

**Protective effects of fish oil and pioglitazone on pancreatic tissue in obese KK mice  
with type 2 diabetes**

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**Disclosure Summary**

The authors have nothing to declare.

## **Abstract**

n-3 Polyunsaturated fatty acids, such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), have protective effects against the pancreatic  $\beta$ -cell dysfunction through several mechanisms. Thiazolidines are insulin sensitizers and are used in treating patients with type 2 diabetes. Our previous study demonstrated that a combination of fish oil, which is rich with EPA and DHA, and pioglitazone exerts beneficial effects on obesity and diabetes through their actions on the liver and adipose tissue. However, it remains largely unknown whether such combination therapy affects the pancreas. To answer this question, KK mice, which serve as a model for obesity and type 2 diabetes, were treated for 8 weeks with fish oil and pioglitazone. The combined regimen suppressed pancreatic islet hypertrophy (mean islet area decreased by an average of 49% vs. control) compared with mice treated with fish oil or pioglitazone alone (decreased by an average of 21% and 32% vs. control, respectively). Compared with the controls, individual or combined treatment significantly increased the percentage of  $\beta$ -cell area in the pancreatic islets, significantly decreased endoplasmic reticulum stress, and reduced the percentage of apoptotic cell death in the pancreatic islets. These findings suggest that fish oil and/or pioglitazone prevents  $\beta$ -cell dysfunction by improving the insulin resistance and decreasing the ER stress.

Keywords: Fish oil; Pioglitazone; Protective effect; Islet hypertrophy;  $\beta$ -cell dysfunction

## **1. Introduction**

Chronic hyperglycemia causes overproduction of reactive oxygen species (ROS) by the mitochondria of pancreatic cells and gradually causes deterioration of  $\beta$ -cell functions such as insulin production and secretion [1, 2]. Hyperinsulinemia that is attributed to insulin resistance (IR) triggers  $\beta$ -cell dysfunction through enhanced endoplasmic reticulum (ER) stress that is induced by accumulation of unfolded proteins in response to intense insulin biosynthesis [3]. These factors ultimately result in  $\beta$ -cell damage and apoptosis, which are referred to as glucotoxicity, that contribute to further deteriorate type 2 diabetes [1, 4]. Pancreatic inflammation and infiltration of macrophages of the pancreatic islets are also closely related to  $\beta$ -cell dysfunction through obesity-related IR [3, 5]. These observations suggest that protection against functional deterioration of pancreatic  $\beta$ -cells is a beneficial and desirable clinical treatment for patients with type 2 diabetes that leads to the prevention of diabetic complications.

Thiazolidines (TZDs) are selective agonists of peroxisome proliferator-activated

receptor- $\gamma$  that are insulin sensitizers and are used to treat patients with type 2 diabetes. Evidence indicates that TZDs protect against pancreatic  $\beta$ -cell dysfunction. By improving IR with TZDs treatment, high insulin production is reduced and over production of ROS and ER stress levels are prevented [6-9]. However, with improving abnormal lipid metabolism (lipotoxicity) by TZDs treatment, visceral and ectopic fat in insulin-sensitive non-adipose tissues, including the liver, skeletal muscle, and pancreas, are redistributed in subcutaneous fat [6, 10, 11]. Animal and clinical studies have demonstrated that despite these protective effects of TZDs on  $\beta$ -cells, TZDs increase body weight via the accumulation of subcutaneous fat [12, 13].

In our previous study, fish oil, which contains n-3 polyunsaturated fatty acids (PUFAs) such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), suppressed the increase in body weight that was associated with subcutaneous fat accumulation because of TZD treatment, which is considered to occur through the effect of n-3 PUFA on hepatic endogenous lipogenesis [14]. n-3 PUFAs protect against  $\beta$ -cell dysfunction by ameliorating the lipotoxicity in non-adipose tissues, excess insulin secretion, and cytokine-induced inflammation [15, 16]. Kato et al. reported that EPA recovered insulin secretion defects on the pancreatic islets in diabetic model mice [17]. Moreover, combined treatment with TZDs and n-3 PUFAs exerts beneficial effects for

obesity and diabetes through their effects on the liver and skeletal muscle [18-20].

We hypothesized that the treatment with fish oil and pioglitazone contributes to maintaining islet function and mitigates IR in patients with type 2 diabetes. To address this question, we conducted immunohistochemical and morphometrical analyses and quantified gene expression in the pancreas of obese KK mice that serve as an animal model of patients with mild diabetes.

## **2. Materials and Methods**

### **2.1. Animals**

The animal 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 was approved by the Institutional Animal Care and Use Committee of the Josai University. KK/Ta mice (males, 6-week-old) were obtained from the Tokyo Laboratory Animals Science Co. (Tokyo, Japan) and were fed a commercial standard diet (MF, Oriental Yeast Co., Tokyo, Japan) for 1 week to stabilize their metabolism. The mice were divided into four weight-matched groups (n = 5 per group) and were individually housed and fed the experimental diets for 8 weeks at

the Josai University Life Science Center. Mice were maintained at  $22^{\circ}\text{C} \pm 2^{\circ}\text{C}$  temperature and  $55\% \pm 10\%$  humidity with a 12-h light–dark cycle (7:00 AM–7:00 PM). The mice were allowed free access to water and food. Mice were fasted for 12 h before collecting blood samples and performing computed tomography (CT).

## 2.2. Diets

All diets comprised 60 energy% (en%) carbohydrates, 20 en% fats, and 20 en% protein (Supplementary Table 1). The safflower oil diet included 20 en% safflower oil (Benibana Foods Co., Ltd., Tokyo, Japan) as a lipid source, and the fish oil diet was prepared with 10 en% safflower oil and 10 en% fish oil (NOF Co., Tokyo, Japan). These diets were designated the SO and FO diets, respectively. The SO and FO diets were each supplemented with 0.012 weight% pioglitazone hydrochloride (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and were designated the SP and FP diets, respectively. These experimental diets, which were supplemented with *t*-butyl hydroquinone (Wako Pure Chemical Industries) to prevent fatty acid oxidation, were stored at  $-30^{\circ}\text{C}$ .

