Diabetic Macular Edema-like Ocular Lesions in Male Spontaneously Diabetic Torii Fatty Rats

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Short title

DME-like lesions in SDT fatty rats

Summary

Diabetic macular edema (DME) is a major factor contributing to visual disabilities in diabetic patients, and the number of patients is increasing. Animal models play a key role in the development of novel therapies. In this study, pathophysiological analyses of ocular lesions in Spontaneously Diabetic Torii (SDT) fatty rats were performed. First, vascular endothelial growth factor (VEGF) concentrations in vitreous humor, retinal vascular permeability and retinal thickness were measured in SDT fatty rats (Experiment 1). Furthermore, the pharmacological effects of two anti-diabetic drugs, phlorizin and pioglitazone, on retinal lesions were evaluated (Experiment 2). As results, the SDT fatty rats exhibited VEGF increase in vitreous humor at 8 and 16 weeks of age, and both retinal vascular hyperpermeability and retinal thickening at 16 weeks of age. In particular, the layers between the retinal internal limiting membrane and the outer nuclear layer were thickened. Phlorizin treatment from 4 to 16 weeks of age improved hyperglycemia and normalized retinal thickness; however, the effect of pioglitazone on retinal thickness was not strong despite the normalization of hyperglycemia. These data demonstrate that the male SDT fatty rat is a useful model for developing new therapeutic approaches in DME.

Key words

SDT fatty rat, VEGF, diabetic retinopathy, diabetic macular edema, retinal thickening

Introduction

Diabetic macular edema (DME) is a frequent finding associated with diabetic retinopathy (DR) and is a major factor contributing to visual disabilities in patients with diabetes mellitus (Cheung *et al.* 2010). The current worldwide estimates are 92.6 million patients with DR and 20.6 million DME patients, with the number of sufferers expected to rise in the future (Yau *et al.* 2012). The relationship between DR and Hemoglobin A1c (HbA1c) was revealed in the landmark Diabetes Control and Complications Trial (DCCT) study (The Diabetes Control and Complications Trial Research Group 1996). Therefore, sustained lowering of blood glucose levels can prevent the risk of progression of DR and other diabetic complications. As such, numerous anti-diabetic drugs have been developed by pharmaceutical companies and seven different types of agents are now available: sulfonylureas, biguanides, α-glucosidase inhibitors, glinides, thiazolidinediones, incretin related drugs and sodium-glucose cotransporter (SGLT) 2 inhibitors (Takamoto and Kadowaki 2011, Chen *et al.* 2016). However, despite the numerous available choices, diabetic complications often develop and lead to both life-threatening and life-altering problems.

Many years of diabetes leads to biochemical and molecular abnormalities, such as the production of advanced glycation end-products (AGEs), generation of reactive oxygen species (ROS) and activation of protein kinase C (PKC) followed by cytokines, including vascular endothelial growth factor (VEGF), and chemokine production in

retina and retinal capillaries (Das 2016).

VEGF, as a permeable factor, induces the alteration of tight junction proteins that contributes to a breakdown of the blood-retinal barrier (BRB), promotes vascular permeability and causes retinal edema (Kim *et al.* 2009, Murakami *et al.* 2009). Hence, neutralizing anti-VEGF antibodies, such as ranibizumab, bevacizumab and VEGF trap, have become standard of care for DME in the past 10 years. However, 25% of DME patients do not respond to anti-VEGF treatment (Singer *et al.* 2016). Therefore, the elucidation of the underlying mechanism is highly anticipated. To reveal the mechanism, a few animal models have been investigated to date. Streptozotocin (STZ) induced rats (Yu *et al.* 2010) or mice (Anand-Apte *et al.* 2010), Otsuka Long-Evans Tokushima Fatty (OLETF) rats (Lu *et al.* 2003), and Zucker Diabetic Fatty (ZDF) rats (Johnson *et al.* 2013) are often used. However, retinal thickening is not observed in these animals, unlike human DME patients.

