

RESEARCH ARTICLE

# Beneficial Effects of Canagliflozin in Combination with Pioglitazone on Insulin Sensitivity in Rodent Models of Obese Type 2 Diabetes

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## Abstract

### Background

Despite its insulin sensitizing effects, pioglitazone may induce weight gain leading to an increased risk of development of insulin resistance. A novel sodium glucose co-transporter 2 (SGLT2) inhibitor, canagliflozin, provides not only glycemic control but also body weight reduction through an insulin-independent mechanism. The aim of this study was to investigate the combined effects of these agents on body weight control and insulin sensitivity.

### Methods

Effects of combination therapy with canagliflozin and pioglitazone were evaluated in established diabetic KK-Ay mice and prediabetic Zucker diabetic fatty (ZDF) rats.

### Results

In the KK-Ay mice, the combination therapy further improved glycemic control compared with canagliflozin or pioglitazone monotherapy. Furthermore, the combination significantly attenuated body weight and fat gain induced by pioglitazone and improved hyperinsulinemia. In the ZDF rats, early intervention with pioglitazone monotherapy almost completely prevented the progressive development of hyperglycemia, and no further improvement was observed by add-on treatment with canagliflozin. However, the combination significantly reduced pioglitazone-induced weight gain and adiposity and improved the Matsuda index, suggesting improved whole-body insulin sensitivity.

Kuriyama, Kiichiro Ueta, Kenji Arakawa, and Masaharu Shiotani are employees of Mitsubishi Tanabe Pharma Corporation. Yoshinori Watanabe and Takaaki Senbonmatsu belong to Saitama Medical University Graduate School of Medicine. Takaaki Senbonmatsu has not received any payments for this study from Mitsubishi Tanabe Pharma Corporation. Canagliflozin was developed by Mitsubishi Tanabe Pharma Corporation in collaboration with Janssen Research & Development, LLC. The authors have declared that no competing interests exist. This does not alter the authors' adherence to PLOS ONE policies on sharing data and materials.

## Conclusions

Our study indicates that combination therapy with canagliflozin and pioglitazone improves insulin sensitivity partly by preventing glucotoxicity and, at least partly, by attenuating pioglitazone-induced body weight gain in two different obese diabetic animal models. This combination therapy may prove to be a valuable option for the treatment and prevention of obese type 2 diabetes.

## Introduction

Obesity is a global pandemic and one of the most important public health concerns in many countries [1,2]. Medical costs for people with obesity are approximately 30% greater than for people with normal weights, because obesity is related to serious health problems such as diabetes, hypertension, and other components of metabolic syndrome [3]. In particular, it has been well recognized that obesity is a risk factor for developing insulin resistance and type 2 diabetes [4–6]. Considering the increasing prevalence of obesity, it is predicted that the total number of people with diabetes in the world will rise to more than 366 million by 2030 [7].

At present, many types of pharmacological agents are available for the treatment of type 2 diabetes [8]. However, almost half of the diabetic adults in the United States still do not meet the targets for glycemic control [9]. It is important to carefully assess the risk–benefit profile before selecting the appropriate pharmacological therapy [10]. Weight gain is one of the side effects of the thiazolidinedione (TZD) insulin sensitizer, pioglitazone [11]. On the other hand, canagliflozin, the first-in-class sodium glucose co-transporter 2 (SGLT2) inhibitor available in the United States, not only lowers blood glucose through an insulin-independent mechanism but also reduces body weight [12,13] via excretion of excess blood glucose into the urine [14–16]. Position statements from the American Diabetes Association and the European Association for the Study of Diabetes recommend combination therapy with two or three drugs if monotherapy does not achieve target HbA1c levels [17]. It was recently reported that add-on treatment with canagliflozin significantly improved glycemic control and reduced body weight in patients with type 2 diabetes inadequately controlled by metformin and pioglitazone [18]. However, little is known about the effects of combination therapy with an SGLT2 inhibitor and TZD on insulin sensitivity.

In this study, we investigated how the combination of canagliflozin and pioglitazone affected body weight control and insulin sensitivity both in established diabetic KK-*A<sup>y</sup>* mice and pre-diabetic Zucker diabetic fatty (ZDF) rats.

## Materials and Methods

### Animals

All experimental procedures were approved by the institutional animal care and use committee of Mitsubishi Tanabe Pharma Corporation (Osaka, Japan) and animal care was conducted in accordance with institutional guidelines. Male KK-*A<sup>y</sup>* mice were purchased from CLEA Japan (Tokyo, Japan), and male Zucker diabetic fatty (ZDF) rats and Zucker lean (ZL) rats were purchased from Charles River Laboratories Japan (Tokyo, Japan). The animals were individually housed in plastic cages with ad libitum access to standard chow (CRF-1; Oriental Yeast Co., Ltd., Tokyo, Japan) and tap water. The animal rooms were controlled for

temperature ( $23 \pm 3^\circ\text{C}$ ), humidity ( $55 \pm 20\%$ ), and a 12-h light/dark cycle. These animals were acclimated for 1 week prior to the start of the studies.

## Drugs

Canagliflozin and pioglitazone hydrochloride were synthesized at the Medicinal Chemistry Laboratory of Mitsubishi Tanabe Pharma Corporation.

## Diabetes intervention study in KK-A<sup>y</sup> mice

Thirteen-week-old established diabetic male KK-A<sup>y</sup> mice were treated with canagliflozin (0.01% w/w food admixture), pioglitazone (0.01% w/w food admixture), and their combination (canagliflozin 0.01% w/w and pioglitazone 0.01% w/w food admixture) for 2 weeks. Average doses were estimated from body weight and daily food intake. Body weight was measured at least once a week. Accumulated food intake was calculated by summing the daily food intake. Plasma glucose and insulin were measured weekly. Blood samples were collected from the tail vein using a heparinized micro-capillary tube, and plasma was obtained by centrifugation at 1800 g for 15 min at 4°C. Twenty-four-hour urine samples were collected on days 14–15 using metabolic cages for the measurement of urinary glucose excretion (UGE). At the end of the study, body composition and histology of mesenteric adipose tissue were analyzed as described below.

## Body composition of KK-A<sup>y</sup> mice

Fat mass and lean mass (whole body) were evaluated at the end of the study using dual-energy X-ray absorptiometry (DEXA) (PIXImus2; GE Medical Systems, Inc., Madison, WI, USA). All images were analyzed using PIXImus2 software. In addition, computed tomography (CT) was performed using an experimental animal CT system (LaTheta LCT-100; Hitachi-Aloka Medical, Ltd., Tokyo, Japan). CT images (3-mm thick) were acquired from the entire abdominal region of each animal under pentobarbital anesthesia. Quantitative assessment of the subcutaneous and visceral fat was performed using LaTheta software. Subcutaneous and visceral fat tissues were distinguished by manual tracing of the abdominal wall in each of the sections.

