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Prophylactic Pattern Scanning Laser Retinal Photocoagulation for Diabetic Retinopathy in Spontaneously Diabetic Torii Fatty Rats: Preliminary Experimental Results

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Abstract

Research Background and Purpose: The number of diabetic patients is rapidly increasing, making it crucial to find methods to prevent diabetic retinopathy (DR), a leading cause of blindness. We investigated the effects of prophylactic pattern scanning laser retinal photocoagulation on DR development in Spontaneously Diabetic Torii (SDT) fatty rats as a new prevention approach. **Methods:** Photocoagulation was applied to the right eyes of 8-week-old Spontaneously Diabetic Torii (SDT) fatty rats, with the left eyes serving as untreated controls. Electroretinography at 9 and 39 weeks of age and pathological examinations, including immunohistochemistry for vascular endothelial growth factor and glial fibrillary acidic protein at 24 and 40 weeks of age, were performed on both eyes. **Results:** There were no significant differences in amplitude and prolongation of the OP waves between the right and left eyes in SDT fatty rats at 39 weeks of age. Similarly, no significant differences in pathology and immunohistochemistry were observed between the right and left eyes in SDT fatty rats at 24 and 40 weeks of age. **Conclusion:** Prophylactic pattern scanning retinal laser photocoagulation did not affect the development of diabetic retinopathy in SDT fatty rats.

Keywords

Diabetes, Pattern Scanning Laser, Diabetic Retinopathy, Spontaneously Diabetic Torii Fatty Rats, Electroretinography

1. Introduction

Diabetes is a chronic disease characterized by hyperglycemia due to impaired glucose tolerance from insulin deficiency or reduced insulin action. It often develops unnoticed and leads to major complications such as retinopathy, nephropathy, and neurological disorders, along with an increased risk of heart disease and stroke. The global diabetic population is on the rise, with the International Diabetes Federation estimating that approximately 537 million people were affected in 2021, and this number is expected to reach 643 million by 2030 [1].

Diabetic retinopathy (DR), a complication of diabetes, results from retinal vascular damage due to chronic hyperglycemia, gradually worsening and potentially leading to blindness [2]. The global prevalence rates of diabetic retinopathy (DR) and its severe form, proliferative DR, are 27% and 1.4%, respectively, in diabetic patients [3]. Patient reports indicate that the prevalence of DR exceeds 30% [4]. In 2020, an estimated 103 million people worldwide were diagnosed with DR [5].

To clarify the pathogenesis of diabetic ocular complications and develop treatments, several animal models of diabetes have been developed. In 1997, the Spontaneously Diabetic Torii (SDT) rat, characterized by severe diabetic ocular complications, was developed through a collaboration between our institute and the Torii Pharmaceutical Research Institute [6]. We developed a new animal model for obese type 2 diabetes, the SDT fatty rat, by introducing the leptin receptor mutation from Zucker fatty rats into the genetic background of the SDT rats [7]. In addition to hyperglycemia, SDT fatty rats exhibit obesity from overeating due to a loss of leptin activity, hyperlipidemia, hypertension, and insulin resistance. These characteristics make them a suitable model for diabetes, closely mimicking diabetic patients in daily clinical practice. In male SDT fatty rats, hyperglycemia is observed from 5 weeks of age, reaching levels of about 600 mg/dl by 8 weeks, with a 100% diabetes development rate by 16 weeks of age [7] [8]. In our previous study, diabetic retinopathy (DR) developed in SDT fatty rats at 16 weeks of age, indicating that DR appears earlier and is more severe in SDT fatty rats compared to SDT rats [9]. Upon the development of DR, symptoms such as retinal thickening and folds emerge, and electroretinography (ERG) reveals prolonged apex latency and reduced amplitude of the oscillatory potential (OP) wave.

No treatment can fully cure diabetic retinopathy (DR), making it crucial to prevent its progression. Preventing the onset of diabetes through lifestyle improvements is key, but if diabetes develops, it's vital to prevent the development of associated diseases as much as possible. DR prevention primarily involves blood sugar control. Standard treatments for DR, based on its stage, include retinal photocoagulation, vitrectomy, and vitreous injection of vascular endothelial growth factor (VEGF) [10].