The Spontaneously Diabetic Torii (SDT) fatty rat was established by introducing the fa allele into the SDT rat genome, and the onset of diabetes is observed at 6 weeks of age (Masuyama *et al.* 2005). Recent studies indicate that this model also exhibits various diabetic complications (Katsuda *et al.* 2015). In particular, in the retina, the peak latencies of oscillatory potentials in electroretinograms (ERGs) at 16 weeks of age are prolonged when compared with the lean rat, demonstrating retinal dysfunction (Matsui *et al.* 2008). In this study, we investigated pathophysiological changes

associated with hyperglycemia in the retina of SDT fatty rats. We evaluated VEGF accumulation in the vitreous humor, retinal vascular permeability, retinal thickening (Experiment 1), and the therapeutic effects of anti-diabetic drugs on retinal thickening in the male SDT fatty rat (Experiment 2).

Materials and Methods

Animals

Male SDT *fa/fa* (fatty) rats and age-matched Sprague-Dawley (SD) rats were purchased from CLEA Japan, Inc. (Tokyo, Japan). Rats were housed in a climate-controlled room at a temperature of 23 ± 3°C, humidity of 55 ± 15%, and a 12 h lighting cycle. A powdered basal diet (CRF-1, Oriental Yeast Co., Ltd., Tokyo, Japan) and water were provided ad libitum. All experiments received prior approval from the committee for human care and use of animals at our laboratory in accordance with the Standards Relating to the Care and Management of Experimental Animals.

Biological parameters

Body weight and biological parameters were evaluated. Blood samples were collected from the tail vein. Glucose, HbA1c, triglyceride and total cholesterol levels were measured using commercial kits (Roche Diagnostics, Basel, Switzerland) and an automatic analyzer (Hitachi 7180; Hitachi, Tokyo, Japan). Plasma insulin levels were

measured using an ELISA kit (Morinaga, Yokohama, Japan).

Experiment 1

VEGF concentration in vitreous humor

Animals at 8 and 16 weeks of age were sacrificed under deep isoflurane anesthesia. Both eyes were enucleated, washed with saline three times, and then wiped with cotton to dry. After pricking the eyes near the optic nerve with a 27-gauge needle, approximately 60 μl of the vitreous humor was collected into 0.5 ml Eppendorf tubes and immediately frozen in liquid nitrogen. Samples were thawed on ice and centrifuged at 10000 x *g* for 5 minutes at 4°C, after which the supernatant was used. VEGF concentrations were measured using a Rat VEGF Quantikine ELISA Kit (R&D Systems Inc., Minneapolis, MN, U.S.A.).

Retinal vascular permeability

Rats were given an intravenous dose of 10 mg/kg of sodium fluorescein. One hour later, blood samples and vitreous humor were collected. After centrifugation (10,000 x *g* for 5 minutes at 4°C), the fluorescein concentration of the serum samples was measured by using a microplate reader (ARVO-X5 2030. Multilabel Reader; Perkin-Elmer) with excitation at 485 nm and emission at 535 nm. Retinal vascular permeability was calculated by dividing fluorescein concentration of vitreous humor by that of blood

plasma.

Ocular histopathology

The eyes were enucleated and fixed in 1% formalin/1.5% glutaraldehyde mix fixative solution, and then embedded in paraffin, sectioned, stained with hematoxylin and eosin (HE), and examined histopathologically.

Measurement of retinal thickness

In the previous report by Toyoda *et al*., mean retinal thickness at 500, 1000, and 1500 μm from the optic nerve disc were almost same (Toyoda *et al.* 2016). Therefore, the total retinal thickness in this experiment was defined as the distance between the retinal internal limiting membrane (ILM) and the photoreceptor layer (PL) 500 μm away from the optic nerve disc. The thickness of the ILM of the ganglion cell layer (GCL), the inner plexiform layer (IPL), inner nuclear layer (INL), outer nuclear layer (ONL) and PL were also measured.