## Histological analysis of adipose tissue in KK-A<sup>y</sup> mice

Mesenteric adipose tissues of KK-A<sup>y</sup> mice were isolated and immediately fixed in 10% neutralized buffered formalin. Formalin-fixed samples were embedded in paraffin, cut into 4- $\mu\text{m}$  sections, and stained with elastic van Gieson without nuclear staining. Whole slide digital images of the adipose tissue were obtained using Scan-Scope XT (Aperio Technologies, Inc., Vista, CA, USA). Five images were extracted in the area of 1,000,000  $\mu\text{m}^2$  from the whole slide images using Image-Scope (Aperio Technologies, Inc.). Each image was converted via 8-bit gray scale processing and binarization. The adipocyte area was then measured (excluding any area of  $<100 \mu\text{m}^2$ ) using Image-Pro Plus (Media Cybernetics, Inc., Bethesda, MD, USA). After manually excluding all non-adipocyte objects, such as intercellular spaces and blood vessels, a standard measure of 100 adipocytes per image was established.

## Diabetes prevention study in prediabetic ZDF rats

Seven-week-old prediabetic male ZDF rats were treated by oral gavage with canagliflozin (daily dose of 10 mg/kg), pioglitazone (daily dose of 10 mg/kg), and their combination at the same dosages for 6 weeks. All drugs were suspended in 0.5% w/v hydroxypropyl methylcellulose

(HPMC) at a concentration of 2 mg/mL. The drugs or vehicle (0.5% HPMC) were administered orally at a volume of 5 mL/kg body weight using a disposable feeding needle (Fuchigami Kikai, Kyoto, Japan) and syringe (Terumo, Tokyo, Japan). Accumulated food intake was calculated by summing the weekly food intake. Body weight, plasma glucose, plasma insulin, and HbA1c were measured every 2 weeks. Blood samples were collected from the tail vein, and plasma was obtained as described above. After the 6-week treatment period, overnight fasted animals were subjected to an oral glucose tolerance test (OGTT) 2 days after the last dosing. Blood samples were collected at 0, 15, 30, 60, and 120 min after oral administration of glucose solution (2 g/kg/5 mL). Whole-body insulin sensitivity during OGTT was assessed according to the Matsuda index, with values derived from fasting plasma glucose (FPG), fasting plasma insulin (FPI), mean plasma glucose during OGTT (MPG), and mean plasma insulin during OGTT (MPI), as previously reported [19]: Matsuda index =  $10,000 / \sqrt{\{(FPG \times FPI) \times (MPG \times MPI)\}}$

After OGTT, the rats were refed and retreated for 3 days. At the end of the study, the rats were euthanized by whole blood collection from the abdominal aorta under isoflurane anesthesia. Mesenteric, epididymal, and perirenal fat, as well as the pancreas, were quickly removed and weighed. The pancreas was immediately frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  to measure insulin.

## Analytical procedures

Plasma and urinary glucose levels were measured using the glucose CII-test Wako (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Pancreatic insulin was extracted by an acid-ethanol solution, as previously reported [14]. Plasma and pancreatic insulin levels were measured by ELISA, using the Ultra Sensitive Mouse Insulin ELISA Kit or Ultra Sensitive Rat Insulin ELISA Kit (Morinaga Institute of Biological Science, Inc., Yokohama, Japan). HbA1c in whole blood was measured using the DCA2000 analyzer (Bayer Medical, Ltd., Tokyo, Japan).

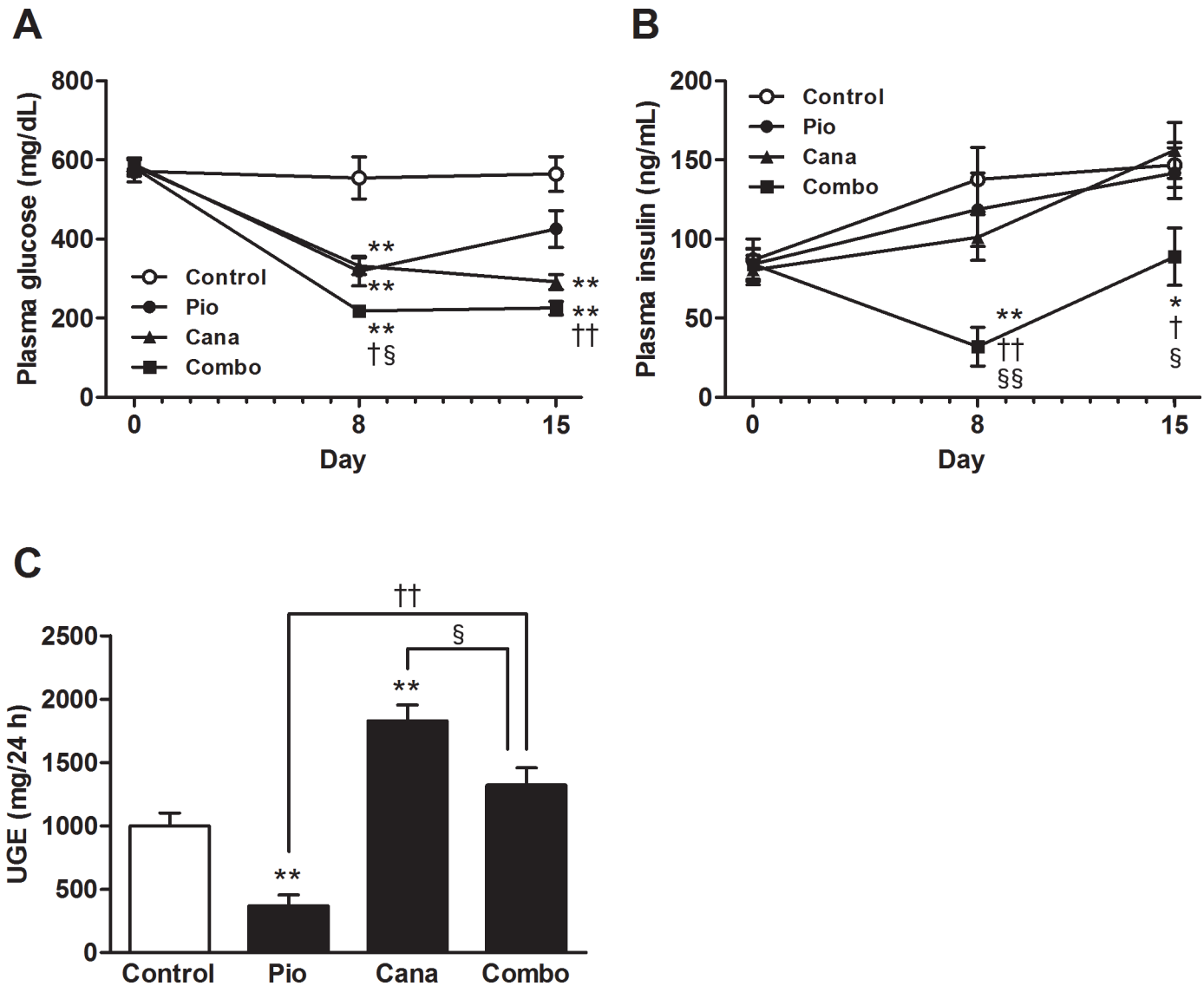
## Statistical analysis

Data are expressed as mean  $\pm$  standard error of the mean (SEM). Significant differences between groups were analyzed using one-way analysis of variance (ANOVA), followed by the Student's *t*-test, as previously reported [20,21]. Probabilities less than 5% ( $P < 0.05$ ) were considered significant. All statistical analyses were performed using the SAS system (SAS Institute Japan, Tokyo, Japan).

