and insulin resistance [19]. In our previous study of young diabetic KK mice, combined treatment with TZDs and fish oil rich in omega-3 polyunsaturated fatty acids (PUFAs), such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), improved IR and suppressed TZD-induced subcutaneous fat accumulation by reducing hepatic lipogenesis [20]. Additionally, combined treatment with TZDs plus fish oil effectively prevented hypertrophy of pancreatic islets and β-cell dysfunction by improving IR and reducing endoplasmic reticulum (ER) stress [21]. The aim of this study is to investigate whether fish oil enhances the antidiabetic efficacy of low-dose TZD to the level of high-dose TZD in aged mice with type 2 diabetes.

2. Research design and methods

2.1. Animals and diets

Five-week-old male KK/Ta mice were purchased from the Tokyo Laboratory Animals Science Co. (Tokyo, Japan) and fed a standard commercial diet (MF, Oriental Yeast Co., Tokyo, Japan). At 40 weeks of age, mice were divided into 6 weight-matched groups (n = 6 per group) and individually housed and fed experimental diets supplemented with the indicated combination for 8 weeks. The diets were designed to maintain total fat energy level at 20 energy% (en%). The Control (Con) diet included 20 en% safflower oil (Benibana Foods Co., Ltd., Tokyo, Japan), and the FO diet included 10 en% safflower oil plus 10 en% fish oil (NOF Co., Tokyo, Japan). The Con and FO groups received these diets accordingly. In the other four treatment groups, the Con and FO diets were supplemented with 0.003 or 0.012 weight% (wt%) pioglitazone hydrochloride (Wako Pure Chemical Industries, Ltd., Osaka, Japan). These are designated PL (Pioglitazone Low-dose) and PH (Pioglitazone High-dose) diets and FO/PL (FO + Pioglitazone Low-dose) and FO/PH (FO + Pioglitazone High-dose) diets, respectively. Safflower oil contained 78% oleic acid and 14% linoleic acid; and fish oil contained about 6.6% EPA and 24.7% DHA. Additional details of these diets are shown in Table 1.

All animals were allowed food and water *ad libitum* and maintained under controlled conditions (22 °C ± 2 °C, 55% ± 10% humidity, 12-h–12-h light–dark cycle [lights on: 7:00 AM–7:00 PM]) at the Josai University Life Science Center. This study was performed in accordance with the "Fundamental Guidelines for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions" (Ministry of Education, Culture, Sports, Science and Technology, Japan, Notice No. 71, dated June 1, 2006) and approved by the Animal Care and Use Committee of the Josai University.

2.2. Abdominal fat analysis

At age 48 weeks, mice were fasted for 12 h and anesthetized by intraperitoneal injection of pentobarbital sodium (Kyoritsu Seiyaku Co., Tokyo, Japan). Anesthetized mice were scanned at 2-mm intervals between the second and fourth lumbar vertebrae by computed tomography (CT) using a La Theta LCT100 scanner (Hitachi Aloka Medical, Ltd., Tokyo, Japan). Subcutaneous and visceral fat masses were estimated from the images using La Theta software (version 2.10).

2.3. Sample collection

After CT scanning, blood glucose level was measured using a blood glucose monitor (One Touch Ultra; Johnson & Johnson, New Brunswick, NJ). Mice were then weighed and dissected. Blood samples were drawn from the inferior vena cava into heparinized tubes (Mochida Pharmaceutical Co., Ltd., Tokyo, Japan) and centrifuged at 900 × g for 15 min to separate plasma. The liver, epididymal white adipose tissue (WAT), brown adipose tissue (BAT), and pancreas were immediately removed. For histopathological and morphometric analyses, tissue samples were collected from 5 or 6 mice per group, fixed in 10% neutral buffered formalin (Wako Pure Chemical Industries), frozen in liquid nitrogen, and stored at −80 °C until further analysis.

2.4. Immunohistochemistry

After fixation, liver, WAT, and pancreas samples were embedded in paraffin and stained with hematoxylin and eosin (H&E). Pancreas samples were also stained with anti-insulin antibody (Takara Bio Inc., Shiga, Japan) and anti-C/EBP homologous protein (CHOP) antibody (Proteintech Group Inc., Chicago, IL). Embedding and staining were performed by Kotobiken Medical Laboratories, Inc. (Tokyo, Japan).

2.5. Morphological analysis

Liver, WAT, and pancreas specimens were examined under light microscopy (100 or 200 × magnification) and images captured with an Olympus DP21 camera system (Olympus, Tokyo, Japan). Image J (Wayne Rasband, NIH) was used for all morphological analyzes. The mean adipocyte size was evaluated from 5 or 6 randomly chosen fields of adipose tissue for each group. Pancreatic islet areas, insulin-positive areas, and

Table 1 – Composition of experimental diets.