VEGF plays a role in the development and progression of DR. Its expression is

triggered by ischemia or hypoxia, yet increased VEGF expression and vascular permeability can occur even before clear signs of retinal vascular occlusion and ischemia are evident [11]. The primary cause is believed to be advanced glycation end products (AGEs), resulting from chronic hyperglycemia. Chronic hyperglycemia increases glucose levels, enhancing the polyol metabolic pathway. This activation leads to the activation of protein kinase C (PKC), enhancement of the hexosamine biosynthetic pathway, and the production of AGEs [12]. These primarily cause retinal microangiopathy. AGEs prompt pericytes to activate the VEGF gene in their cells [11]. However, pigment epithelium-derived factor (PEDF), which possesses an antioxidant effect, inhibits AGEs and thereby reduces the induction of VEGF [11] [13].

It takes about 13 years for DR to develop [14]. In the meantime, a minimally invasive method to prevent DR could significantly reduce the number of patients with DR. According to the Early Treatment Diabetic Retinopathy Study, photocoagulation is recommended for proliferative DR rather than for simple DR, due to its disadvantages such as narrowing of the visual field, decreased dark adaptation, color blindness, and macular edema [15] [16]. In contrast, the current study explored the effectiveness of prophylactic pattern scanning laser as a novel treatment in the early stages of DR. Unlike standard panretinal photocoagulation, which is painful and harms the inner retinal layer and choroid, the pattern scanning laser offers a less invasive alternative. It operates with high power (350 to 475 mW, approximately 3 to 4 times that of conventional photocoagulation) and short pulse irradiation (10 to 30 msec, roughly $1/10^{th}$ to $1/20^{th}$ the duration of conventional photocoagulation). This approach potentially reduces patient discomfort and damage to the inner retinal layer and choroid [17]-[19]. Laser treatment for diabetic retinopathy (DR) has gained significant attention as a method for managing advanced stages of the condition, with numerous reports highlighting its effectiveness [20] [21], but no study has reported the effects and complications of prophylactic pattern scanning lasers in the pre-diabetic retinopathy (DR) stages. We hypothesized that if retinal photocoagulation, already widely used in clinical practice, can suppress DR progression and prevent its onset, it will offer a new, simple, and safe method to prevent DR. Therefore, we evaluated the effect of prophylactic pattern scanning laser on developing DR in SDT fatty rats.

2. Methods

2.1. Animals

Sixteen SDT fatty rats (obese type 2 diabetic model animals), which were purchased from CLEA Japan Inc. (Tokyo, Japan), were used in this study. The care and handling of the animals adhered to the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Visual Research, the Guidelines for Animal Experimentation of Japan Tobacco Inc., and the Guidelines for Animal Experimentation of the Animal Care and Com-

mittee of Jichi Medical University. Additionally, the study was conducted in accordance with the Declaration of Helsinki guidelines and was approved by Jichi Medical University (Jichi Medical University Animal Experiment Approval No. 17105-02 and date of approval 11/3/2019). All SDT fatty rats were diagnosed as diabetic if their non-fasting blood glucose level reached 350 mg/dl or higher. They were fed a standard rat diet (CRF-1, Oriental Yeast, Inc., Tokyo, Japan) and kept in an environment that maintained their body temperature at approximately 37 degrees Celsius. This study builds on previous research indicating that SDT fatty rats develop hyperglycemia by 8 weeks of age but do not exhibit diabetic retinopathy (DR) symptoms, such as retinal thickening, until 16 weeks of age [9]. The animals were divided into two groups: one was sacrificed at 24 weeks of age (24-week SDT fatty rats) and the other at 40 weeks (40-week SDT fatty rats).

2.2. Laser Photocoagulation

All 8-week-old SDT fatty rats underwent pattern scanning retinal laser photocoagulation in their right eyes under anesthesia, administered through intraperitoneal injection of a mixed anesthetic solution containing medetomidine hydrochloride (0.375 mg/kg), midazolam (2 mg/kg), and butorphanol tartrate (2.5 mg/kg). The left eyes were left untreated as controls. Photocoagulation was carried out using a Volk Quad Pediatric Laser Lens with a 1.82× laser spot magnification (Volk Optical Inc., Mentor, OH, USA). The settings included an irradiation power of 360 mW, an irradiation time of 20 msec, and a spot size of 200 μm (resulting in a retinal spot size of 330 μm), utilizing the Novus Spectra green laser photocoagulator (Lumenis Be Ltd., Yokneam Illit, Israel). Since rats lack a macula, coagulation was performed circumferentially, leaving a space equivalent to one coagulation spot around the optic disc. The number of laser applications was approximately 300 shots. However, if rats moved due to the diminishing effect of the anesthetic, the laser procedure was halted. Consequently, the minimum number of laser applications recorded was 240 shots.