## Results

### Glycemic control in KK- $A^y$ mice

The effects of the combination of canagliflozin and pioglitazone on glycemic control were evaluated for 2 weeks in the established diabetic KK- $A^y$  mice. The average daily dose of the drugs (calculated from food intake and body weight) was as follows; pioglitazone 0.01% w/w food admixture, 13.0 mg/kg; canagliflozin 0.01% w/w food admixture, 16.6 mg/kg; and the combination of canagliflozin 0.01% w/w and pioglitazone 0.01% w/w food admixture, canagliflozin 15.1 mg/kg and pioglitazone 15.1 mg/kg. Canagliflozin or pioglitazone monotherapy significantly improved glycemic control, which improved further with the drugs in combination (Fig. 1A). Neither type of monotherapy affected plasma insulin levels, but the combination significantly ameliorated hyperinsulinemia (Fig. 1B). Although remarkable UGE was observed in the control KK- $A^y$  mice, canagliflozin monotherapy significantly enhanced UGE (Fig. 1C). UGE with the combination therapy was also significantly higher than that with pioglitazone



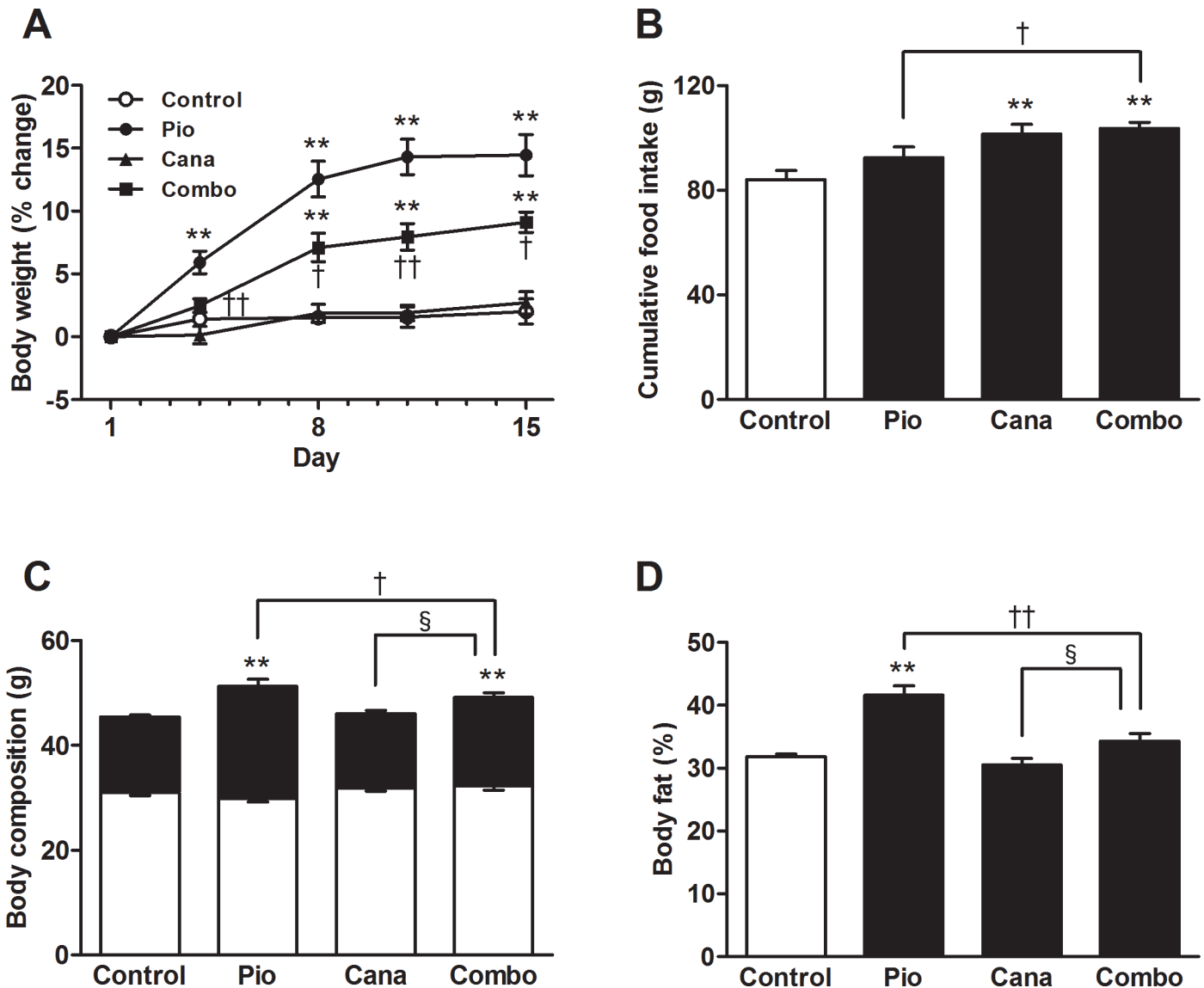
**Figure 1. Glycemic control and urinary glucose excretion (UGE) in KK-A<sup>y</sup> mice.** A: Plasma glucose, B: Plasma insulin, and C: UGE. Canagliflozin (Cana; 0.01% w/w food admixture), pioglitazone (Pio; 0.01% w/w food admixture), and their combination (Combo) were administered for 2 weeks. UGE was measured on days 14–15. Data are expressed as mean ± SEM (n = 7–8). \* *P* < 0.05, \*\* *P* < 0.01 vs. control, † *P* < 0.05, †† *P* < 0.01 vs. Pio, § *P* < 0.05, §§ *P* < 0.01 vs. Cana.

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monotherapy. However, UGE with the combination therapy was significantly lower than that with canagliflozin monotherapy, probably reflecting the contribution of pioglitazone to glycemic control.