## 2.3. Computed tomography

At the end of the experiment, mice were fasted for 12 h and anesthetized using intraperitoneal injections of pentobarbital sodium (Kyoritsu-seiyaku Co., Tokyo, Japan). The mouse mode was used for CT scanning (La Theta LCT100, Hitachi Aloka Medical, Ltd., Tokyo, Japan). Abdominal compositions were estimated from images of fat slices that were acquired at 2-mm intervals between the second and fourth lumbar vertebrae using the La Theta software (version 2.10).

#### 2.4. Sample collection

After CT, we obtained blood samples from the tail vein and measured glucose levels using a blood glucose monitoring system (One Touch Ultra, Johnson & Johnson, New Brunswick, NJ). The mice were immediately weighed and sacrificed. Blood samples were subsequently collected from the inferior vena cava into a tube containing heparin (Mochida Pharmaceutical Co., Ltd., Tokyo, Japan) to prevent coagulation, and the tubes were centrifuged at  $900 \times g$  for 10 min to separate plasma, which was stored at  $-80^{\circ}\text{C}$  until further analysis. White adipose tissue (WAT) around the epididymis was removed and weighed. The pancreas was collected and divided into two sections. The first section was fixed with 10% neutral-buffered formalin (Wako Pure Chemical Industries) for histology. The second section was immersed in an RNA stabilization reagent for

RNA isolation and was stored at  $-80^{\circ}\text{C}$  until further use.

## 2.5. Oxidative stress and evaluation of antioxidative potential

The diacron of reactive oxygen metabolites (d-ROMs) test and the measurement of biological antioxidative potential (BAP) test were performed to evaluate oxidative stress and antioxidative potential using the Free Radical Analytical System (Diacron, Grosseto, Italy). The d-ROMs data were expressed in conventional units (Carratelli Units, U.CARR), and 1 U.CARR corresponds to  $0.8\text{ mg/L H}_2\text{O}_2$ . At 0 and 7 weeks after initiating experimental diet, blood samples from the tail veins of fasted mice were collected into a tube containing heparin and centrifuged at  $900 \times g$  for 10 min. All tests were conducted following the manufacturer's instructions.

## 2.6. Biochemical analyses of plasma

Plasma insulin and adiponectin concentrations were determined using enzyme-linked immunosorbent assay (ELISA) kits (Insulin ELISA kit, Morinaga Institute of Biological Science, Tokyo, Japan; Mouse/rat adiponectin ELISA kit, Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan). The homeostasis model assessment of insulin resistance (HOMA-IR) index was calculated as follows: fasting blood glucose (mg/dL)



× fasting plasma insulin ( $\mu\text{U}/\text{mL}$ )/405.

## 2.7. Immunohistochemical and morphological analyses

After fixation, pancreas samples were embedded in paraffin and were stained with hematoxylin and eosin (H&E). Immunohistochemical staining of  $\alpha$ -cells,  $\beta$ -cells, and CCAAT/enhancer-binding protein homologous protein (CHOP) were performed in the Kotobiken Medical Laboratories, Inc. (Tokyo, Japan) using anti-glucagon antibody (Takara Bio Inc., Shiga, Japan), anti-insulin antibody (Takara Bio Inc.), and anti-CHOP antibody (Proteintech Group Inc., Chicago, IL). Specimens were examined using a microscope (200× magnification). Images were captured using an Olympus DP21 camera system (Olympus, Tokyo, Japan), and immunoreactivities were quantified using image analysis software (Image J, United States National Institutes of Health). The mean area ( $\mu\text{m}^2$ ) of the pancreatic islets and their distribution were determined in all H&E-stained sections. The percentages of  $\alpha$ - and  $\beta$ -cell areas were calculated as follows: {glucagon- or insulin-positive area ( $\mu\text{m}^2$ )/islet area ( $\mu\text{m}^2$ )} × 100. The glucagon- and insulin-positive areas were quantitated using an appropriate threshold after altering and adjusting color images to gray scale.

## 2.8. Apoptosis assay

TdT-mediated dUTP nick-end labeling (TUNEL) assays were performed in Kotobiken Medical Laboratories, Inc.. TUNEL-positive nuclei and the total number of nuclei in the pancreatic islets were counted using the Image J software. The proportion of apoptotic cells to total islet cells was calculated as follows:  $\{\text{number of TUNEL-positive nuclei}/\text{total number of nuclei}\} \times 100$ . Cells with undetectable TUNEL labeling were designated not detected.

## 2.9. RNA isolation and quantitative real-time PCR

TRIzol reagent and a PureLink RNA Mini Kit (Thermo Fisher Scientific Inc., Carlsbad, CA) were used according to the manufacturer's instructions to extract total RNA from pancreatic samples immersed in an RNA stabilization reagent. Quantitative real-time polymerase chain reactions were performed using total RNA, gene-specific primers, QuantiTect SYBR Green RT-PCR kits (QIAGEN N. V. Inc., Hilden, Germany), and an ABI Prism 7500 Sequence Detection System (Applied Biosystems, Carlsbad, CA). The sense and antisense sequences of primers are shown in Supplementary Table 2. Reaction conditions were previously described [14]. The mRNA levels are represented as the ratios of each group to those of the SO group.

## 2.10. Statistical methods

Data are represented as the mean  $\pm$  standard error of the mean. A significant difference ( $P < 0.05$ ) between groups was identified using one-way ANOVA, followed by the Tukey–Kramer test. Values or groups sharing different letters are significantly different. All statistical analyses were performed using the Ekuseru-Toukei 2015 (Social Survey Research Information Co., Ltd., Tokyo, Japan).

## 3. Results

### 3.1. Total food intake, body weight gain, WAT weight, and abdominal fat mass

The data for total food intake, body weight gain, epididymal WAT weight, and visceral and subcutaneous fat mass (Supplementary Table 3) were similar to those of a previous study [14].

### 3.2. Evaluation of IR

The blood glucose level was significantly higher in the SP group than in other groups. In contrast, the plasma insulin levels of the SP and FP groups were 82% ( $P < 0.05$ ) and 66% ( $P = 0.0565$ ) lower, respectively, than those of the SO group (Table 1).

Similarly, the HOMA-IR indices of the SP and FP groups decreased by 74% ( $P < 0.05$ ) and 68% ( $P = 0.0569$ ), respectively, than the index of the SO group, indicating that IR was reduced by pioglitazone treatment (Table 1). The plasma adiponectin levels significantly increased in the pioglitazone-treated groups compared with the untreated groups, and the plasma adiponectin level in the FP group was 183% greater than that in SP group (Table 1).