2.3. Electroretinography

Electroretinography (ERG) was used to assess the development of diabetic retinopathy (DR) in the left eye and the effect of laser photocoagulation on DR in the right eye of SDT fatty rats. In previous experiments, the use of three types of mixed anesthetic agents resulted in the death of some SDT fatty rats. To minimize stress from repeated anesthesia in a short timeframe, ERG was conducted only in the 40-week-old SDT fatty rat group. Thus, ERG was carried out at 9 weeks of age immediately after laser photocoagulation in eight rats and at 39 weeks of age before euthanasia in four surviving rats. Full-field ERG responses were recorded using a Ganzfeld dome, an acquisition system, and light-emitting diode stimulators (PuREC, MAYO Corporation, Inazawa, Japan). We measured the peak latency of the OP1 wave, noting that the frequency of apex latency pro-

longation is especially pronounced in the OP1 wave of diabetic patients [22]. The amplitude of the OP wave was defined as the sum of amplitudes from OP1 to OP4 (ΣOPs).

2.4. Preparation of Eye Tissue Specimens

At 24 and 40 weeks of age, the SDT fatty rats in each group were euthanized under anesthesia for pathological examination. The animals were euthanized by intraperitoneal injection of pentobarbital (85 mg/kg), and blood was drawn from the aorta. Subsequently, the eyes were enucleated, immediately fixed in Super Fix solution (KY-500, Kurabo, Japan), embedded in paraffin, and sectioned into 4-μm slices using a microtome. These sections were then stained with hematoxylin and eosin.

2.5. Measurement of Retinal Thickness and Retinal Folds

Retinal thickness and the number of retinal folds were measured in eye tissue specimens. Retinal thickness was measured at three different points (**Figure 1(a)**). A retinal fold was defined as deformed tissue extending from the photoreceptor layer to the outer retinal granular layer.

2.6. Measurement of the Area of Immunostained GFAP and VEGF

Immunohistochemical studies included immunostaining for VEGF and glial fibrillary acidic protein (GFAP). In diabetic retinopathy (DR), both VEGF and GFAP levels increase in ganglion cells. Immunostaining utilized the conventional avidin-biotin horseradish peroxidase method, with AEC Substrate-Chromogen (DakoCytomation, Carpinteria, CA, USA) serving as the chromogenic substrate. The Hybrid Cell Count Module/BZ-H3C software (Keyence, Chicago, IL, USA) quantitatively analyzed the immunostained areas within 1500 μm from the optic disc, calculating the area ratio. Positive areas were color-coded in dark blue, negative areas in magenta, and the positive area ratio for each specimen was determined.

2.7. Statistical Analysis

We used the F test to check the variance and analyzed the equality of variances using the Student's t-test, while the Welch t-test was used for unequal variances. Microsoft® Excel (Microsoft Corporation, Redmond, WA, USA) was employed for statistical analysis, with $P < 0.05$ deemed significant.

3. Results

SDT fatty rats consistently exhibit blood sugar levels above 350 mg/dl, increasing their risk of sudden death as they age. Initially, 16 SDT fatty rats were split into two groups based on age: 24-week and 40-week. Eventually, the number of rats in the 24-week group dropped to six, and in the 40-week group to four. Each rat underwent pattern scanning retinal laser photocoagulation in the right eye, with the left eye remaining untreated.