Group	Con	PL	PH	FO	FO/PL	FO/PH
Safflower oil (g)	8	8	8	4	4	4
Fish oil (g)	–	–	–	4	4	4
Casein (g)	20	20	20	20	20	20
Sucrose (g)	10.37	10.37	10.37	10.37	10.37	10.37
β-starch (g)	51.83	51.83	51.83	51.83	51.83	51.83
Vitamin mix[a] (g)	1	1	1	1	1	1
Mineral mix[a] (g)	3.5	3.5	3.5	3.5	3.5	3.5
Cellulose powder (g)	5	5	5	5	5	5
L-cystin (g)	0.3	0.3	0.3	0.3	0.3	0.3
t-Butylhydroquinone (g)	0.0016	0.0016	0.0016	0.0016	0.0016	0.0016
Pioglitazone (g)	–	0.003	0.012	–	0.003	0.012
Total (g)	100.00	100.00	100.01	100.00	100.00	100.01
Energy (kcal/100g)	374.02	374.01	373.99	374.02	374.01	373.99
Fat energy (%)	19.70	19.70	19.70	19.70	19.70	19.70

[a] Vitamin and mineral mix were based on the AIN-93G formation. Vitamin mix substituted 0.25% sucrose for choline bitartrate.

numbers of CHOP-positive nuclei in islets were determined in pancreas sections from each group. The % of β-cell area was calculated as follows: insulin-positive area (μm^2)/total islet area (μm^2) × 100. The number of CHOP-positive nuclei is expressed as a percentage of the total number of nuclei in pancreatic islets.

2.6. Biochemical assays

Hepatic lipids were extracted from approximately 100 mg of liver tissue per mouse in accordance with the method of Folch et al. [22]. Triacylglycerol (TG), total cholesterol (TC), and free fatty acid (FFA) levels in liver and plasma were assayed by an enzymatic colorimetric method using commercial kits (Wako E-Test kits; Wako Pure Chemical Industries Ltd.). Plasma insulin and adiponectin levels were measured by enzyme-linked immunosorbent assay (ELISA) kits (Insulin ELISA from Morinaga Institute of Biological Science, Tokyo, Japan; Mouse/rat adiponectin ELISA kit from Otsuka Pharmaceutical, Tokyo, Japan).

2.7. Calculation of homeostasis model assessment of insulin resistance index

The homeostasis model assessment of insulin resistance (HOMA-IR) index was calculated by the following formula: fasting blood glucose (mg/dl) × fasting plasma insulin (μU/ml)/405.

2.8. Real-time polymerase chain reaction

Total RNA was extracted from liver and WAT using TRIzol® reagent (ThermoFisher Scientific Inc., Carlsbad, CA) following the manufacturer's protocol. The concentration of RNA was measured by a NanoDrop 2000c spectrophotometer (ThermoFisher). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed and used primer sequences in previously described [20,21]. The relative expression levels of target genes in each treatment group are presented as ratios relative to Con group expression.

2.9. Statistical analysis

Data are presented as mean ± standard error (SE). Group means were compared by one-way analysis of variance (ANOVA) with Tukey–Kramer post hoc tests for pair-wise comparisons using the Ekuseru-Toukei 2015 program (Social Survey Research Information Co., Ltd., Tokyo, Japan). Statistical significance was defined as $P < 0.05$.

3. Results

3.1. Low-dose pioglitazone plus fish oil enhanced insulin sensitivity to the same extent as high-dose pioglitazone monotherapy without subcutaneous fat accumulation and body weight gain

We compared various IR-related indices among Con, FO, PL, PH, FO/PL, and FO/PH diet groups of aged KK mice. There was no significant difference in plasma insulin levels or HOMA-IR index between Con and PL groups, indicating that low-dose TZD monotherapy was ineffective against these IR-related changes. However, both plasma insulin and HOMA-IR were significantly reduced in the PH group as well as the FO/PL group compared to the Con group, indicating enhanced low-dose TZD efficacy with addition of dietary fish oil. Moreover, the FO/PH group exhibited lower plasma insulin ($P = 0.0718$) and HOMA-IR index ($P = 0.0669$) than the Con group. Plasma adiponectin level was significantly higher in PH and FO/PH groups, and numerically higher in the FO/PL group ($P = 0.0514$) compared to the Con group (Table 2). Thus, dietary fish oil supplementation appears to augment the benefits of low-dose pioglitazone on IR.

CT scans performed to evaluate the effects of dietary fish oil on body weight gain under pioglitazone treatment revealed significant subcutaneous fat mass increases of 19% and 44% in PL and PH groups compared to the Con group, respectively (Fig. 1B). These increases were significantly suppressed by combined fish oil treatment. In fact, while final body weight was significantly greater in the PH group than the Control

Table 2 – Blood glucose, HOMA-IR, and plasma parameters in 48-week-old KK male mice.