### 3.3. Plasma oxidative stress and anti-oxidative potential markers

At 0 and 7 weeks after initiating experimental periods, d-ROMs and BAP levels did not significantly differ among all groups (Fig. 1A and 1B). However, after 7 weeks, there was a 118% ( $P = 0.0536$ ) increase than the d-ROMs value of the SO group at 0 week and a 24% – 43% (FO,  $P < 0.05$ ; FP,  $P < 0.05$ ) increase in that of the other groups compared with those at 0 week (Fig. 1A). With respect to time, no significant change was found in BAP levels with or without fish oil feeding and pioglitazone treatment (Fig. 1B).

### 3.4. Pancreatic islet morphology

Pancreatic sections that were stained with H&E are shown in Fig. 2A. Numerous

hypertrophic pancreatic islets were observed in the SO group. Consistent with plasma insulin levels, the islet areas decreased by 32%, 21%, and 49% ( $P = 0.0948$ ) in the SP, FO, and FP groups, respectively, compared with that of the SO group (Fig. 2D). The SO group had the lowest percentage of small pancreatic islets ( $<9999 \mu\text{m}^2$ ) and the highest percentage of large pancreatic islets ( $>50000 \mu\text{m}^2$ ). The proportions of small pancreatic islets were higher in the FO and FP groups, and the proportions of large pancreatic islets were lower in the pioglitazone-treated SP and FP groups than in the SO group. In particular, the FP group data were the opposite of the SO group. Moreover, the proportion of large pancreatic islets was lower in the FP group than in the SP group (Fig. 2E).

The results of the immunohistochemical analyses of glucagon and insulin expression in the pancreatic islets are shown in Fig. 2B and 2C, and the percentages of areas of  $\alpha$ - and  $\beta$ -cells in the pancreatic islets are shown in Fig. 2F and 2G. The percentages of the  $\alpha$ -cell areas in the pancreatic islets were significantly higher in the SP and FO groups, and including tendency ( $P = 0.0824$ ) in the FP group than in the SO group (Fig. 2F). The percentages of  $\beta$ -cell areas in the pancreatic islets were significantly increased in the SP, FO, and FP groups compared with the SO group, whereas no change was observed among these three groups (Fig. 2G).

### 3.5. Morphometric analysis of pancreatic islet cells

Immunohistochemical analysis of the expression of CHOP, a marker of ER stress, in the pancreatic islets is shown in Fig. 3A. CHOP-positive nuclei were frequently observed in sections of the SO group. The percentages of CHOP-positive cells were significantly lower in the SP, FO, and FP groups than in the SO group and were <0.1% in the FP group (Fig. 3C). TUNEL-positive nuclei were detected in sections of the SO and SP groups but not in those of the FO and FP groups (Fig. 3B). There was a decrease in the percentage of apoptotic cells in the pancreatic islets of the SP group compared with that of the SO group (Fig. 3D).

### 3.6. Analysis of gene expression in pancreatic tissue

Among proinflammatory cytokines, the mean levels of mRNA encoding tumor necrosis factor- $\alpha$  (TNF $\alpha$ ) were higher in the SP group than in the SO group, but no significant difference was observed among all groups (Table 2). Furthermore, the levels of the mRNAs encoding interleukin-1 $\beta$  (IL1 $\beta$ ), monocyte chemoattractant protein-1 (MCP1), and toll-like receptor 4 (TLR4) were not affected by the fish oil or pioglitazone treatment diet. The levels of the mRNA encoding insulin (INS) were 43%

and 75% ( $P = 0.0849$ ) lower in the SP and FP groups, respectively, but were 70% higher in the FO group compared with the SO group, even these differences were not significant (Table 2). The levels of the mRNA encoding pancreatic and duodenal homeobox 1 (PDX1), which regulate the transcription of INS, were lower in the SP group ( $P = 0.0792$ ) and significantly lower in the FP group compared with the SO group (Table 2). The levels of the mRNA encoding aquaglyceroporin 7 (AQP7), a glycerol channel that regulates insulin production and secretion, were significantly at least three-times higher in the pioglitazone-treated groups than in the untreated groups (Table 2). The levels of the mRNA encoding CHOP were 37% and 38% lower in the SP and FP groups, respectively, than in the SO group (Table 2).

#### **4. Discussion and Conclusions**

The structural and functional features of the pancreatic islets change to adapt to pathological conditions and to the severity of IR in patients with type 2 diabetes, which consider to hypothetical phases of deterioration. In the early stage of IR, expansion of pancreatic  $\beta$ -cell mass and increased insulin secretion are induced to produce higher insulin levels, which are required to maintain normal blood glucose levels, leading to hypertrophy of the pancreatic islets [21]. The overproduction of insulin under conditions

of IR causes ER stress through increased the concentration of misfolded proteins in response to the high demand for insulin synthesis and excess ER folding capacity [22]. Under prolonged intense ER stress in pancreatic  $\beta$ -cells, an apoptotic pathway is activated, resulting in  $\beta$ -cell dysfunction that is attributed to a decrease in the  $\beta$ -cell mass [23].

Here we demonstrated that after the combination treatment of fish oil and pioglitazone in KK mice, the mean islet area was lower compared with treatment of fish oil or pioglitazone alone. Moreover, fish oil, pioglitazone, or both prevented a reduction in the number of insulin-positive  $\beta$ -cells and an enhancement of ER stress in pancreatic islets. Furthermore, no apoptotic cells were detected in the islet of the groups that were fed fish oil. These results suggest that the combination of fish oil and pioglitazone may inhibit  $\beta$ -cell dysfunction and decrease IR thorough different pathways that are specific for each treatment without a pioglitazone-induced accumulation of subcutaneous fat and increase in body weight.