Experiment 2

Experiment grouping design for anti-diabetic drugs

Based on the biological parameters, SD rats and SDT fatty rats at 4 weeks of age were divided into four subgroups; Group 1 (normal control): SD rats subcutaneously

injected with 20% propylene glycol and fed powdered CRF-1 chow; Group 2 (DME vehicle): SDT fatty rats subcutaneously injected with 20% propylene glycol and fed powdered CRF-1 chow; Group 3 (DME pioglitazone): SDT fatty rats subcutaneously injected with 20% propylene glycol and administered 10 mg/kg/day pioglitazone (synthesized at Japan Tobacco Co.) through powdered CRF-1 chow (food admixture); and Group 4 (DME phlorizin): SDT fatty rats subcutaneously injected with 150 mg/kg/day phlorizin (Sigma-Aldrich Co., St. Louis, MO, U.S.A.) and fed powdered CRF-1 chow.

Statistical Analysis

All values were expressed as the mean ± standard deviation. If there were 2 groups to compare, an F-test for homogeneity of variance followed by Student's t-test or Aspin-Welch's t-test was performed. To compare the means of the three groups (Experiment 2), Dunnett's test or Steel's test was performed after Bartlett's test was performed to assess the homogeneity of variances. All statistical analyses were performed using Statlight 2000 (Yukms Corp., Tokyo, Japan) statistical software. A P-value < 0.05 was considered statistically significant.

Results

Experiment 1

Body weight and blood chemical parameters, such as glucose, insulin, triglyceride (TG) and total cholesterol (TC) levels, are shown in Table 1. At 8 weeks of age, the SDT fatty rats exhibited obesity, hyperglycemia, hyperinsulinemia and hyperlipidemia. Although hyperglycemia was observed in SDT fatty rats that were 16 weeks of age, body weight, insulin and TG levels showed decreases.

The vitreous humor from SD rats and SDT fatty rats were collected. Compared with normal values, the mean vitreous VEGF concentration value in SDT fatty rats was significantly higher at 8 weeks of age (50.5 ± 19.2 pg/ml for SD rats versus 200.1 ± 41.9 pg/ml for SDT fatty rats) and showed tendency to increase at 16 weeks of age (9.0 ± 2.7 pg/ml for SD rats versus 28.6 ± 32.6 pg/ml for SDT fatty rats) in Figure 1A. Furthermore, the retinal permeability of SDT fatty rats at 16 weeks of age also showed tendency to increase (p value = 0.07) in Figure 1B.

The eye specimens from SD rats (Fig. 2A) and SDT fatty rats (Fig. 2B) were observed under a microscope. The mean values for total retinal thickness, defined as the distance from the ILM to the PL 500 μm away from the optic nerve disc, were measured. The retinas of SDT fatty rats were significantly thicker than that of SD rats in Figure 2C (157.5 ± 19.8 μm for SD rats versus 218.4 ± 16.9 μm for SD rats). The layers between the ILM and the GCL, the IPL, INL and ONL of SDT fatty rats were significantly thicker than those of SD rats. On the other hand, the thickness of the PL in SDT fatty rats was not significant when compared with that in SD rats, as shown in Figure 2D.

Experiment 2

SDT fatty rats were treated from 4 to 16 weeks of age with anti-diabetic drugs, pioglitazone (10 mg/kg/day) or phlorizin (150 mg/kg/day). Pioglitazone significantly increased body weight at 8 and 16 weeks of age and phlorizin at 16 weeks of age (Figure 3A). Both drugs strongly reduced plasma glucose levels (Figure 3B), HbA1c (Figure 3C) and TG levels (Figure 3E) at 8 and 16 weeks of age. From 8 to 16 weeks of age, vehicle groups exhibited sharp declines in plasma insulin levels, whereas both treatment groups retained higher levels (Figure 3D), indicating the preservation of pancreatic β-cell function. For plasma TC levels, treatment with pioglitazone resulted in a tendency toward decreases and significant decreases at 16 weeks of age (Figure 3F) with phlorizin treatment.