### Body weight and adiposity in KK-A<sup>y</sup> mice

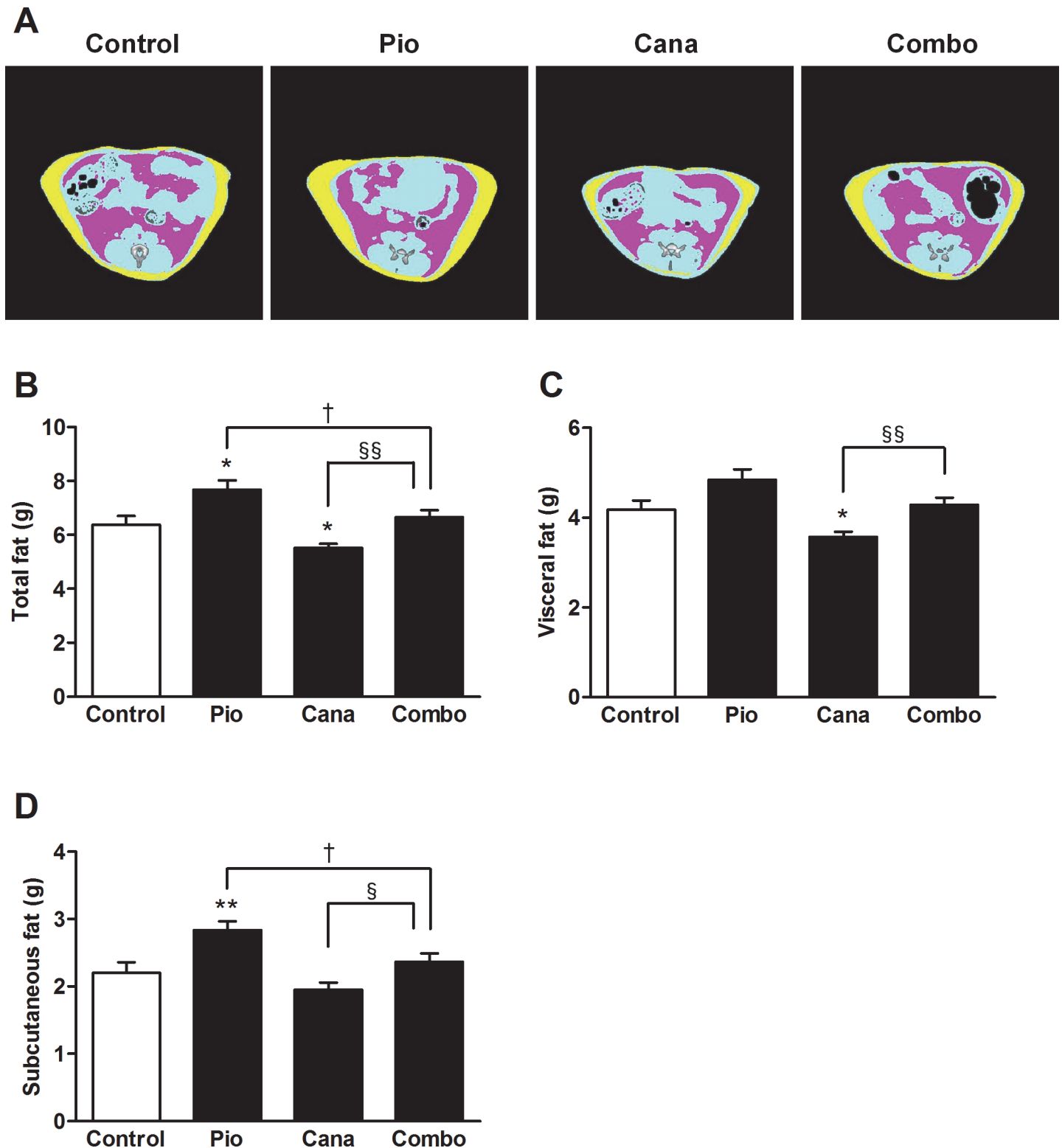
The 2-week treatment with pioglitazone monotherapy induced significant body weight gain in the KK-A<sup>y</sup> mice (Fig. 2A). Although canagliflozin monotherapy did not reduce body weight, the add-on treatment with canagliflozin significantly attenuated the pioglitazone-induced body weight gain. During the treatment period, canagliflozin monotherapy and the combination therapy each stimulated a slight increase in food intake (Fig. 2B). Body composition was



**Figure 2. Body weight, food intake, and body composition by dual-energy X-ray absorptiometry (DEXA) in KK-A<sup>y</sup> mice.** A: Body weight percent change, B: Cumulative food intake, C: Body composition (lean mass, open column; fat mass, closed column), and D: Body fat. Canagliflozin (Cana; 0.01% w/w food admixture), pioglitazone (Pio; 0.01% w/w food admixture), and their combination (Combo) were administered for 2 weeks. Body composition and body fat were measured at the end of the study using the DEXA scanner. Data are expressed as mean ± SEM (n = 7). \*\* *P* < 0.01 vs. control, † *P* < 0.05, †† *P* < 0.01 vs. Pio, § *P* < 0.05 vs. Cana.

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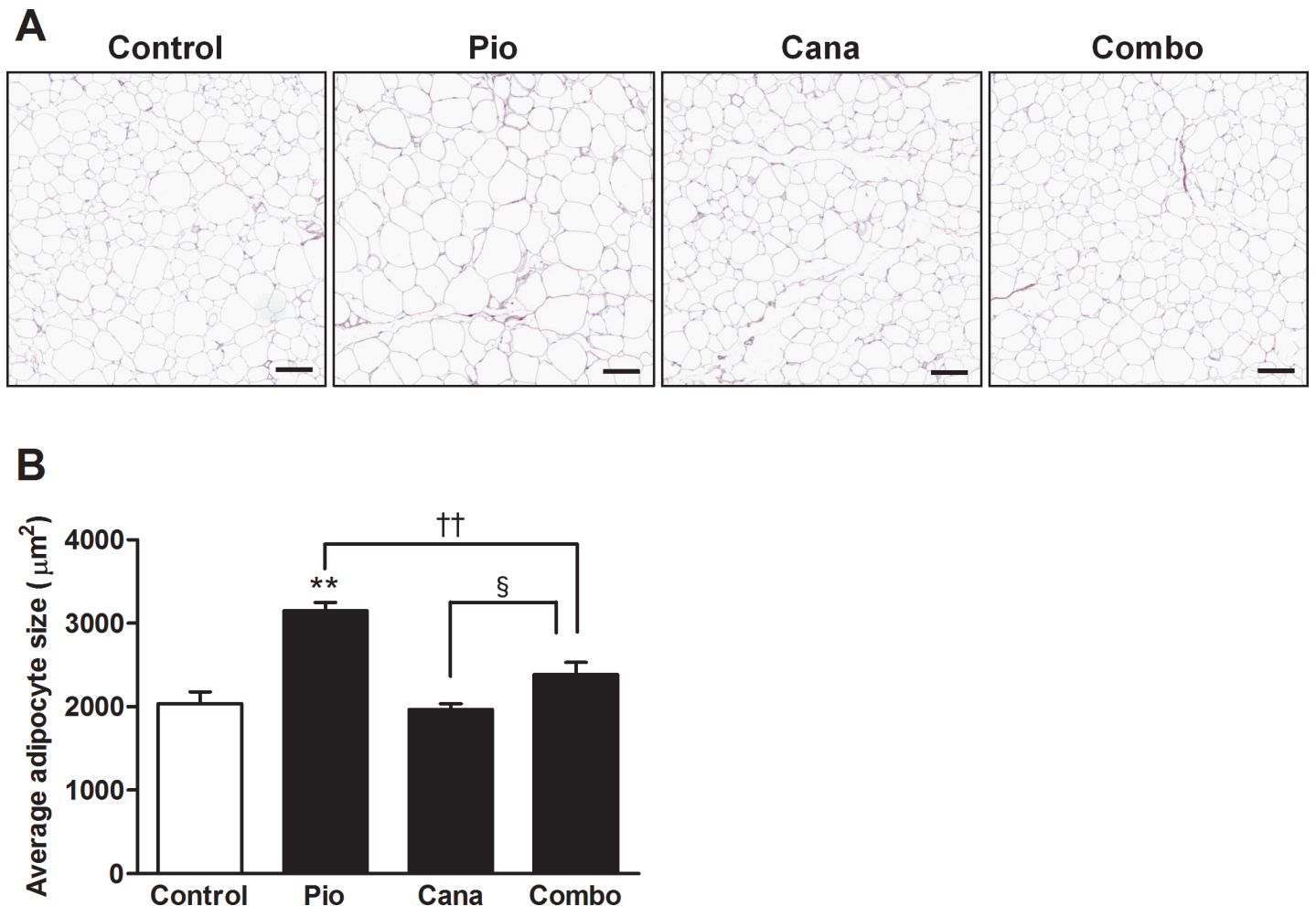
measured at the end of the study by DEXA scanning. Canagliflozin monotherapy did not affect body composition; however, the add-on treatment markedly attenuated any increase in pioglitazone-induced fat mass without affecting lean mass (Fig. 2C and 2D). The effects on adiposity were further confirmed by CT scanning, which showed that pioglitazone-induced fat gain was markedly reduced by the add-on treatment (Fig. 3A-D). Furthermore, adipocyte size was analyzed in the mesenteric adipose tissue of the KK-A<sup>y</sup> mice. Pioglitazone monotherapy clearly induced significant adipocyte hypertrophy (Fig. 4A and 4B). Canagliflozin monotherapy did not affect adipocyte size, whereas the add-on treatment significantly attenuated pioglitazone-induced adipocyte hypertrophy.