Consistent with the results of our previous study [14], the combination of fish oil and pioglitazone significantly increased the plasma adiponectin level compared with pioglitazone alone. Adiponectin, which is an adipocytokine that is mainly secreted by small adipocytes, acts as an insulin-sensitizing hormone to increase insulin secretion,



stimulate insulin signaling, and decrease ER stress [24]. TZDs induce the differentiation of pre-adipocytes to mature adipocytes in subcutaneous fat, which contribute to the redistribution of accumulated fat in non-adipose tissue or that of visceral fat into subcutaneous fat [25, 26]. In addition, TZDs are associated with decreasing IR and upregulating adiponectin expressions in WAT and increasing plasma adiponectin levels [27]. Other studies demonstrated that TZDs decrease ER stress and preserve pancreatic morphology and function via several mechanisms, such as the inhibition of  $\beta$ -cell overwork by decreasing IR, reducing lipotoxicity, directly upregulating PDX1 expression, and inhibiting islet cell apoptosis [6, 24, 28].

Our results demonstrated that pioglitazone decreased the insulin level, improving the IR and increasing the plasma adiponectin level, all of which prevented  $\beta$ -cell dysfunction that was induced by ER stress. Moreover, the pioglitazone-treated SP and FP groups expressed low PDX1 and INS mRNA levels compared with untreated groups. On the other hand, fish oil feeding reduced plasma insulin and lowered the HOMA-IR index that was associated with the decreased visceral fat mass, and the reduced mean islet area. But, there was no detectable increase in plasma adiponectin levels that were detected in the SP group that was administered a single pioglitazone treatment. Nascimento et al. found that a diet including fish oil prevents hypertrophy of the

pancreatic islets because of reduced fat pad adiposity and body mass in mice that were fed a high-fat and -sucrose diet [29]. Altogether, these and the present findings indicate that the improvement of islet hypertrophy and  $\beta$ -cell dysfunction by the combination of fish oil and pioglitazone are mainly associated with the amelioration of IR through increasing the blood adiponectin level by pioglitazone treatment and inhibiting the pancreatic  $\beta$ -cell apoptosis by fish oil.

We were interested to determine that the plasma adiponectin level corresponded with the AQP7 mRNA level, which was 1.62-fold higher in the FP group than in the SP group, suggesting an inverse association between these parameters and mean islet area. Other studies revealed that adiponectin protected against pancreatic  $\beta$ -cell failure by increasing the regeneration of  $\beta$ -cells, which is mediated by improved lipid metabolism [30]. Moreover, adiponectin counteracted  $\beta$ -cell lipotoxicity that was induced by inflammatory cytokines or fatty acids [31]. AQP7 is a channel-forming membrane protein that is expressed in the pancreas of mice and rats. AQP7-mediated transport of water and glycerol through the membrane contributes to insulin production and secretion [32]. Notably, Matsumura et al. found that insulin secretion is impaired in *Aqp7*<sup>-/-</sup> mice and is associated with the accumulation of triacylglycerol and elevated INS mRNA levels in the pancreatic islets and blood insulin levels [33]. We suggest that

the combination of fish oil and pioglitazone may contribute an antihypertrophic effect on the pancreatic islets, in part, through an adiponectin- and AQP7-mediated mechanism of  $\beta$ -cell lipotoxicity and insulin secretion.

Inflammation and oxidative stress imposed on the pancreas or pancreatic islets are critical factors that contribute to  $\beta$ -cell dysfunction [3, 34]. Although n-3 PUFAs and TZDs reduce the proinflammatory process [35, 36], we did not observe significant differences in the expression of pancreatic genes related to inflammation, such as TNF $\alpha$ , IL1 $\beta$ , MCP1, and TLR4. These findings suggested that the improvement of  $\beta$ -cell functions by fish oil and pioglitazone treatment of obese KK mice may not be relevant to pancreatic inflammation. In contrast, the d-ROMs test, a systemic evaluation of oxidative stress, demonstrated that fish oil and pioglitazone treatment suppressed the adverse effects of oxidative stress, which were consistent with decreased epididymal WAT weight, visceral fat mass, CHOP expression, and TUNEL activity in the pancreatic islets. Visceral fat accumulation is strongly associated with increased oxidative stress, which causes systemic oxidative stress disorder [37, 38]. The reduction of  $\beta$ -cell dysfunction by fish oil and pioglitazone may be led to the suppression of systemic oxidative stress because of decreased visceral fat levels.

In conclusion, this study demonstrated that the combination of fish oil and

pioglitazone effectively prevented hypertrophy of the pancreatic islets and apoptosis-induced cell death through increased insulin sensitivity and decreased ER stress, which contributed to the maintenance of pancreatic  $\beta$ -cell functions.

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### **Abbreviations**

AQP7, aquaglyceroporin 7; BAP, biological antioxidative potential; CHOP, CCAAT/enhancer-binding protein homologous protein; CT, computed tomography; DHA, docosahexaenoic acid; d-ROMs, diacron of reactive oxygen metabolites; ELISA, enzyme-linked immunosorbent assay; en%, energy%; EPA, eicosapentaenoic acid; ER, endoplasmic reticulum; H&E, hematoxylin and eosin; HOMA-IR, homeostasis model assessment of insulin resistance; IL1 $\beta$ , interleukin-1 $\beta$ ; IR, insulin resistance; MCP1, monocyte chemoattractant protein-1; PDX1, pancreatic and duodenal homeobox 1; PUFAs, polyunsaturated fatty acids; ROS, reactive oxygen species; TLR4, toll-like receptor 4; TNF $\alpha$ , tumor necrosis factor- $\alpha$ ; TUNEL, TdT-mediated dUTP nick-end labeling; TZDs, thiazolidines; WAT, white adipose tissue