Group	Con	PL	PH	FO	FO/PL	FO/PH
Blood glucose (mg/dl)	159 ± 16^{a}	157 ± 29^{a}	161 ± 14^{a}	82 ± 9^{b}	151 ± 21^{ab}	114 ± 5^{ab}
Plasma insulin (ng/ml)	28.0 ± 7.2^{a}	15.1 ± 6.1^{ab}	3.5 ± 1.2^{b}	19.3 ± 6.0^{ab}	4.5 ± 1.3^{b}	8.3 ± 2.9^{ab}
HOMA-IR	1.00 ± 0.30^{a}	0.62 ± 0.30^{ab}	0.12 ± 0.04^{b}	0.38 ± 0.12^{ab}	0.15 ± 0.03^{b}	0.23 ± 0.09^{ab}
Plasma adiponectin (μg/ml)	8.3 ± 0.6^{c}	11.4 ± 0.8^{c}	32.3 ± 5.2^{b}	8.7 ± 0.9^{c}	19.5 ± 1.8^{c}	60.0 ± 4.1^{a}
Plasma TG (mg/dl)	78 ± 7^{a}	83 ± 6^{a}	75 ± 5^{a}	71 ± 7^{ab}	69 ± 3^{ab}	51 ± 2^{b}
Plasma TC (mg/dl)	127 ± 7^{ab}	114 ± 7^{bc}	157 ± 18^{a}	93 ± 4^{cd}	79 ± 2^{d}	64 ± 3^{d}
Plasma FFA (mEq/l)	1.01 ± 0.18^{a}	0.73 ± 0.03^{ab}	0.72 ± 0.06^{ab}	0.56 ± 0.07^{b}	0.50 ± 0.03^{b}	0.45 ± 0.05^{b}

Data are represented as mean ± SE, n = 5–6.
Groups sharing different superscripts in a row are significantly different: $P < 0.05$ by Tukey–Kramer test.

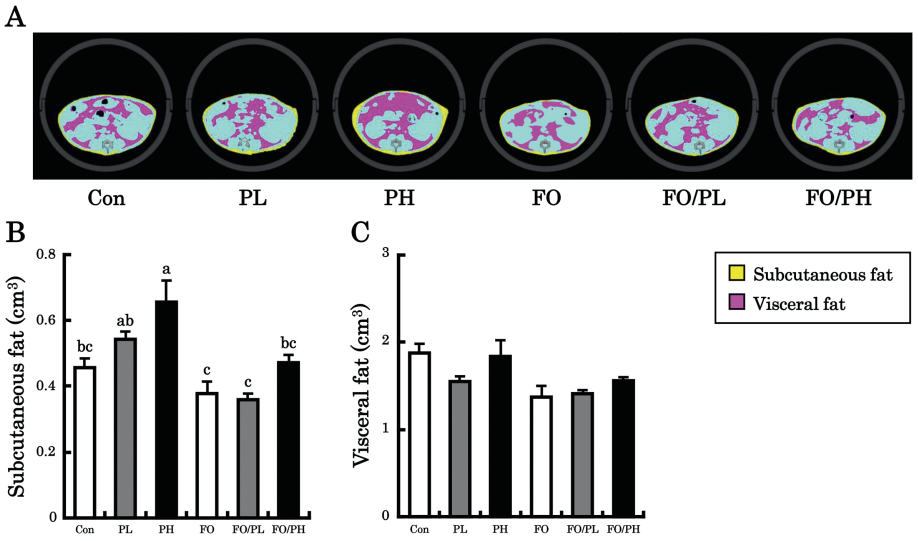

Fig. 1 – X-ray CT images and abdominal fat masses in 48-week-old KK male mice. X-ray CT images (A), subcutaneous fat masses (B), and visceral fat masses (C). Representative images show X-ray CT scanned cross-sections from mice fed Con, PL, PH, FO, FO/PL, and FO/PH diets for 8 weeks. The different colored areas show subcutaneous fat and visceral fat corresponding to yellow, pink, respectively. Subcutaneous fat and visceral fat are evaluated in abdominal area between second lumbar vertebra and forth lumbar vertebra. Data are shown as mean ± SE, n = 4–5. Groups sharing different letters are significantly different: $P < 0.05$ by Tukey–Kramer test.

group, it was unchanged in the PL and FO groups (Table 3). These results suggest that the body weight gain associated with high-dose pioglitazone is reduced by lowering the dose, while adding fish oil to low-dose pioglitazone results in anti-diabetic efficacy similar to that of the high-dose without concomitant body weight gain.