**Figure 3. Body fat analysis by computed tomography (CT) in KK-A<sup>y</sup> mice.** A: Representative CT images (red, visceral fat; yellow, subcutaneous fat), B: Total fat, C: Visceral fat, and D: Subcutaneous fat. Canagliflozin (Cana; 0.01% w/w food admixture), pioglitazone (Pio; 0.01% w/w food admixture), and their combination (Combo) were administered for 2 weeks. Adipose tissue weight was measured at the end of the study using an experimental animal CT system. Data are expressed as mean  $\pm$  SEM (n = 7–8). \*  $P < 0.05$ , \*\*  $P < 0.01$  vs. control, †  $P < 0.05$  vs. Pio, §  $P < 0.05$ , §§  $P < 0.01$  vs. Cana.

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**Figure 4. Histological analysis of adipose tissue in KK-*A<sup>y</sup>* mice.** A: Representative images of mesenteric adipose tissue and B: Average size of adipocytes. Canagliflozin (Cana; 0.01% w/w food admixture), pioglitazone (Pio; 0.01% w/w food admixture), and their combination (Combo) were administered for 2 weeks. Mesenteric adipose tissue was stained with elastic van Gieson without nuclear staining at the end of the study. The size of 500 adipocytes was analyzed using Image-Pro Plus software. Data are expressed as mean  $\pm$  SEM (n = 8). \*\*  $P < 0.01$  vs. control, ††  $P < 0.01$  vs. Pio, §  $P < 0.05$  vs. Cana. Scale bar = 100  $\mu$ m.

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### Diabetes prevention in ZDF rats

[Table 1](#) summarizes the effects of the combination of canagliflozin and pioglitazone on the development of diabetes in the prediabetic ZDF rats. At the beginning of the study, the ZDF rats exhibited marked hyperinsulinemia and subsequently developed progressive hyperglycemia as indicated by plasma glucose and HbA1c levels. Plasma insulin levels in the vehicle-treated ZDF rats gradually decreased to levels similar to those in the ZL rats. At the end of the study, pancreatic insulin content in the vehicle-treated ZDF rats was significantly lower than that in the ZL rats. Conversely, 6-week treatment with canagliflozin or pioglitazone monotherapy almost completely prevented the development of hyperglycemia; no further improvement was observed with the add-on treatment. The pancreatic insulin content after each monotherapy and the combination therapy was significantly higher than that of the vehicle-treated ZDF rats. Marked hyperinsulinemia was present in ZDF rats at the start of the treatment period. Pioglitazone monotherapy markedly reduced plasma insulin levels throughout the study period. In contrast, the insulin level was lower in the canagliflozin monotherapy group only at week



**Table 1. Effects of combination therapy on the development of diabetes in ZDF and ZL rats.**

		ZDF Vehicle	ZDF Pio	ZDF Cana	ZDF Combo	ZL Vehicle
Plasma glucose (mg/dL)	0 wk	139.7 ± 2.3 ##	137.7 ± 2.4	140.5 ± 3.6	141.6 ± 3.1	125.6 ± 1.0
	2 wk	241.5 ± 20.8 ##	117.7 ± 3.3 **	143.8 ± 6.5 **	118.6 ± 3.8 ** §§	125.0 ± 5.3
	4 wk	412.6 ± 7.6 ##	128.1 ± 6.2 **	147.1 ± 5.5 **	132.2 ± 4.8 **	122.4 ± 1.4
	6 wk	518.4 ± 12.8 ##	143.9 ± 3.4 **	195.5 ± 11.8 **	142.6 ± 4.5 ** §§	127.5 ± 3.9
HbA1c (%)	0 wk	2.7 ± 0.03 #	2.7 ± 0.02	2.7 ± 0.02	2.7 ± 0.03	2.6 ± 0.02
	2 wk	3.6 ± 0.06 ##	2.8 ± 0.03 **	3.3 ± 0.04 **	3.0 ± 0.03 ** §§	3.0 ± 0.04
	4 wk	5.8 ± 0.20 ##	3.1 ± 0.03 **	3.5 ± 0.03 **	3.0 ± 0.04 ** §§	3.1 ± 0.03
	6 wk	8.1 ± 0.17 ##	3.5 ± 0.09 **	3.8 ± 0.08 **	3.4 ± 0.11 ** §§	3.4 ± 0.03
Plasma insulin (ng/mL)	0 wk	13.9 ± 1.3 ##	12.7 ± 1.1	13.8 ± 0.8	13.0 ± 0.9	0.4 ± 0.1
	2 wk	15.5 ± 2.0 ##	3.9 ± 0.3 **	7.7 ± 1.2 **	4.0 ± 0.4 ** §	1.0 ± 0.2
	4 wk	6.9 ± 1.3 ##	5.8 ± 0.6	9.4 ± 0.8	3.9 ± 0.5 † §§	0.9 ± 0.1
	6 wk	3.5 ± 0.2 ##	5.2 ± 0.4 **	12.5 ± 1.3 **	4.0 ± 0.3 † §§	1.7 ± 0.1
Pancreatic insulin (µg/g pancreas)	6 wk	10.4 ± 2.0 ##	99.7 ± 14.6 **	76.4 ± 11.5 **	79.3 ± 15.1 **	59.7 ± 7.9
Body weight (g)	0 wk	207.4 ± 2.4 ##	207.3 ± 2.1	207.8 ± 2.3	207.4 ± 2.0	162.0 ± 2.6
	2 wk	300.2 ± 2.9 ##	329.1 ± 2.4 **	283.2 ± 2.2 **	305.1 ± 2.5 †† §§	223.4 ± 2.9
	4 wk	338.1 ± 7.2 ##	432.6 ± 3.8 **	349.8 ± 3.7	399.5 ± 4.3 ** †† §§	253.9 ± 4.1
	6 wk	363.3 ± 6.4 ##	533.6 ± 5.7 **	403.5 ± 4.8 **	480.7 ± 8.5 ** †† §§	281.4 ± 5.0
Food intake (g/day)	2 wk	27.7 ± 0.5 ##	36.0 ± 0.5 **	27.7 ± 0.2	34.0 ± 0.3 ** †† §§	17.4 ± 0.4
	4 wk	32.1 ± 0.8 ##	38.6 ± 0.6 **	33.5 ± 0.6	40.8 ± 0.9 ** §§	17.6 ± 0.4
	6 wk	37.8 ± 1.3 ##	38.3 ± 0.6	35.3 ± 0.6	38.5 ± 2.3	17.3 ± 0.4
Cumulative food intake (g)		1109.8 ± 22.6 ##	1298.2 ± 17.5 **	1126.4 ± 15.5	1337.7 ± 20.4 ** §§	613.8 ± 12.6