### **Legends for figures**

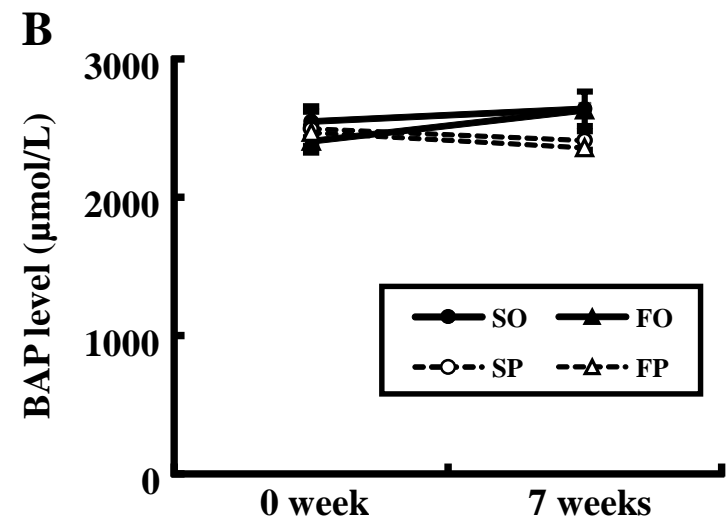
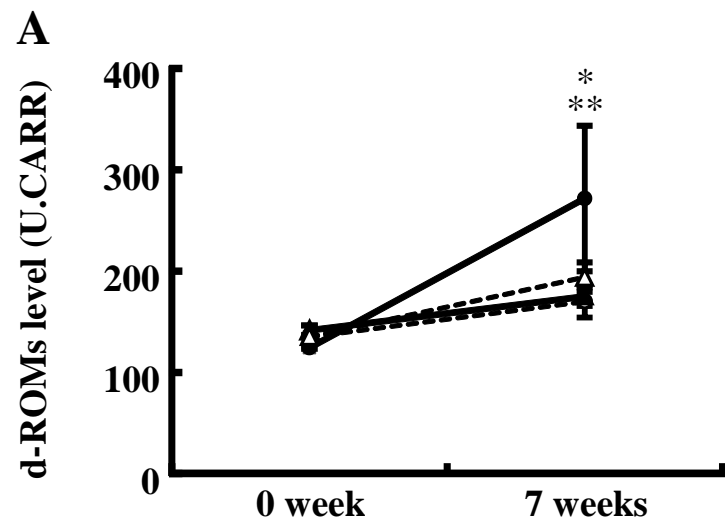
**Figure 1** Plasma diacron of reactive oxygen metabolites (d-ROMs) level (A) and biological anti-oxidative potentials (BAP) level (B) at 0 and 7 weeks after the beginning of experimental period in KK mice fed SO, SP, FO and FP. Data are represented as

mean  $\pm$  SEM, n = 4 or 5. \*:  $P < 0.05$ , FO group in comparison with 0 week; \*\*:  $P < 0.05$ , FP group in comparison with 0 week by Tukey–Kramer test.

**Figure 2** Islet images of H&E staining (A), glucagon immunostaining (B), insulin immunostaining (C), mean islet area (D), islet area proportion (E), percentage of  $\alpha$ -cell area (F) and percentage of  $\beta$ -cell area (G) in KK mice fed SO, SP, FO and FP for 8 weeks. Magnification,  $\times 200$ . Scale bar,  $100\mu\text{m}$ . Data except for (E) are represented as mean  $\pm$  SEM, n = 4 or 5. Groups sharing different letters are significantly different:  $P < 0.05$  by Tukey–Kramer test.

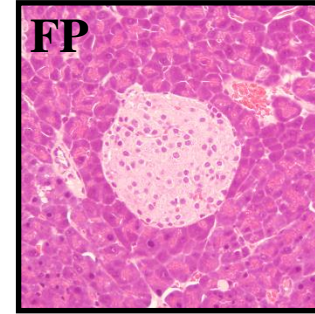
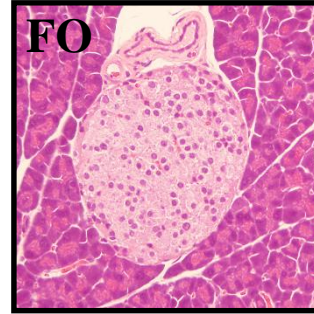
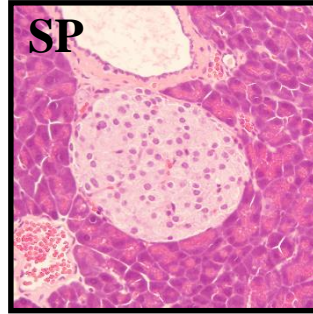
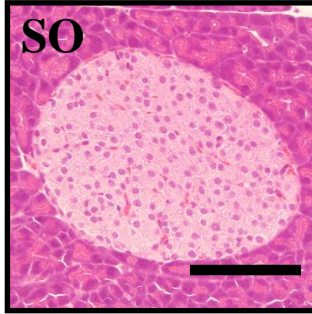
**Figure 3** Islet images of CHOP immunostaining (A), TUNEL staining (B), mean CHOP positive ratio (C) and mean TUNEL positive ratio (D) in KK mice fed SO, SP, FO and FP for 8 weeks. Arrows indicate positive nuclei with CHOP or TUNEL staining. Magnification,  $\times 200$ . Scale bar,  $100\mu\text{m}$ . Data are represented as mean  $\pm$  SEM, n = 4 or 5. Group undetected apoptotic cell in all islet are expressed as not-detected (N.D.). Groups sharing different letters are significantly different:  $P < 0.05$  by Tukey–Kramer test.

**Figure 1**

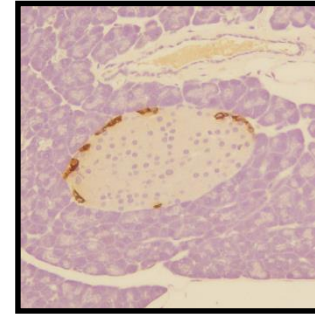
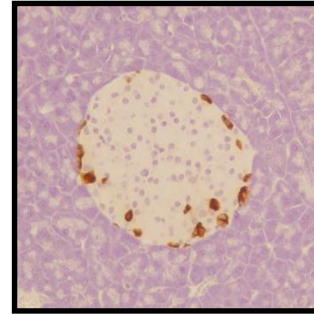
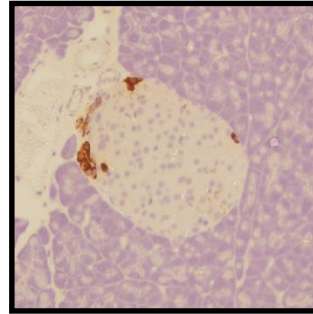
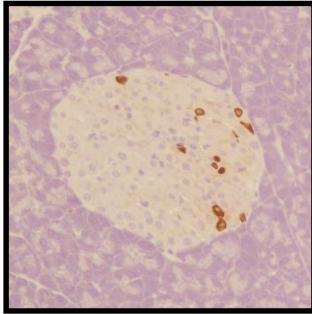