The total retina of SDT fatty rats was thicker than that of SD rats, and both pioglitazone and phlorizin decreased retinal thickness in Figure 4A. The layers between the ILM and GCL, the IPL, INL and ONL in SDT fatty rats were significantly thicker than those of SD rats and these layers showed a tendency to decrease when treated using either of the drugs in Figure 4B.

Discussion

To reveal the mechanism and develop new therapies, many researchers have

investigated animal models. STZ-induced animals are recognized as the most common model and retinal hyperpermeability was reported. Nevertheless, in this model, retinal thickness becomes thinner than control, unlike in human patients (Zhang *et al.* 2008). Although the OLETF rat and ZDF rat show morphological changes in the retinal capillaries or pericytes, retinal thickening has unfortunately not been reported (Lu *et al.* 2003, Johnson *et al.* 2013, Lai and Lo 2013).

The SDT rat is a known non-obese type 2 diabetes model and VEGF overexpression, vascular lesions, such as acellular capillaries and pericyte loss, leakage of fluorescein around the optic disc, retinal thickening, neovascularization, and tractional retinal detachment with fibrous proliferation around 60 weeks of ages have been reported by Kakehashi *et al*. (Shinohara *et al.* 2000, Kakehashi *et al.* 2006, Sasase *et al.* 2013, Toyoda *et al.* 2016). However, these retinal abnormalities develop over the course of a year. Because SDT fatty rat was originally produced by introducing *fa* gene to SDT rat to accelerate the development of diabetic complications, similar ocular lesions are basically expected between these two strains. However, the effect of aging is quite important for pathophysiology of eyes – actually, SD rats demonstrate spontaneous ocular abnormalities, such as corneal or lenticular opacities, choroidal or retinal atrophy, retinal fold, and hemorrhage increased with greater frequency with increasing age (Ban *et al.* 2008, Tomomi 2008). Therefore, advancing the development of ocular complications is meaningful for studying diabetic retinopathy using experimental

animal model. In addition, according to obesity and following insulin resistance, inflammation is found in eyes in SDT fatty rat at older age (Kemmochi *et al.* 2014). Also, further studies are needed to unveil the difference of response to various drugs, such as SGLT2 inhibitor, between SDT and SDT fatty rat.

The SDT fatty rat is recognized as a diabetes model, and in this study, exhibited severe hyperglycemia from 8 weeks of age followed by VEGF overproduction, retinal vascular hyperpermeability, and retinal thickening. Generally, rapid increases in blood glucose may enhance glycolysis and the diacylglycerol pathway involving PKC activation followed by VEGF production (Xia *et al.* 2007). Furthermore, oxidative stress induced by hyperglycemia also causes VEGF production, matrix metalloproteinase (MMP) activation and breakdowns in the BRB (El-Remessy *et al.* 2013). These changes can be one of the reasons of the peak latency in ERGs of male SDT fatty rats at 16 weeks of age (Matsui *et al.* 2008).

In this study, we have chosen pioglitazone and phlorizin to evaluate the effect of controlling blood glucose level. These drugs are cheap and easy to purchase, and above all, these drugs have already reported to lower blood glucose levels well without any side effects in SDT fatty rats. Interestingly, although the glucose-lowering effect of pioglitazone and phlorizin is the same, pioglitazone could not normalize retinal thickness. Recently, Dennis et al. reported that reductions in serum glucose concentrations with phlorizin treatment led to normalized VEGF concentrations in the

retina of STZ-induced rats (Dennis *et al.* 2015). Therefore, glucose-lowering effects can contribute to decreases in retinal VEGF expression.

Generally, glitazones have side effects, such as edema, which is likely caused by renal sodium retention (Colucciello 2005, Ryan *et al.* 2006, Horita *et al.* 2015), and in actual human DME patients, some studies have indicated the adverse effect of pioglitazone (Oshitari *et al.* 2008, Idris *et al.* 2012). Hence, the effect of pioglitazone must be weakened against the retinal thickness despite plasma glucose normalization. As mentioned above, lowering blood glucose levels normalize VEGF concentrations in retina. On the other hands, pioglitazone causes edema as its side effect. Therefore, we speculate that the imbalance of effect and side effect of pioglitazone may cause different output on retinal thickness between human and SDT fatty rat. Given these facts, SDT fatty rat is a prominent animal model, which enables us to evaluate not only positive effects but also side effects in advance without clinical trials.