Canagliflozin (Cana; daily dose of 10 mg/kg), pioglitazone (Pio; daily dose of 10 mg/kg), and their combination (Combo) were administered by oral gavage for 6 weeks. All values were measured in the nonfasting state. Data are expressed as mean ± SEM (n = 8). # *P* < 0.05, ## *P* < 0.01 vs. ZL rats, \*\* *P* < 0.01 vs. vehicle-treated ZDF rats, † *P* < 0.05, †† *P* < 0.01 vs. Pio-treated ZDF rats, § *P* < 0.05, §§ *P* < 0.01 vs. Cana-treated ZDF rats.

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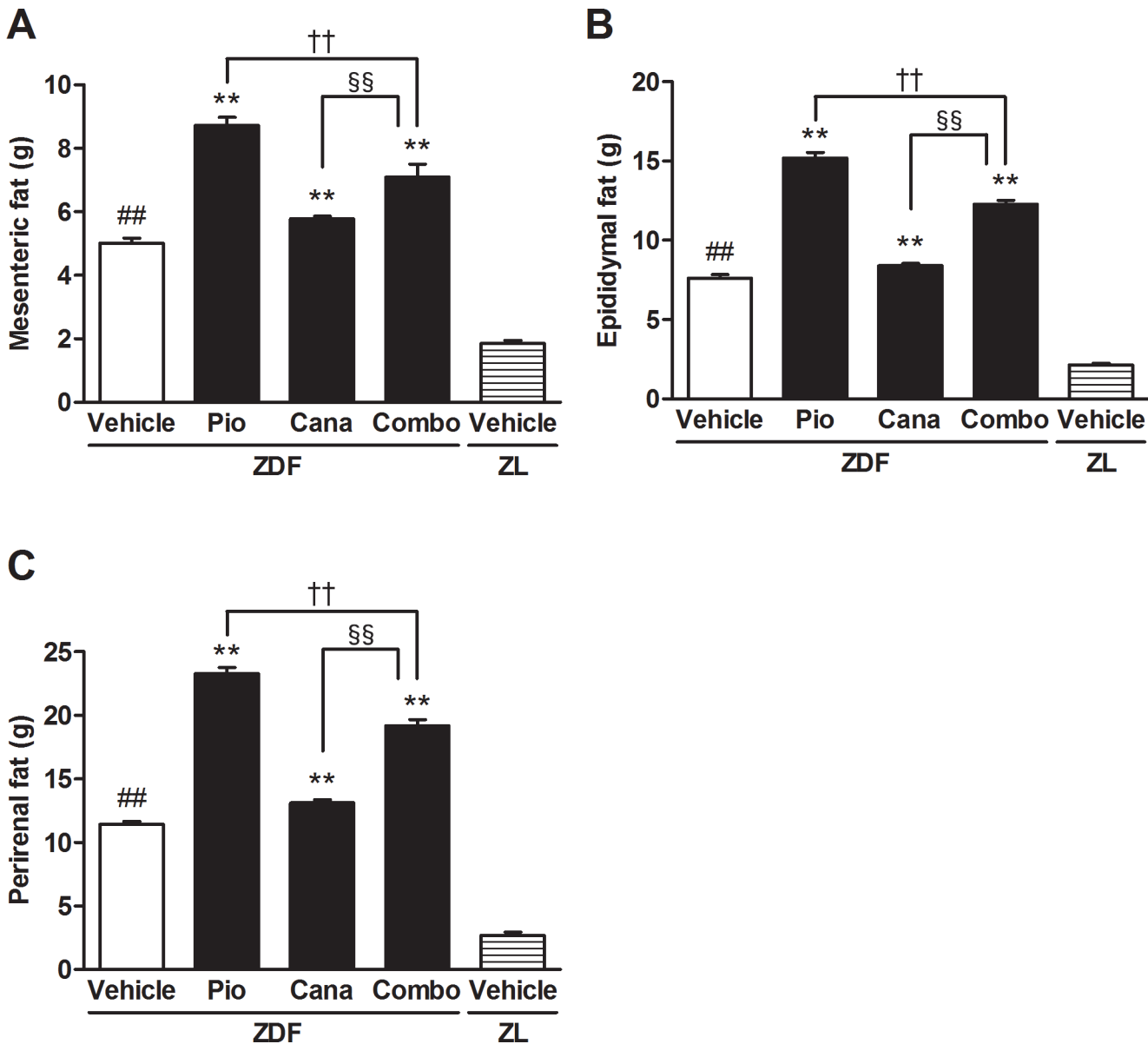
2. Add-on treatment of canagliflozin further reduced plasma insulin levels at weeks 4 and 6 compared with pioglitazone monotherapy.

### Body weight and adiposity in ZDF rats

Pioglitazone monotherapy significantly increased body weight throughout the study in the ZDF rats, whereas canagliflozin monotherapy slightly but significantly reduced body weight at week 2 and then increased it at week 6 (Table 1). However, the add-on treatment significantly attenuated pioglitazone-induced body weight gain. During the treatment period, pioglitazone monotherapy and the combination therapy significantly increased food intake, but canagliflozin monotherapy did not affect food intake (Table 1). At the end of the study, mesenteric, epididymal, and perirenal fat weights were measured. Pioglitazone monotherapy significantly increased all of these adipose tissue weights (Fig. 5A-C). Although canagliflozin monotherapy slightly increased these fat weights, the add-on treatment significantly attenuated the pioglitazone-induced adiposity.