**Figure 2A-2C**

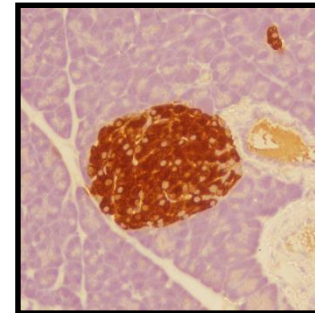
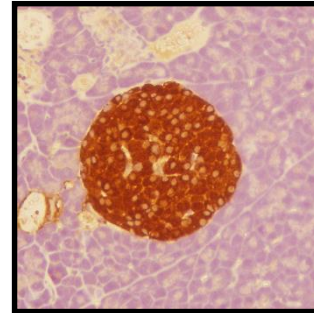
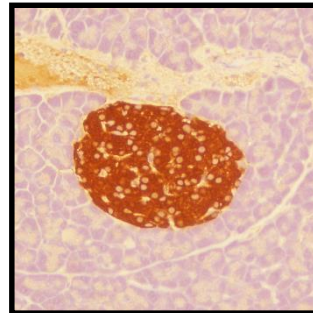
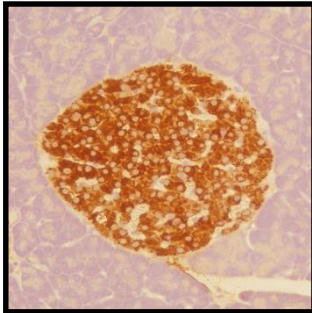
**A**