3.2. *Fish oil protected against low-dose pioglitazone-induced hepatic TG accumulation and reduced hepatic TC and FFA*

To investigate the mechanisms by which fish oil treatment reduced pioglitazone-induced accumulation of subcutaneous

Table 3 – Total food intake, body weight, and tissue weights in 48-week-old male KK mice.

Group	Con	PL	PH	FO	FO/PL	FO/PH
Total food intake (g/mouse)	240 ± 4	243 ± 5	242 ± 7	237 ± 7	228 ± 3	230 ± 3
Initial body weight (g)	36.5 ± 0.9	36.5 ± 1.0	36.5 ± 0.9	36.5 ± 0.8	36.5 ± 0.8	36.5 ± 0.8
Final body weight (g)	42.3 ± 0.3^{bc}	43.6 ± 0.5^{ab}	45.5 ± 1.4^{a}	40.2 ± 0.3^{c}	41.3 ± 0.4^{bc}	44.0 ± 0.6^{ab}
Body weight gain (g)	+5.9 ± 1.0^{abc}	+7.1 ± 0.7^{abc}	+9.1 ± 1.1^{a}	+3.7 ± 0.9^{c}	+4.8 ± 0.4^{bc}	+7.5 ± 0.2^{ab}
Liver weight (g)	1.55 ± 0.09^{b}	1.87 ± 0.11^{a}	1.45 ± 0.06^{b}	1.40 ± 0.04^{b}	1.40 ± 0.08^{b}	1.30 ± 0.04^{b}
Epididymal WAT weight (g)	1.37 ± 0.20	1.03 ± 0.07	1.13 ± 0.10	0.96 ± 0.11	1.01 ± 0.04	1.04 ± 0.08
BAT weight (g)	0.24 ± 0.02^{c}	0.40 ± 0.02^{c}	0.96 ± 0.14^{a}	0.26 ± 0.02^{c}	0.49 ± 0.04^{bc}	0.69 ± 0.07^{ab}

Data are represented as mean ± SE, n = 5–6.
Groups sharing different superscripts in a row are significantly different: $P < 0.05$ by Tukey–Kramer test.

fat in aged KK mice, we focused on changes in the liver that may alter lipid metabolism and the transportation of FFAs into WAT. On histological examination, the largest lipid droplets were observed in liver sections from the PL group, with no differences among other groups (Fig. 2A). Consistent with these histological observations, hepatic TG content and liver weight were significantly higher in the PL group, while other treatment groups did not differ significantly from the Con group (Fig. 2B and Table 3). Although no difference in hepatic TC content was observed between Con and PL groups, TC content was significantly reduced in the PH, FO, FO/PL, and FO/PH groups (Fig. 2C). Hepatic FFA content was significantly higher in the PL group and significantly lower in all FO groups, including the FO/PL group, compared to the Con group (Fig. 2D). Thus, addition of fish oil to low-dose pioglitazone reproduced the hepatic lipid-lowering effect of high-dose pioglitazone, while low-dose pioglitazone monotherapy was not effective.

3.3. *Fish oil treatment, but not pioglitazone, improved plasma lipid profiles*

In light of the reduced hepatic lipid levels in high-dose pioglitazone- and fish oil-treated groups, we examined whether these treatments can also improve plasma lipid profiles (Table 2). Plasma TG level was not affected by pioglitazone monotherapy, but was significantly lower in the FO/PH group than in fish oil-untreated groups. Moreover, plasma TC and FFA levels were significantly lower in fish oil-treated groups than the Con group.

3.4. *Fish oil treatment inhibited hepatic lipogenesis*

To assess the molecular mechanism underlying these effects of pioglitazone and fish oil on hepatic lipid accumulation, we examined expression levels of hepatic genes related to lipogenesis and lipid catabolism (Table 4). The sterol regulatory element binding protein (SREBP)-1c transcription factor regulates expression of genes involved in lipogenesis, such as acetyl-CoA carboxylase (ACC), fatty acid synthase (FAS), and stearoyl-CoA desaturase (SCD-1). SREBP-1c mRNA level was not significantly changed by pioglitazone or fish oil alone, but was significantly reduced by the combined treatment compared to the Con group.

ACC mRNA levels were significantly lower in FO and FO/PH groups than in the Con group, and also tended to be lower in the FO/PL group ($P = 0.0760$). Expression levels of FAS and SCD-1 mRNAs were significantly higher in the PH group than the Con group, but markedly lower in all three fish oil-treated groups than untreated groups (Con and pioglitazone monotherapy groups). Thus, fish oil (with or without pioglitazone) reduced the expression of multiple hepatic genes that promote lipogenesis.