### Insulin sensitivity in ZDF rats

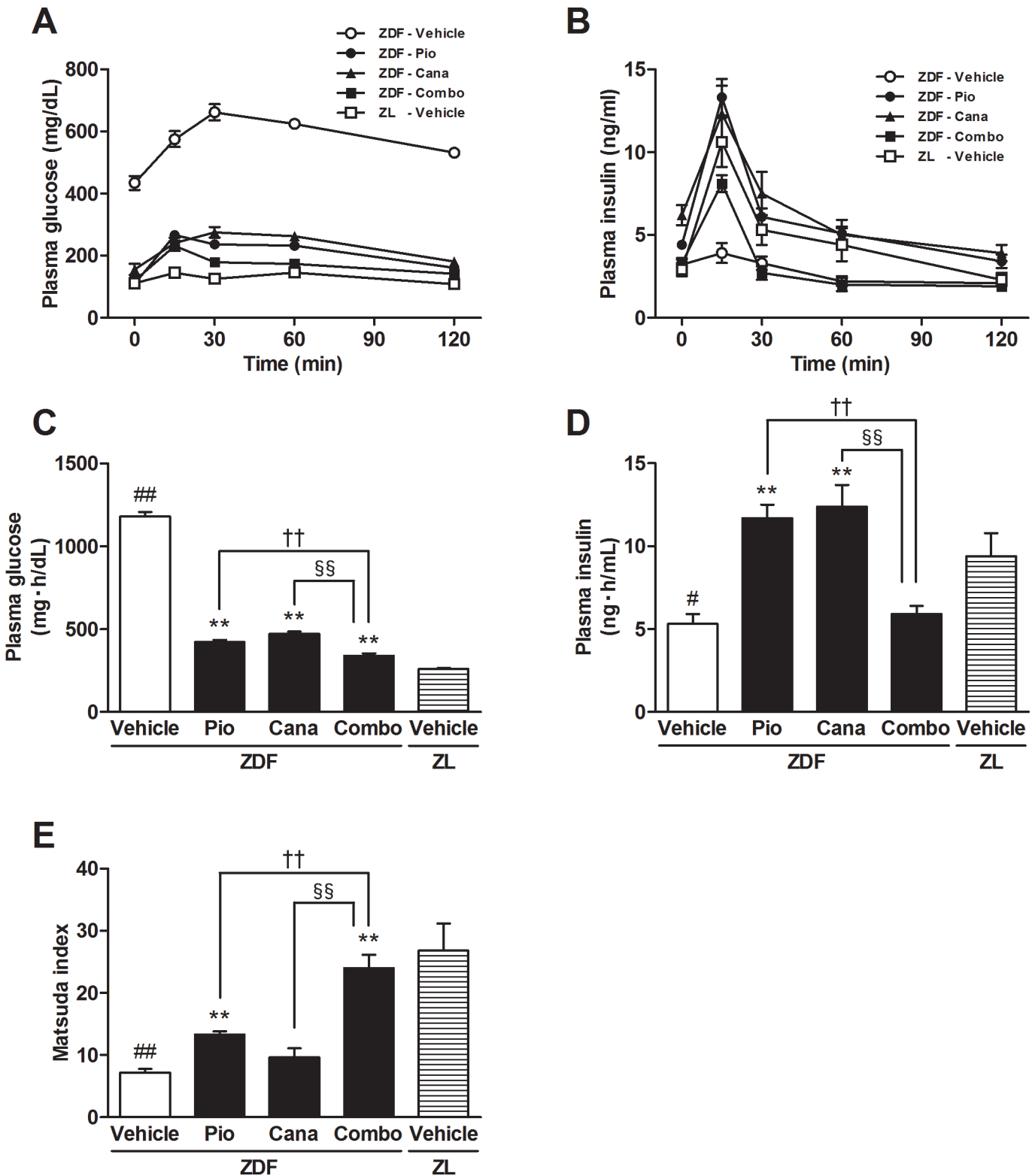
OGTT was performed after the 6-week treatment in overnight fasted ZDF rats (Fig. 6A-D and Table 2). The AUC<sub>0-2h</sub> of plasma glucose levels during OGTT was significantly higher in the vehicle-treated ZDF rats than in the ZL rats. Each monotherapy significantly improved glucose intolerance, and further amelioration was achieved by the combination therapy. Although



**Figure 5. The adipose tissue weight of ZDF and ZL rats.** A: Mesenteric fat, B: Epididymal fat, and C: Perirenal fat. Canagliflozin (Cana; daily dose of 10 mg/kg), pioglitazone (Pio; daily dose of 10 mg/kg), and their combination (Combo) were administered by oral gavage for 6 weeks. Adipose tissue weight was measured at the end of the study. Data are expressed as mean  $\pm$  SEM ( $n = 8$ ). ##  $P < 0.01$  vs. ZL rats, \*\*  $P < 0.01$  vs. vehicle-treated ZDF rats, ††  $P < 0.01$  vs. Pio-treated ZDF rats, §§  $P < 0.01$  vs. Cana-treated ZDF rats.

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glucose-stimulated insulin secretion almost disappeared in the vehicle-treated ZDF rats, insulin secretion during the first 30 min was markedly improved by each monotherapy and the combination therapy. The  $AUC_{0-2h}$  of plasma insulin levels was significantly higher with both monotherapies, but not with the combination therapy, reflecting the effects of basal insulin and insulin secretion during OGTT. The Matsuda index revealed that pioglitazone monotherapy significantly improved insulin sensitivity (Fig. 6E). Whereas canagliflozin monotherapy slightly but not significantly improved insulin sensitivity, the combination therapy significantly improved insulin sensitivity compared with the respective monotherapies.



**Figure 6. The oral glucose tolerance test (OGTT) and Matsuda index in ZDF and ZL rats.** A: Plasma glucose, B: Plasma insulin, C: AUC<sub>0-2 h</sub> of plasma glucose, D: AUC<sub>0-2 h</sub> of plasma insulin, and E: Matsuda index. Canagliflozin (Cana; daily dose of 10 mg/kg), pioglitazone (Pio; daily dose of 10 mg/kg), and their combination (Combo) were administered by oral gavage for 6 weeks. OGTT was performed 2 days after the last dosing. Data are expressed as mean  $\pm$  SEM (n = 8). #  $P < 0.05$ , ##  $P < 0.01$  vs. ZL rats, \*  $P < 0.05$ , \*\*  $P < 0.01$  vs. vehicle-treated ZDF rats, ††  $P < 0.01$  vs. Pio-treated ZDF rats, §§  $P < 0.01$  vs. Cana-treated ZDF rats.

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**Table 2. Effects of combination therapy on glucose and insulin excursions during OGTT in ZDF and ZL rats.**

		ZDF Vehicle	ZDF Pio	ZDF Cana	ZDF Combo	ZL Vehicle
Plasma glucose (mg/dL)	0 min	434.3 ± 22.4 ##	109.8 ± 4.2 **	155.8 ± 18.2 **	119.4 ± 2.8 **	110.7 ± 3.7
	15 min	575.3 ± 25.9 ##	266.4 ± 8.8 **	241.1 ± 17.0 **	231.4 ± 16.0 **	144.7 ± 7.2
	30 min	661.8 ± 25.7 ##	236.1 ± 12.1 **	275.3 ± 17.1 **	178.8 ± 13.5 ** †† §§	125.8 ± 3.2
	60 min	624.2 ± 13.0 ##	232.0 ± 9.3 **	262.5 ± 11.9 **	173.4 ± 9.4 ** †† §§	145.6 ± 3.8
	120 min	531.7 ± 10.0 ##	160.9 ± 4.9 **	180.7 ± 10.9 **	141.7 ± 8.1 ** §	108.3 ± 2.4
Plasma insulin (ng/mL)	0 min	3.2 ± 0.3	4.4 ± 0.2 **	6.2 ± 0.6 **	3.3 ± 0.3 † §§	2.9 ± 0.4
	15 min	3.9 ± 0.6 ##	13.3 ± 1.1 **	12.3 ± 1.7 **	8.1 ± 0.5 ** †† §	10.6 ± 1.5
	30 min	3.3 ± 0.4	6.1 ± 0.5 **	7.5 ± 1.3 **	2.7 ± 0.4 †† §§	5.3 ± 0.9
	60 min	2.2 ± 0.3	5.1 ± 0.8 **	5.0 ± 0.5 **	2.0 ± 0.4 †† §§	4.4 ± 1.0
	120 min	2.1 ± 0.2	3.4 ± 0.4 *	3.9 ± 0.5 **	1.9 ± 0.2 †† §§	2.3 ± 0.4

Canagliflozin (Cana; daily dose of 10 mg/kg), pioglitazone (Pio; daily dose of 10 mg/kg), and their combination (Combo) were administered by oral gavage for 6 weeks. Overnight-fasted animals were subjected to OGTT 2 days after the last dosing. Data are expressed as mean ± SEM (n = 8). ## *P* < 0.01 vs. ZL rats, \* *P* < 0.05, \*\* *P* < 0.01 vs. vehicle-treated ZDF rats, † *P* < 0.05, †† *P* < 0.01 vs. Pio-treated ZDF rats, § *P* < 0.05, §§ *P* < 0.01 vs. Cana-treated ZDF rats.