In summary, we identified an increase in VEGF in vitreous humor, retinal vascular hyperpermeability, and retinal thickening, in particular from the ILM to ONL of SDT fatty rats at 16 weeks of age. Furthermore, the retinal thickening improved with treatment with anti-diabetic drugs, pioglitazone and phlorizin. However, unlike phlorizin, the effect of pioglitazone is not strong despite the normalization of blood glucose levels. Altogether, we confirmed that the male SDT fatty rat is a suitable preclinical model for testing new therapeutic approaches in DME.

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Figure Legends

Fig. 1. VEGF concentrations in the vitreous humor (A) and retinal vascular permeability (B) of SD rats and SDT fatty rats at 8 and 16 weeks of age were collected and measured. Each value represents the mean ± S.D (A: n=4, B: n=8). **: $p < 0.01$, significantly different from SD rats.

Fig. 2. Photomicrograph of the eyes of male SD rats (A, representative example) and SDT fatty rats (B, representative example) at 16 weeks of age. The layer in the retina that was 500 μm from the optic disc was enlarged. The total retinal thickness (C) and the layer between the ILM (inter limiting membrane) and GCL (ganglion cell layer), the IPL (inner plexiform layer), INL (inner nuclear layer), ONL (outer nuclear layer) and PL (photoreceptor layer) (D) were evaluated. Each value represents the mean ± S.D (n=5). **: $p < 0.01$, significantly different from SD rats.

Fig. 3. Effect of pioglitazone and phlorizin on body weight and blood biological parameters. SDT fatty rats were treated with pioglitazone (10 mg/kg/day) or phlorizin (150 mg/kg/day) from 4 to 16 weeks of age. Body weight (A) and plasma glucose level (B), HbA1c level (C), plasma insulin (D), triglyceride (TG) (E) and total cholesterol (TC) levels (F) at 8 and 16 weeks of ages were evaluated. Each value represents the mean ± S.D (n=4). *: $p < 0.05$, **: $p < 0.01$, significantly different from SD rats. #: $p < 0.05$, ##: $p < 0.01$, significantly different from the vehicle group.

Fig. 4. Effect of pioglitazone and phlorizin on retinal thickening. The total retinal thickness (A) and layers, such as between the ILM and GCL, the IPL, INL, ONL and PL (B) that is 500 μm from the optic disc, were measured. Pioglitazone and phlorizin improved total retinal thickness, in particular, from the ILM to the ONL. Each value

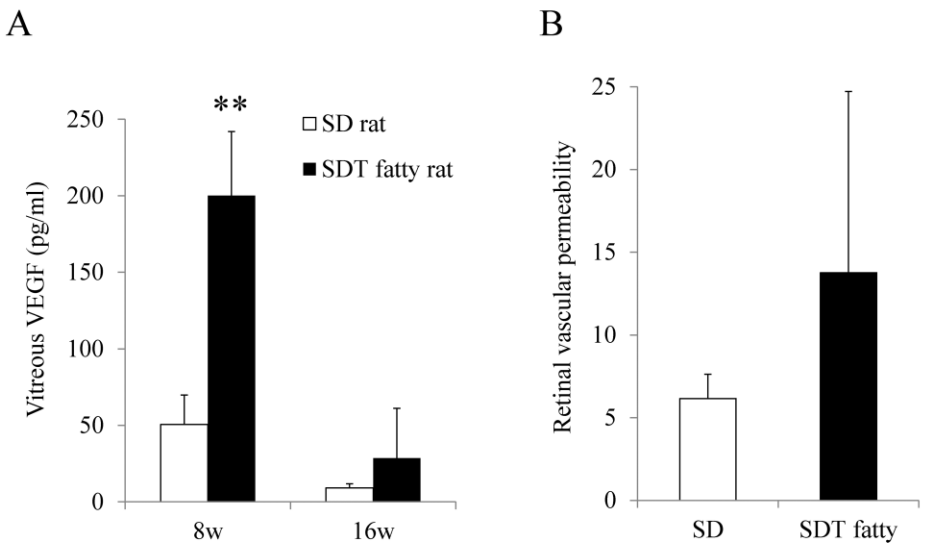
represents the mean ± S.D (n=4). **: $p < 0.01$, significantly different from the control group. #: $p < 0.05$, ##: $p < 0.01$, significantly different from the vehicle group.