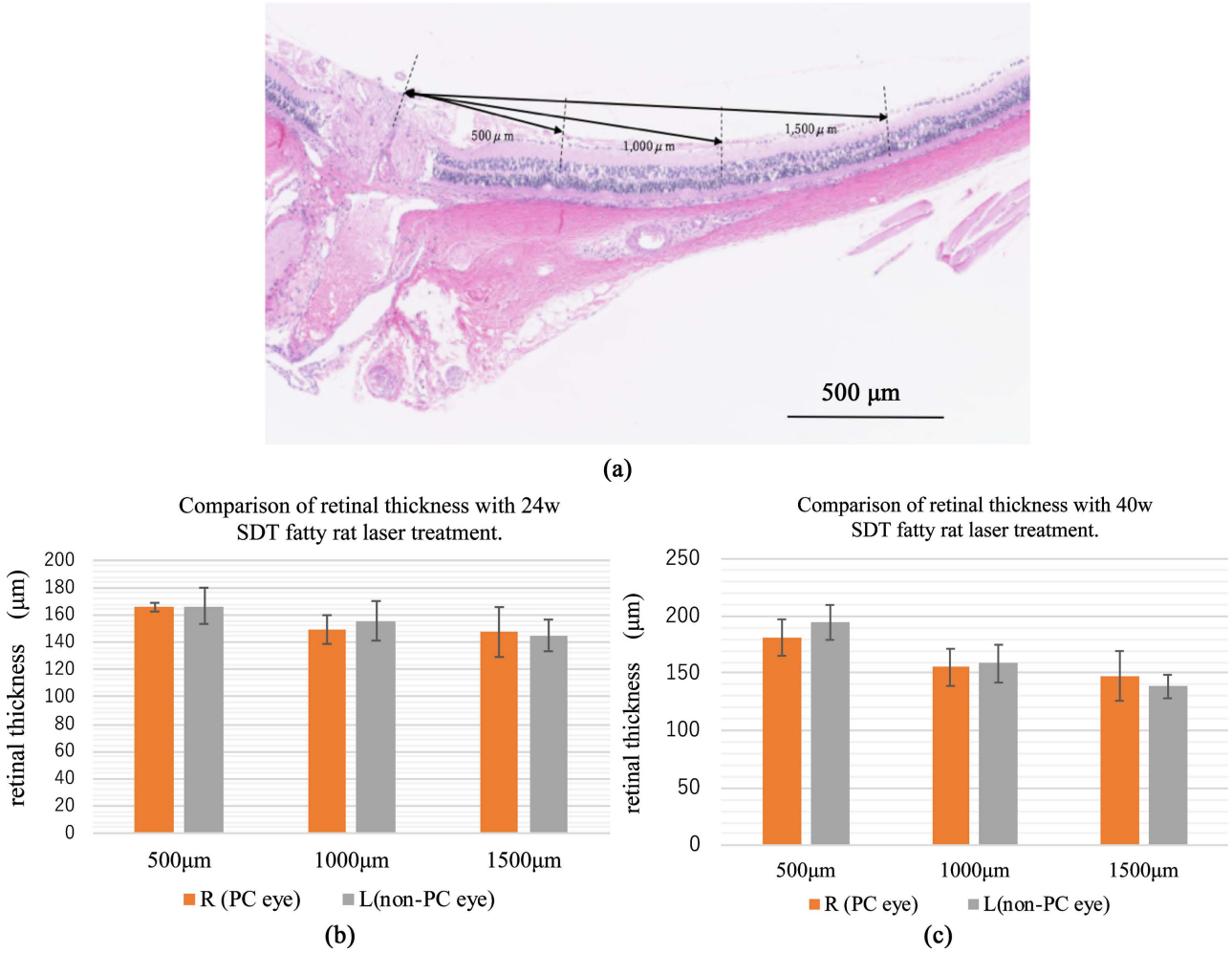


Figure 1. (a) Retinal thicknesses are measured at distances of 500, 1000, and 1500 μm from the optic disc in each specimen. (b) Comparison of Retinal Thicknesses from the Optic Disc in Treated and Untreated 24-Week-Old SDT Fatty Rats. (c) Comparison of retinal thicknesses at the optic disc in treated and untreated 40-week-old SDT fatty rats. R, right; L, left; PC, photocoagulation.

3.1. ERG

Figure 2(a) displays the ERG waveforms of the SDT fatty rat before and after the onset of retinopathy. ERGs recorded at 9 weeks of age were obtained before retinopathy onset, while ERGs at 39 weeks were recorded after. The peak latency of the OP wave is prolonged, and the amplitude of the OP wave is reduced in the ERGs following the development of DR.

At 9 weeks, immediately following laser photocoagulation, the peak latencies of the OP1 wave in the right eyes treated with photocoagulation and the untreated left eyes were 21.3 ± 1.08 ms in the treated eyes and 21.0 ± 1.54 ms in the untreated eyes. The amplitudes of the ΣOPs waves were 401.1 ± 176.41 μv in the treated eyes and 413.5 ± 150.62 μv in the untreated eyes. At 39 weeks, before slaughter, the peak latencies of the OP1 wave in both the treated and untreated eyes were 22.9 ± 1.37 ms, and the amplitudes of the ΣOPs waves were 247.6 ± 142.56 μv in the treated eyes and 284.2 ± 77.04 μv in the untreated eyes. The experiment demonstrated that the amplitude of the ΣOPs waves decreased in the