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## Discussion

We demonstrated that the combination therapy with canagliflozin and pioglitazone markedly improved hyperinsulinemia in established diabetic KK-*A<sup>y</sup>* mice, implying improved insulin sensitivity. Furthermore, the effects on insulin sensitivity were evaluated in the ZDF rats after early pharmacological intervention with the combination therapy. Several insulin resistance/sensitivity indices are calculated from plasma glucose and insulin in the fasting state and during OGTT. These include HOMA-IR and the Matsuda index, although the former may not represent appropriate insulin resistance in a subject with severely impaired β-cell function [22]. Conversely, the Matsuda index highly correlates with whole-body insulin sensitivity, as determined by the euglycemic insulin clamp technique, which is the gold standard for measuring insulin resistance [19]. In this context, we used the Matsuda index to estimate whole-body insulin sensitivity in the ZDF rats with progressive β-cell dysfunction [23]. We observed that the Matsuda index was significantly improved by early intervention with the combination therapy, which strongly suggests that combination therapy improves whole-body insulin sensitivity.

It is well known that the ability of insulin to stimulate glucose uptake and inhibit hepatic glucose production is impaired at an early stage in type 2 diabetes [24,25]. As a consequence, pancreatic β-cells respond by increasing insulin secretion to maintain normoglycemia [26]. In the presence of β-cell deficits, this compensatory response is progressively lost, yielding overt hyperglycemia [27]. Thereafter, sustained hyperglycemia leads to glucotoxicity, which further worsens insulin sensitivity and secretion [28–30]. Correction of hyperglycemia with a non-selective SGLT inhibitor, phlorizin, normalizes insulin sensitivity in partially pancreatectomized diabetic rats with moderate glucose intolerance [31] and non-insulin-dependent diabetic rats induced by neonatal streptozotocin [32]. In the present study, we observed that the combination therapy with a selective SGLT2 inhibitor, canagliflozin, and an insulin sensitizer, pioglitazone, further improved glycemic control in the KK-*A<sup>y</sup>* mice with established diabetes. This finding implies that insulin resistance may be improved by the prevention of glucotoxicity. On the other hand, early intervention with either canagliflozin or pioglitazone monotherapy

almost completely prevented the progressive development of hyperglycemia in the ZDF rats. At the end of the study, each monotherapy and the combination therapy maintained pancreatic insulin content and glucose-stimulated insulin secretion during OGTT at levels comparable with those in the ZL rats, probably due to the attenuation of glucotoxicity. These results strongly suggest that early intervention is favorable to maintain pancreatic  $\beta$ -cell function. This is consistent with a previous report that states early intervention with antidiabetic agents can prevent type 2 diabetes [33]. In addition, whereas no further improvement in glycemic control was achieved by the add-on treatment with canagliflozin, the combination therapy significantly improved whole-body insulin sensitivity compared with the respective monotherapies. Taken together, these results imply the existence of mechanisms independent of blood glucose lowering that underlie the improvement of insulin sensitivity observed with the combination therapy.

As obesity is a risk factor for developing insulin resistance [4,5], control of body weight is important for the prevention and treatment of type 2 diabetes. In the present study, we observed that add-on treatment with canagliflozin significantly attenuated pioglitazone-induced body weight and fat gain. Such a response may partly contribute to the improvement of insulin sensitivity independent of blood glucose lowering. This attenuation of weight gain was explained by enhanced urinary calorie loss via canagliflozin-induced UGE, as observed in KK- $A^Y$  mice. In addition, our previous study showed that canagliflozin monotherapy significantly reduces the respiratory exchange ratio in DIO mice [15], suggesting that increased fatty acid metabolism may account for the reduction in body weight gain.

Certain limitations of this study should be considered when interpreting the results. First, body weight reduction may be attenuated by compensatory hyperphagia in response to increased UGE. This has been previously reported in DIO rats treated with the SGLT2 inhibitor dapagliflozin [34]. We observed that canagliflozin induced compensatory hyperphagia in the KK- $A^Y$  mice, possibly explaining the lack of reduction of body weight gain with canagliflozin monotherapy. Second, plasma insulin may affect body weight via its anabolic effects. We recently reported that plasma insulin levels are transiently elevated at 6–7 weeks of age in vehicle-treated ZDF rats, and then gradually decrease because of pancreatic exhaustion [35]. In addition, the above study showed that canagliflozin attenuates the pancreatic exhaustion and subsequently increases plasma insulin levels, resulting in body weight gain. In the present study, canagliflozin monotherapy induced this paradoxical weight gain without compensatory hyperphagia. In contrast, the pioglitazone monotherapy induced body weight gain despite lower plasma insulin levels via its insulin-sensitizing effects. Thus, canagliflozin and pioglitazone both increase body weight gain in ZDF rats, although plasma insulin levels differ between canagliflozin- and pioglitazone-treated animals because of their different modes of action. The combination of these two drugs achieved the lowest plasma insulin levels among the drug treatment groups and attenuated pioglitazone-induced body weight gain. Finally, there are differences between rodent and human type 2 diabetes. Compensatory hyperphagia was observed in canagliflozin-treated KK- $A^Y$  mice but was not reported in clinical trials of canagliflozin. Furthermore, the SGLT2 inhibitor-induced increase in plasma insulin levels and subsequent weight gain is limited to animal models such as ZDF rats and db/db mice, which develop severe pancreatic dysfunction within a short period [35–37].

In conclusion, our findings suggest that combination therapy with canagliflozin and pioglitazone markedly improves hyperglycemia and hyperinsulinemia in obesity-associated type 2 diabetes. Furthermore, the addition of canagliflozin to pioglitazone therapy improves insulin sensitivity partly by preventing glucotoxicity and, at least partly, by attenuating pioglitazone-induced body weight gain. This combination therapy may represent a valuable option for the treatment and prevention of obesity-associated type 2 diabetes.

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## Author Contributions

Conceived and designed the experiments: YW KN NT CK KU MS. Performed the experiments: YW KN NT YH KU. Analyzed the data: YW KN NT YH. Contributed reagents/materials/analysis tools: YW KN NT YH. Wrote the paper: YW KA TS.

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