Table 1. Body weight and serum biochemical parameters

Parameters	SD rat		SDT fatty rat	
	8 weeks	16 weeks	8 weeks	16 weeks
Body weight (g)	342.5±8.6	625.8±40.3 ##	390.6±27.4 *	510.4±36.7 **, ##
Glucose (mg/dl)	148.8±10.5	196.2±14.0 ##	520.8±162.4 **	741.6±76.9 **, #
Insulin (ng/ml)	0.73±0.17	0.84±0.17	8.3±5.4 *	1.1±0.2 #
TG (mg/dl)	154.6±58.1	215.3±72.8	764.0±150.8 **	330.5±86.7 ##
TC (mg/dl)	77.2±8.9	64.7±4.8 #	106.9±7.1 **	136.5±27.7 **

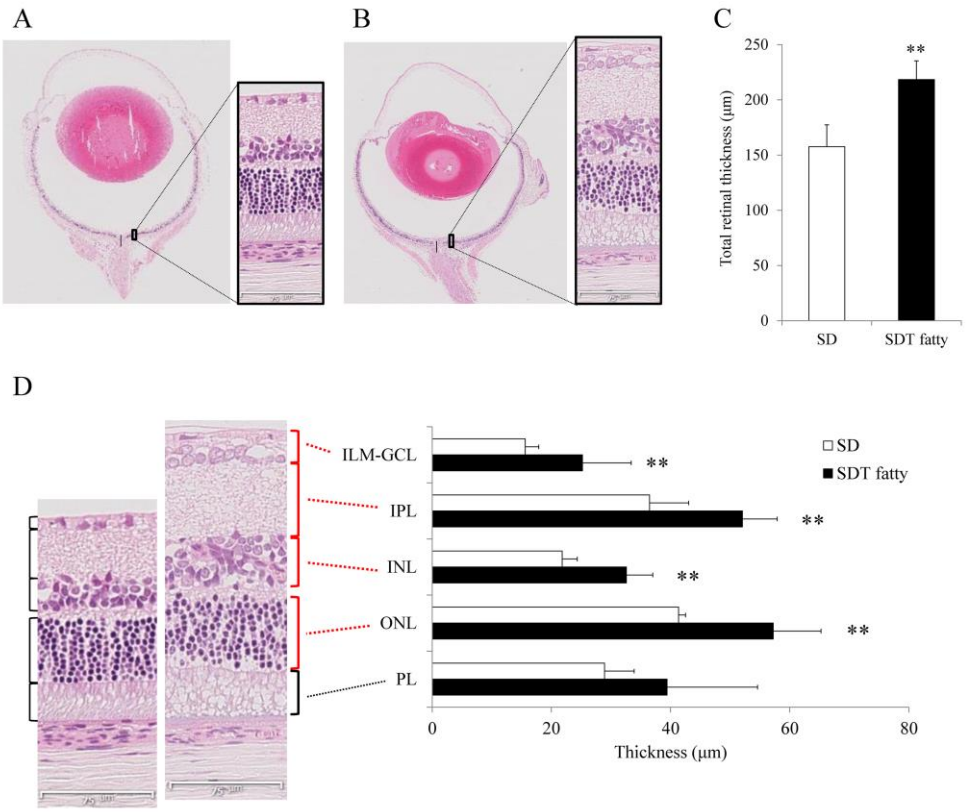
Body weight and plasma glucose, insulin, triglyceride (TG) and total cholesterol (TC) levels in SD rats and SDT fatty rats were measured at 8 and 16 weeks of age. SDT fatty rats exhibited significant increases in body weight, plasma glucose, insulin, TG and TC at 8 weeks of age. At 16 weeks of age, SDT fatty rats exhibited severe hyperglycemia with decreases in plasma insulin and TG levels. Data are presented as the mean ± S.D (n=5). *: $p < 0.05$, **: $p < 0.01$ versus age-matched SD rats (t-test). #: $p < 0.05$, ##: $p < 0.01$, significantly different from rats 8 weeks of age