3.5. *Fish oil treatment stimulated hepatic fatty acid β-oxidation with pioglitazone*

We next investigated the effects of fish oil treatment on mRNAs associated with hepatic fat catabolism (Table 4). Acyl-CoA oxidase (AOX) mRNA levels were significantly enhanced in fish oil-treated groups compared to the Con group. Pioglitazone monotherapy groups also exhibited a moderate increase in AOX expression, but this effect was further enhanced by addition of dietary fish oil. Moreover, medium chain acyl-CoA dehydrogenase (MCAD) mRNA levels were higher in the FO/PL and FO/PH groups than the PL and PH groups, respectively. Thus, fish oil alone appears to enhance hepatic lipid catabolism and also augments the effects of pioglitazone.

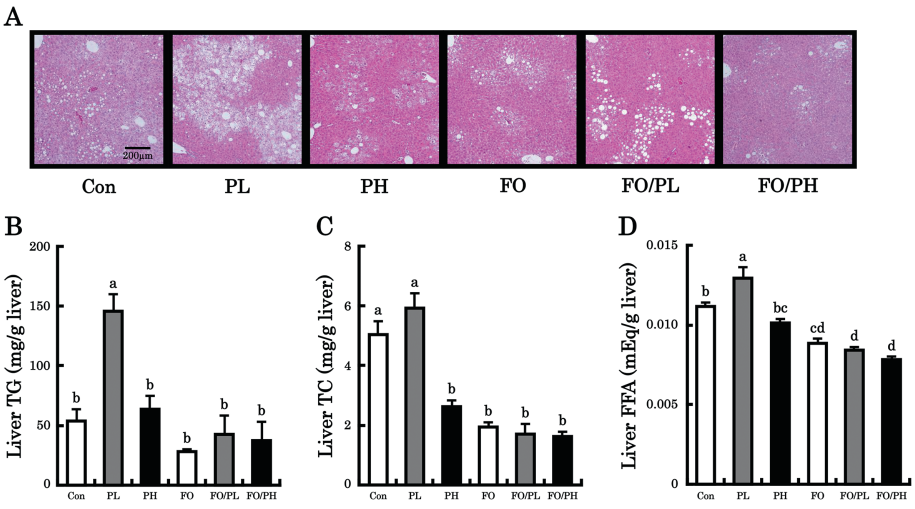

Fig. 2 – Liver histological sections and lipid parameters in 48-week-old male KK mice. Liver histology (A), liver triacylglycerol levels (B), liver total cholesterol levels (C), and liver FFA levels (D). Representative macroscopic liver images and tissue sections from mice fed Con, PL, PH, FO, FO/PL, and FO/PH diets for 8 weeks. Sections were stained by H&E and examined under a microscope at 100-fold magnification. Scale bar, 200 μm. Data are represented as mean ± SE, n = 5–6. Groups sharing different letters are significantly different: $P < 0.05$ by Tukey–Kramer test.

Table 4 – Liver and WAT gene expressions in 48-week-old male KK mice.

Group	Con	PL	PH	FO	FO/PL	FO/PH
Liver						
Lipogenesis						
SREBP-1c	1.00 ± 0.25^{a}	0.69 ± 0.18^{ab}	0.81 ± 0.16^{ab}	0.42 ± 0.11^{ab}	0.25 ± 0.09^{b}	0.14 ± 0.05^{b}
FAS	1.00 ± 0.07^{b}	0.87 ± 0.12^{bc}	1.50 ± 0.10^{a}	0.47 ± 0.05^{d}	0.55 ± 0.06^{cd}	0.46 ± 0.02^{d}
SCD-1	1.00 ± 0.21^{b}	1.56 ± 0.24^{ab}	1.91 ± 0.20^{a}	0.23 ± 0.06^{c}	0.21 ± 0.03^{c}	0.24 ± 0.04^{c}
ACC	1.00 ± 0.09^{ab}	0.94 ± 0.09^{abc}	1.08 ± 0.06^{a}	0.59 ± 0.06^{d}	0.67 ± 0.02^{bcd}	0.61 ± 0.10^{cd}
Fatty acid β-oxidation and energy consumption						
AOX	1.00 ± 0.10^{d}	1.45 ± 0.10^{cd}	1.71 ± 0.14^{cd}	1.95 ± 0.17^{c}	2.87 ± 0.20^{b}	3.60 ± 0.23^{a}
MCAD	1.00 ± 0.23^{b}	1.18 ± 0.26^{b}	1.83 ± 0.24^{ab}	1.88 ± 0.16^{ab}	2.63 ± 0.14^{a}	2.62 ± 0.35^{a}
UCP-2	1.00 ± 0.26	1.55 ± 0.16	1.25 ± 0.14	0.94 ± 0.14	1.03 ± 0.16	1.38 ± 0.19
WAT						
Inflammation						
TNF-α	1.00 ± 0.25	1.17 ± 0.50	1.22 ± 0.38	1.27 ± 0.52	2.47 ± 0.89	0.84 ± 0.17
IL-6	1.00 ± 0.55	0.31 ± 0.13	0.34 ± 0.13	0.32 ± 0.14	0.47 ± 0.17	0.67 ± 0.28
MCP-1	1.00 ± 0.48	0.48 ± 0.13	0.45 ± 0.13	0.45 ± 0.16	0.39 ± 0.10	0.52 ± 0.16

Results are expressed as the ratio of the obtained value to that of the Con group. Data are represented as mean ± SE, n = 5–6. Groups sharing different superscripts in a row are significantly different: $P < 0.05$ by Tukey–Kramer test.