**B**

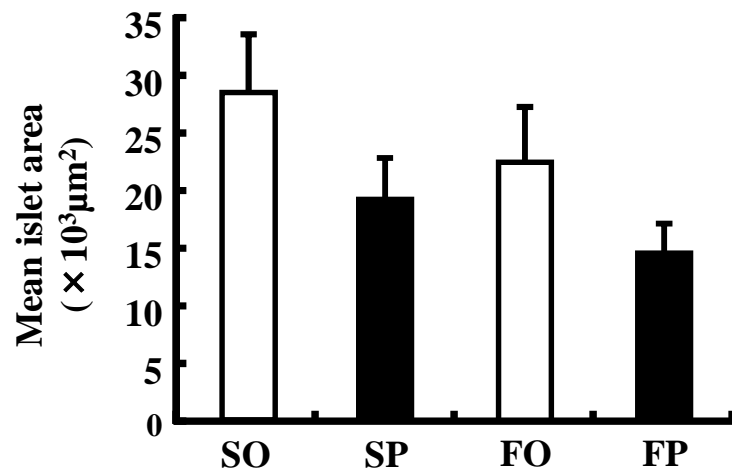


**C**

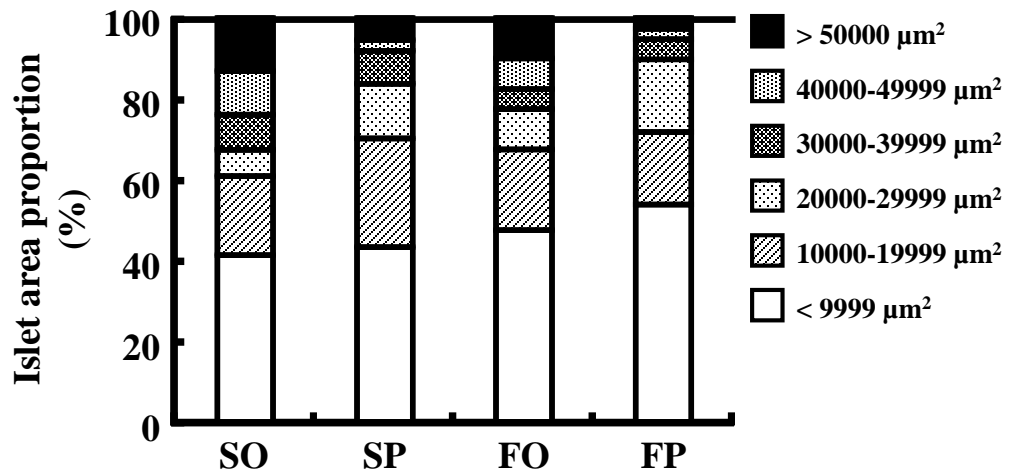


**Figure 2D-2G**

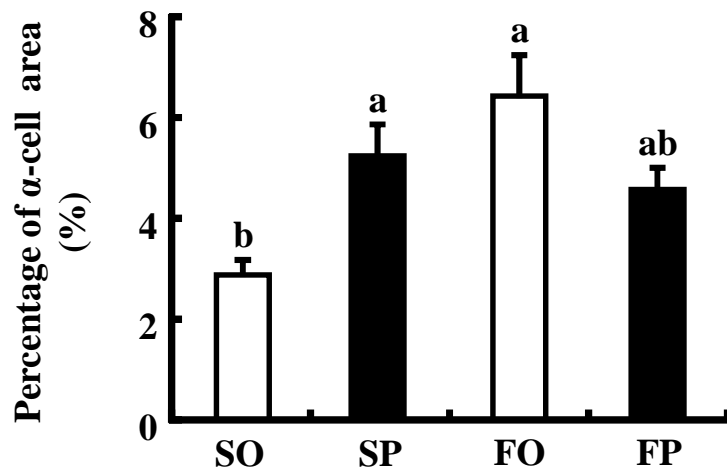
**D**



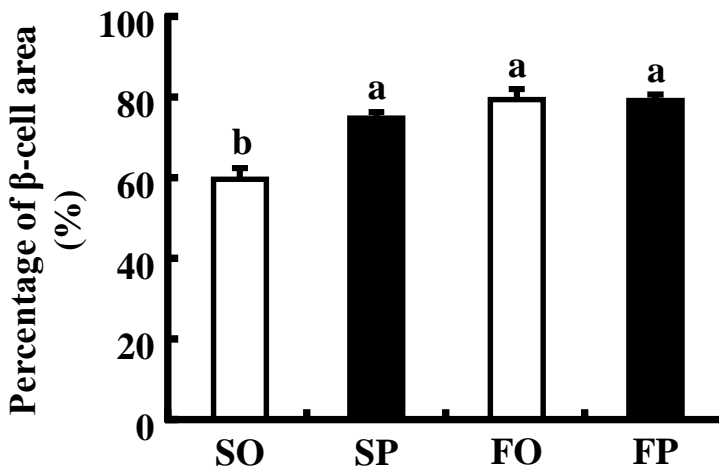
**E**



**F**

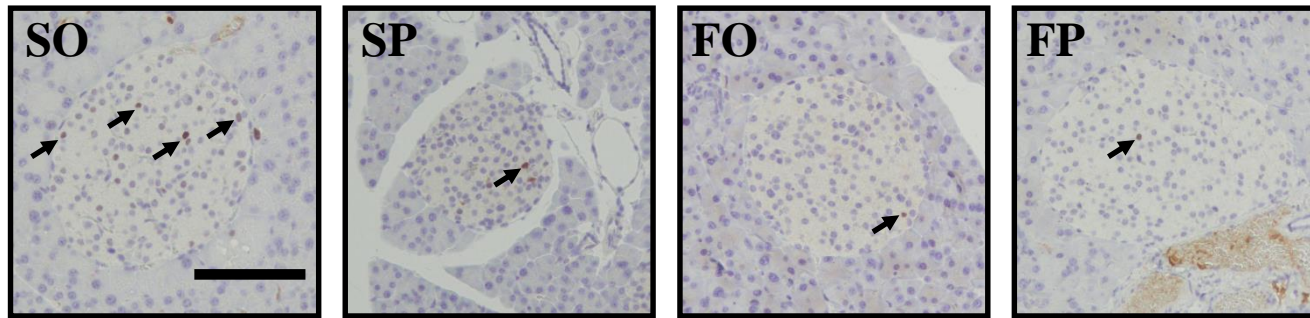


**G**

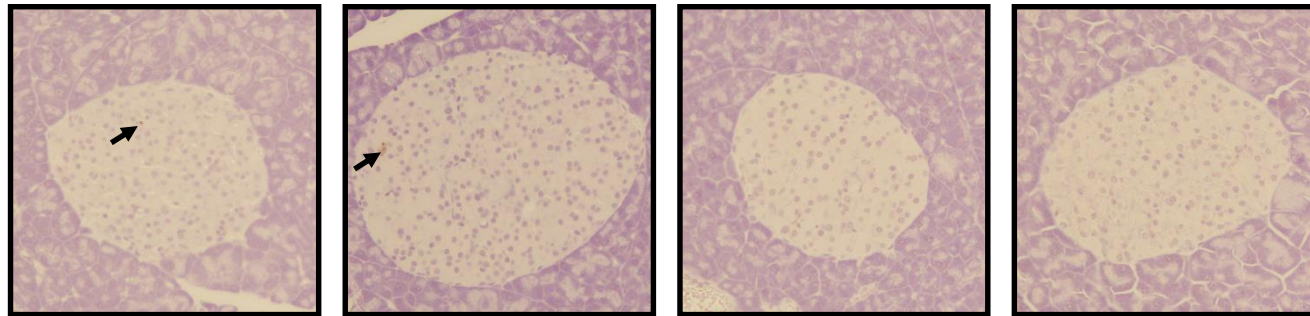


**Figure 3**

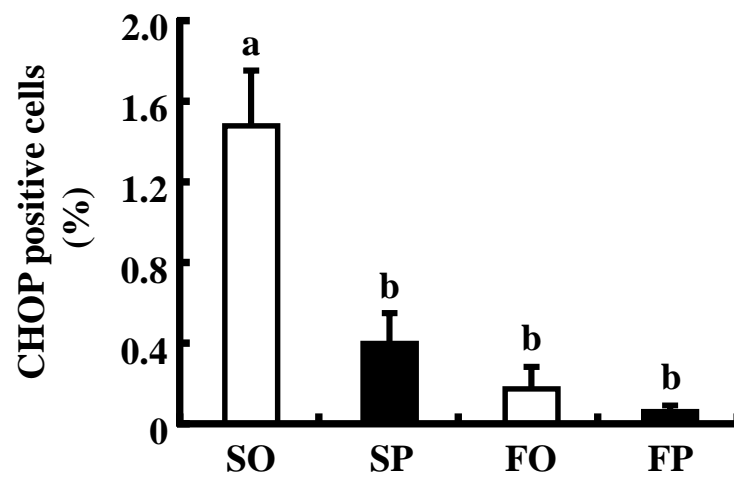
**A**



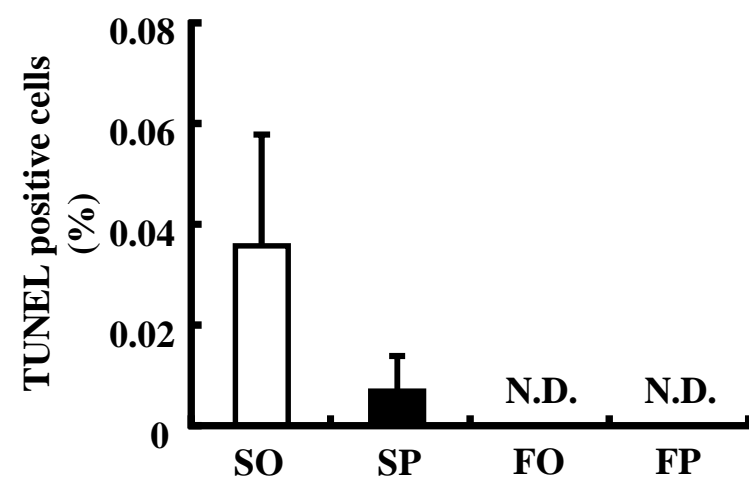
**B**



**C**



**D**





**Table 1** Blood glucose, plasma parameters and HOMA-IR index in KK mice fed SO, SP, FO and FP for 8 weeks<sup>1</sup>

Group	SO	SP	FO	FP
Blood glucose (mg/dL)	161 ± 20 <sup>b</sup>	243 ± 32 <sup>a</sup>	144 ± 3 <sup>b</sup>	144 ± 5 <sup>b</sup>
Plasma insulin (ng/mL)	15.0 ± 4.4 <sup>a</sup>	2.7 ± 0.8 <sup>b</sup>	9.0 ± 1.8 <sup>ab</sup>	5.1 ± 0.6 <sup>ab</sup>
HOMA-IR index <sup>2</sup>	147 ± 44 <sup>a</sup>	38 ± 13 <sup>b</sup>	83 ± 16 <sup>ab</sup>	47 ± 6 <sup>ab</sup>
Plasma adiponectin (µg/mL)	9.1 ± 1.3 <sup>c</sup>	37.2 ± 4.1 <sup>b</sup>	11.9 ± 0.7 <sup>c</sup>	68.0 ± 8.5 <sup>a</sup>

<sup>1</sup> Data are represented as mean ± SEM, n = 4 or 5. Values sharing different superscript letters in a row are significantly different:  $P < 0.05$  by Tukey–Kramer test.