1 Fig.1


A
B
**
SD rat
SDT fatty rat
Vitreous VEGF (pg/ml)
250
200
150
100
50
0
8w
16w
Retinal vascular permeability
25
20
15
10
5
0
SD
SDT fatty

2
3

1 Fig.2


A
B
C
250
200
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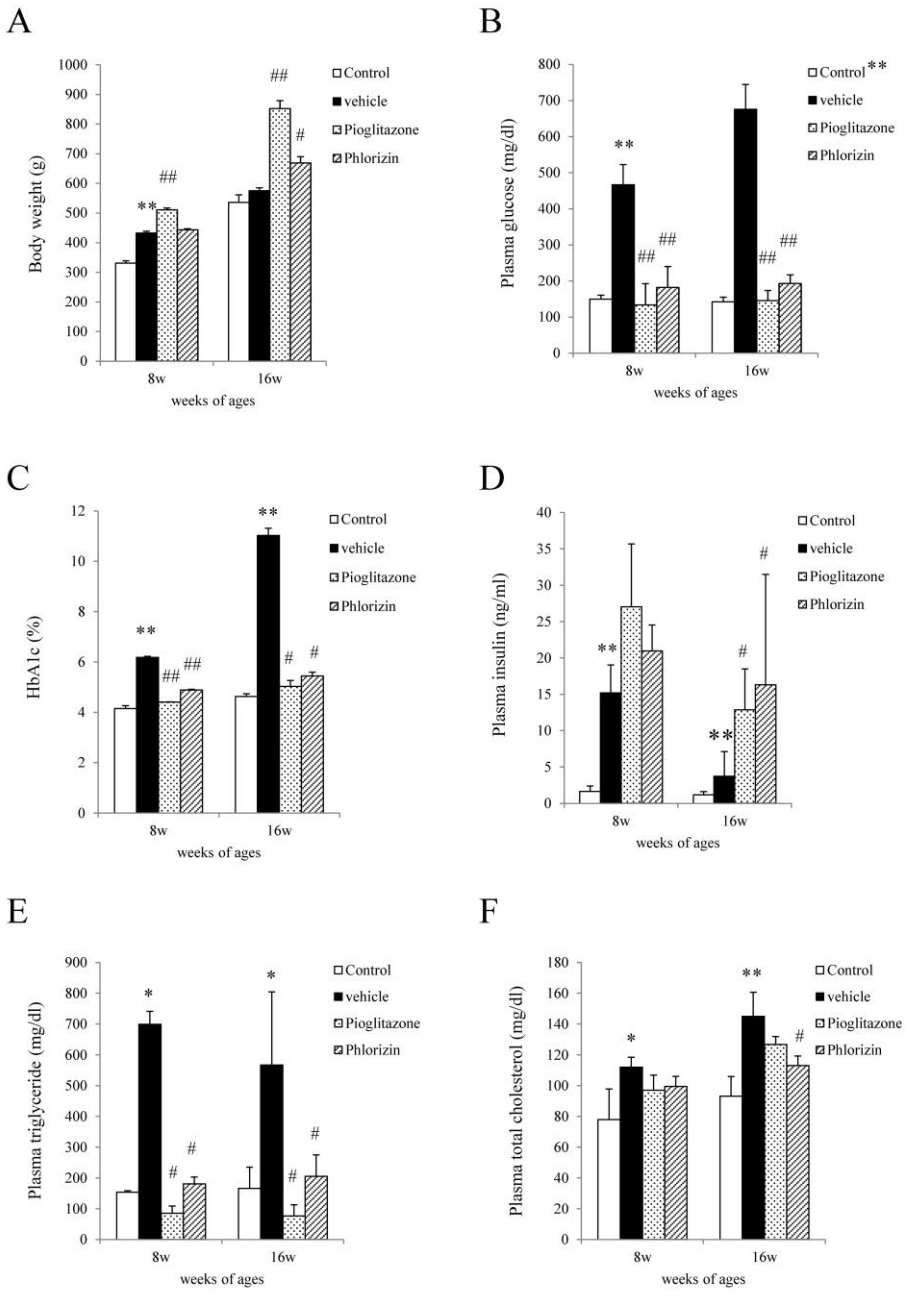
100
50
0
Total retinal thickness (μm)
**
SD
SDT fatty
D
ILM-GCL
IPL
INL
ONL
PL
SD
SDT fatty
**
**
**
**
0
20
40
60
80
Thickness (μm)