3.6. *Combined pioglitazone and fish oil additively ameliorated adipocyte hypertrophy, but did not affect inflammation of visceral WAT in aged KK mice*

To examine possible mechanisms underlying the amelioration of IR by the combination of pioglitazone and fish oil in aged KK mice, we performed morphological analysis and measured mRNA expression of inflammatory cytokines in epididymal WAT (Fig. 3 and Table 4). Large adipocytes (>6400 μm^2) were observed most frequently in the Con group. The peak size distribution was shifted to smaller adipocytes in FO, FO/PL, and FO/PH groups (2500–3600 μm^2) compared to the Con group (6400–8100 μm^2) and compared to the PL and PH groups (3600–4900 μm^2) (Fig. 3B). Mean adipocyte area was significantly smaller in both pioglitazone- and fish oil-treated groups than in the Con group, with larger decreases in the FO/PL and FO/PH groups compared to the PL and PH groups, respectively (Fig. 3C). No significant differences in tumor necrosis factor (TNF)-α mRNA levels were observed among groups. Interleukin (IL)-6 and monocyte chemoattractant protein (MCP)-1 mRNA levels in pioglitazone- and fish oil-treated groups were lower than in the Con group, but these changes did not reach statistical significance (Table 4). Therefore, fish oil reduced adipocyte hypertrophy, a sign of fat accumulation, in WAT both in the presence and absence of pioglitazone, but had no marked effects on the expression levels of inflammatory cytokines.

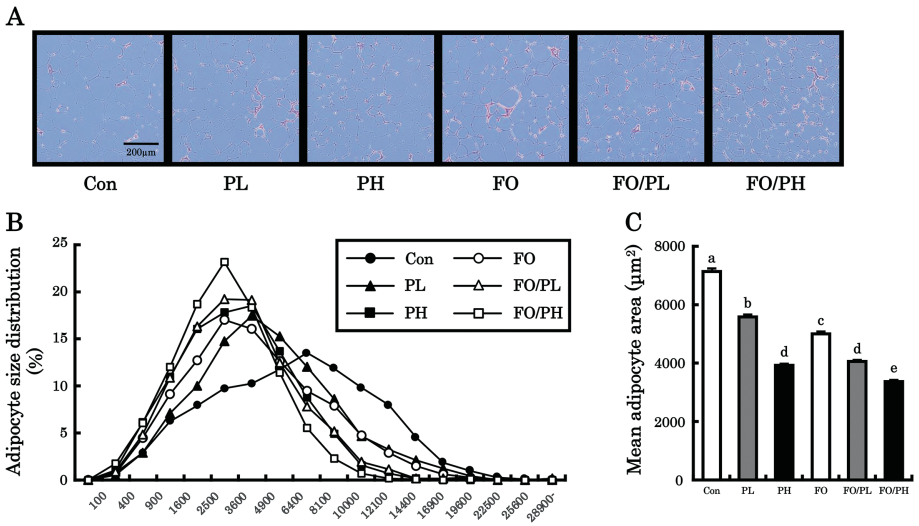

Fig. 3 – Morphological analysis of epididymal white adipose tissue in 48-week-old male KK mice. Epididymal white adipose tissue histological sections (A), adipocyte size distributions (B), and mean adipocyte areas (C). Representative sections from mice fed Con (black circles), PL (black triangles), PH (black squares), FO (white circles), FO/PL (white triangles), and FO/PH (white squares) diets for 8 weeks. Sections were stained by H&E and examined under a microscope at 100-fold magnification. Data except for (B) are represented as mean ± SE, n = 5–6. Groups with different letters are different at $P < 0.05$ by Tukey–Kramer test.