<sup>2</sup> HOMA-IR index was calculated as follows: fasting blood glucose (mg/dL) × fasting plasma insulin (µU/mL)/405.

**Table 2** Pancreatic gene expression in KK mice fed SO, SP, FO and FP for 8 weeks<sup>1, 2</sup>

Group	SO	SP	FO	FP
<b><i>Inflammation</i></b>				
TNF $\alpha$	1.00 $\pm$ 0.19	1.63 $\pm$ 0.32	1.44 $\pm$ 0.20	1.28 $\pm$ 0.13
IL1 $\beta$	1.00 $\pm$ 0.03	1.07 $\pm$ 0.03	1.11 $\pm$ 0.02	1.08 $\pm$ 0.05
MCP1	1.00 $\pm$ 0.37	0.79 $\pm$ 0.17	0.76 $\pm$ 0.24	0.82 $\pm$ 0.11
TLR4	1.00 $\pm$ 0.22	1.25 $\pm$ 0.13	1.14 $\pm$ 0.15	1.26 $\pm$ 0.15
<b><i>Insulin synthesis and secretion</i></b>				
INS	1.00 $\pm$ 0.22 <sup>ab</sup>	0.57 $\pm$ 0.14 <sup>b</sup>	1.70 $\pm$ 0.31 <sup>a</sup>	0.25 $\pm$ 0.02 <sup>b</sup>
PDX1	1.00 $\pm$ 0.08 <sup>ab</sup>	0.74 $\pm$ 0.04 <sup>bc</sup>	1.08 $\pm$ 0.09 <sup>a</sup>	0.66 $\pm$ 0.02 <sup>c</sup>
AQP7	1.00 $\pm$ 0.19 <sup>c</sup>	3.11 $\pm$ 0.36 <sup>b</sup>	0.93 $\pm$ 0.13 <sup>c</sup>	5.07 $\pm$ 0.48 <sup>a</sup>
<b><i>Endoplasmic reticulum stress</i></b>				
CHOP	1.00 $\pm$ 0.24	0.63 $\pm$ 0.07	0.95 $\pm$ 0.12	0.62 $\pm$ 0.10

<sup>1</sup> Data are represented as mean  $\pm$  SEM, n = 4 or 5. Values sharing different superscript letters in a row are significantly different:  $P < 0.05$  by Tukey–Kramer test.

<sup>2</sup> The relative mRNA levels are represented as ratios to SO group.



**Supplementary Table 2** Primers used for real time PCR

Genes	Sense (5'→3')	Antisense (3' →5')
TNF $\alpha$	TTCATCAGTTCTATGGCCC	GGGAGTAGACAAGGTACAAC
IL1 $\beta$	CTCGGCCAAGACAGGTCGCTC	CCCCCACACGTTGACAGCTAGG
MCP1	GGCTCAGCCAGATGCAGT	GAGCTTGGTGACAAAACTACAG
TLR4	CTTCATTCAAGACCAAGCCTTTC	AACCGATGGACGTGTAAACCAG
INS	AGCAGGAAGGTTATTGTTTC	ACATGGGTGTGTAGAAGAAG
PDX1	GATGAAATCCACCAAAGCTC	TAAGAATTCTTCTCCAGCTC
AQP7	TGGGTTTTGGATTCGGAGT	TGTTCTTCTTGTCGGTGATGG
CHOP	AGTCCCTGCCTTTCACCTT	GCTTTGGGATGTGCGTGTG
GAPDH <sup>1</sup>	TGTGTCCGTCGTGGATCTGA	CCTGCTTCACCACCTTCTTGAT

<sup>1</sup> GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

**Supplementary Table 3** Total food intake, body weight, epididymal WAT weight and abdominal fat masses in KK mice fed SO, SP, FO and FP for 8 weeks<sup>1</sup>

Group	SO	SP	FO	FP
Total food intake (g/mouse)	281 ± 10 <sup>ab</sup>	290 ± 7 <sup>ab</sup>	299 ± 10 <sup>a</sup>	266 ± 5 <sup>b</sup>
Initial body weight (g)	26.1 ± 0.7	26.1 ± 0.6	26.1 ± 0.6	26.1 ± 0.6
Final body weight (g)	38.0 ± 0.4 <sup>b</sup>	42.2 ± 1.4 <sup>a</sup>	36.6 ± 0.7 <sup>b</sup>	38.8 ± 1.0 <sup>ab</sup>
Body weight gain (g)	+11.9 ± 0.6 <sup>b</sup>	+16.1 ± 0.8 <sup>a</sup>	+10.5 ± 0.8 <sup>b</sup>	+12.7 ± 0.5 <sup>b</sup>
Epididymal WAT weight (g)	1.34 ± 0.04 <sup>a</sup>	0.97 ± 0.05 <sup>b</sup>	0.91 ± 0.04 <sup>b</sup>	0.83 ± 0.05 <sup>b</sup>
Visceral fat mass (cm <sup>3</sup> )	1.59 ± 0.13 <sup>a</sup>	1.17 ± 0.13 <sup>ab</sup>	1.07 ± 0.07 <sup>b</sup>	1.04 ± 0.07 <sup>b</sup>
Subcutaneous fat mass (cm <sup>3</sup> )	0.44 ± 0.02 <sup>c</sup>	0.82 ± 0.04 <sup>a</sup>	0.40 ± 0.03 <sup>c</sup>	0.57 ± 0.03 <sup>b</sup>

<sup>1</sup> Data are represented as mean ± SEM, n = 4 or 5. Values sharing different superscript letters in a row are significantly different:  $P < 0.05$  by Tukey–Kramer test.