2

3

4

1 Fig.3


A
Body weight (g)
1000
900
800
700
600
500
400
300
200
100
0
8w
16w
weeks of ages
Control
vehicle
Pioglitazone
Phlorizin
B
Plasma glucose (mg/dl)
800
700
600
500
400
300
200
100
0
C
HbA1c (%)
12
10
8
6
4
2
0
D
Plasma insulin (ng/ml)
40
35
30
25
20
15
10
5
0
E
Plasma triglyceride (mg/dl)
900
800
700
600
500
400
300
200
100
0
F
Plasma total cholesterol (mg/dl)
180
160
140
120
100
80
60
40
20
0

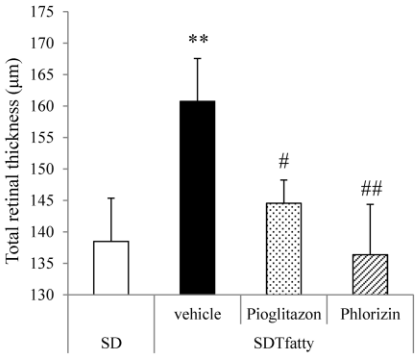
2

3

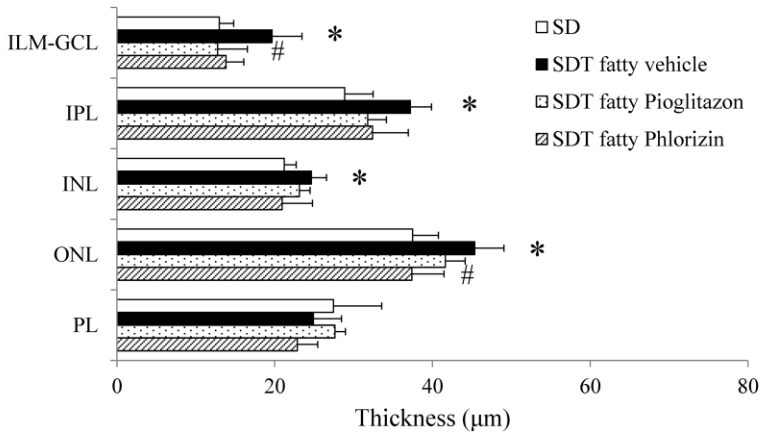
4

1 Fig.4

A


Total retinal thickness (μm)
175
170
165
160
155
150
145
140
135
130
**
#
##
vehicle
Pioglitazon
Phlorizin
SD
SDTfatty

B


ILM-GCL
IPL
INL
ONL
PL
*
#
SD
SDT fatty vehicle
SDT fatty Pioglitazon
SDT fatty Phlorizin
0
20
40
60
80
Thickness (μm)

2

3

4