3.7. *Pioglitazone, fish oil, and combined treatment protected against pancreatic β-cell dysfunction in aged KK mice*

We performed histopathological and morphometric analysis of pancreatic sections to investigate whether combined pioglitazone plus fish oil protects against pancreatic β-cell dysfunction. In the Con and FO groups, there were many hypertrophic pancreatic islets (>50,000 μm^2) and fewer small islets (<9999 μm^2) compared to PH, FO/PL, and FO/PH groups (Fig. 4D). Further, mean islet area tended to be lower in the PL, PH, FO/PL, and FO/PH groups compared to the Con group (Fig. 4E). Pioglitazone, fish oil, and co-administration significantly increased immunohistochemical expression of insulin in islets compared to the Con group (Fig. 4F). Conversely, the proportion of cells positive for the ER stress marker CHOP was significantly lower in pioglitazone-treated groups, with and without fish oil co-treatment, than in the Con group (Fig. 4G).

4. Discussion

Combined treatment with low-dose pioglitazone plus dietary fish oil improved IR to the same extent as high-dose pioglitazone monotherapy and ameliorated subcutaneous fat accumulation and body weight gain, which are observed with pioglitazone treatment, in aged KK mice. These results suggest that fish oil allows for a reduction in pioglitazone dose without a decrease in antidiabetic efficacy. Aging is closely associated with serious adverse effects from TZDs, such as heart failure, bone fracture, and bladder cancer [15]. Therefore, reducing dose could contribute to minimize several adverse events from TZDs, which enhances therapeutic efficacy in elderly type 2 diabetes.

Adiponectin is a physiological enhancer of insulin sensitivity secreted primarily by small adipocytes [23]. Thus, adipocyte hypertrophy associated with obesity decreases adiponectin secretion and increases the release of proinflammatory cytokines such as TNF-α, IL-6, and MCP-1, which in turn impairs IR and worsens type 2 diabetes [24]. TZDs increase blood adiponectin levels by promoting both the differentiation of pre-adipocytes and the apoptosis of hypertrophic adipocytes [25,26]. EPA and DHA have also been reported to promote adipocyte differentiation and increase adiponectin secretion [27–29]. In the present study, low-dose pioglitazone and fish oil monotherapy increased the proportion of small adipocytes and decreased mean adipocyte size but had no effects on plasma adiponectin level and IR, while combined low-dose pioglitazone and fish oil additively reduced adipocyte size, elevated plasma adiponectin, and improved IR.

Hepatic lipid accumulation in patients with nonalcoholic steatohepatitis (NASH) and obesity is strongly associated with IR [30]. Low-dose pioglitazone-treated mice exhibited markedly increased hepatic TG as well as slightly increased subcutaneous fat accumulation, and both effects were reversed by addition of dietary fish oil. Therefore, preservation of insulin sensitivity in aging diabetic mice may stem in part from prevention of hepatic TG accumulation.

Fatty acids derived from de novo synthesis in liver are esterified to TG and exported into adipose tissues for storage in the form of very low density lipoproteins (VLDLs) [31]. We previously reported that combined pioglitazone plus fish oil prevented body weight gain and subcutaneous fat accumulation induced by pioglitazone in young adult KK mice, mainly by suppressing hepatic lipogenesis [20]. In the present study, fish oil not only reduced mRNA levels of factors related to lipogenesis, but also enhanced mRNA levels of factors related to fatty acid β-oxidation in the liver. There is evidence that the concentrations of EPA and DHA in plasma and WAT increase with aging. The mechanisms were speculated to relate in part to an endogenous production due to the change of hormones,

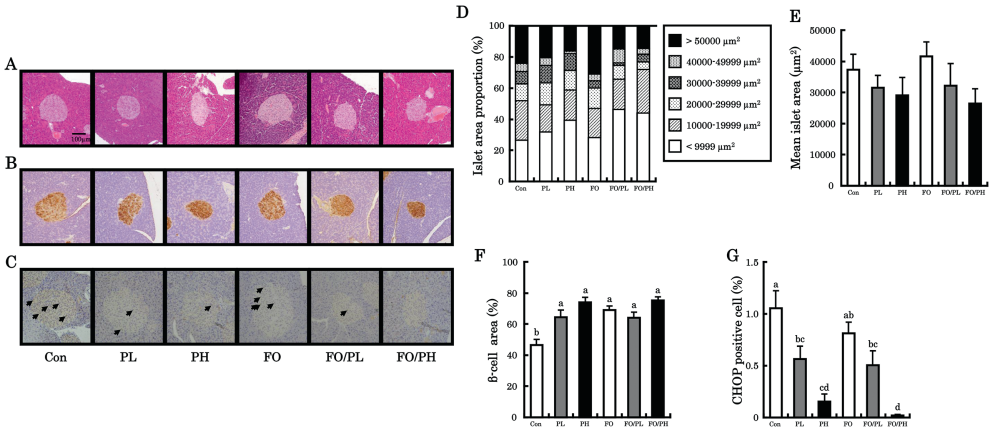

Fig. 4 – Morphological analysis of pancreatic islets in 48-week-old male KK mice. Islet images of H&E staining (A), insulin immunostaining (B), CHOP immunostaining (C), islet area proportions (D), mean islet areas (E), percentages of β-cell area (F), and mean CHOP positive ratio (G). Representative pancreatic tissue sections from mice fed Con, PL, PH, FO, FO/PL, and FO/PH diets for 8 weeks. All sections were examined under a microscope at 200-fold magnification. Scale bar, 100 μm. Data except for (D) are represented as mean ± SE, n = 5–6. Groups with different letters are significantly different: $P < 0.05$ by Tukey–Kramer test.

hormone sensitivity, body consumption and physical activity in older adults [32]. Therefore, the antilipidemic effects of fish oil may be enhanced in aged KK mice due to alterations in EPA and DHA metabolism and storage by age-related endogenous changes, such as hormones and hormone sensitivity.

Several clinical reports have proposed that TZDs are useful for patients with NASH, and that the antisteatotic effect is associated with redistribution of FFAs from the liver to TZD-induced new adipocytes [33–35]. However, hepatic PPARγ expression is highly upregulated in obese and diabetes mouse models, and this phenotype contributes to the development of TZD-induced steatosis [36,37]. As hepatic PPARγ is stimulated by TZDs, the liver is continuously exposed to high FFA levels due to activation of FFA uptake from blood in response to upregulated PPARγ-responsive genes related to fatty acid transportation [38]. In this study, accumulation of hepatic TG observed in aged KK mice treated with low-dose pioglitazone monotherapy. On the other hand, high-dose pioglitazone monotherapy improved IR, reduced adipocyte size, and increased plasma adiponectin level; however, body weight gain and subcutaneous fat accumulation were more strongly induced than under low-dose pioglitazone monotherapy. In addition, combined treatment with fish oil markedly suppressed accumulation of hepatic TG and subcutaneous fat at both doses. It was reported that PPARγ agonists strongly increased lipoprotein lipase activity and promoted uptake of TG-derived FFA into subcutaneous fat and BAT and lesser extent into visceral fat [39]. These results suggest that IR is improved in aged KK mice only by pioglitazone doses sufficient to enhance the transport of excess fatty acids from visceral fat and liver into subcutaneous fat and BAT. Although pioglitazone responses may be dose-dependent due to distinct effects on liver, WAT, and BAT, combined treatment with fish oil markedly suppressed accumulation of hepatic TG and subcutaneous fat at both doses. This could be due to decreased FFA in the liver associated with inhibition of lipogenesis and activation of fatty acid β-oxidation by low-dose pioglitazone plus fish oil, which resulted in FFA transport to other tissues. This finding strongly suggests that the combination of pioglitazone with EPA and DHA may be useful for elderly type 2 diabetes patients with fatty liver.

Another beneficial effect of combination therapy for elderly type 2 diabetes is protection against pancreatic β-cell dysfunction, which is strongly associated with clinical deterioration. Hyperinsulinemia in the type 2 diabetes triggers accumulation of unfolded proteins in response to intense insulin biosynthesis [40]. This situation induces β-cell dysfunction through enhanced ER stress, which ultimately results in β-cell damage and apoptosis that contribute to further deteriorate type 2 diabetes [41,42]. Combined low-dose pioglitazone plus fish oil protected against pancreatic islet hypertrophy to about the same extent as high-dose pioglitazone monotherapy. In addition, low-dose pioglitazone with or without fish oil reduced the number of islet cells expressing the ER stress marker CHOP, but the effect was greater for high-dose pioglitazone. In a previous study of young adult KK mice, we demonstrated that the combination of 0.012 wt% pioglitazone (the high-dose used in this study) with fish oil effectively prevented pancreatic islet hypertrophy and β-cell dysfunction by improving IR and reducing ER stress [21]. This finding indicates an additional benefit of fish oil, augmenting the effects of low-dose pioglitazone against pancreatic islet ER stress in aged KK mice with type 2 diabetes.

Moreover, it was investigated that the combination of pioglitazone and red mold dioscorea improved oral glucose tolerance in the streptozotocin (STZ)-induced type 1 diabetic rats [43]. Further studies are needed to clarify the benefits of functional foods in pioglitazone treatment.

In conclusion, addition of fish oil to low-dose pioglitazone effectively improved signs of type 2 diabetes in aged KK mice without the subcutaneous fat accumulation associated with therapeutic pioglitazone doses. These results could contribute to the basis of an alternative therapeutic method using combination of pioglitazone with fish oil for type 2 diabetes in older patients by not only suppressing subcutaneous fat accumulation in therapeutic pioglitazone doses but enhancing therapeutic efficacy of type 2 diabetes with the reduced pioglitazone dose.

Conflict of interest

No potential conflicts of interest relevant to this article were reported.

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