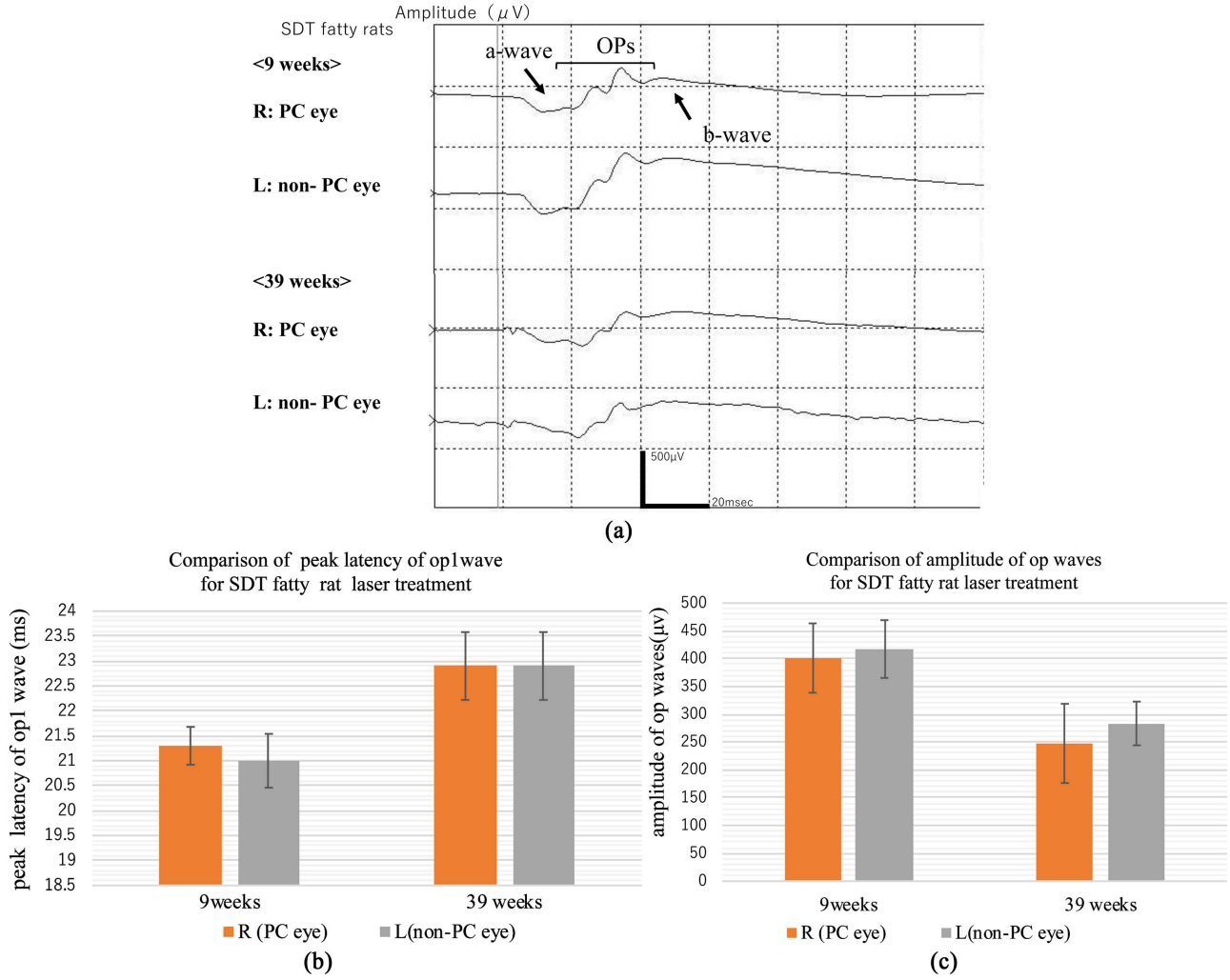


Figure 2. (a) ERG waveforms of SDT fatty rats before and after laser application. (b) Comparison of OP1 wave peak latency in treated and untreated SDT fatty rats. (c) Comparison of OP wave amplitude in treated and untreated SDT fatty rats. R, right; L, left; PC, photocoagulation.

39-week rats compared to the 9-week rats, although the change in peak latency of the OP wave was not clear. However, there were no significant differences between the groups in terms of the amplitude of the ΣOPs wave ($p = 0.0894$) and the peak latency of the OP1 wave ($p = 0.0617$).

The ERG waveform clearly showed no significant differences in the peak latency of OP1 or the amplitude of ΣOPs between the right eyes treated with photocoagulation and the untreated left eyes of SDT fatty rats (amplitude of ΣOPs wave, $p = 0.844$ at 9 weeks, $p = 0.674$ at 39 weeks; peak latency of OP1, $p = 0.672$ at 9 weeks, $p = 1$ at 39 weeks) (Figure 2(a)-(c)).

3.2. Pathology

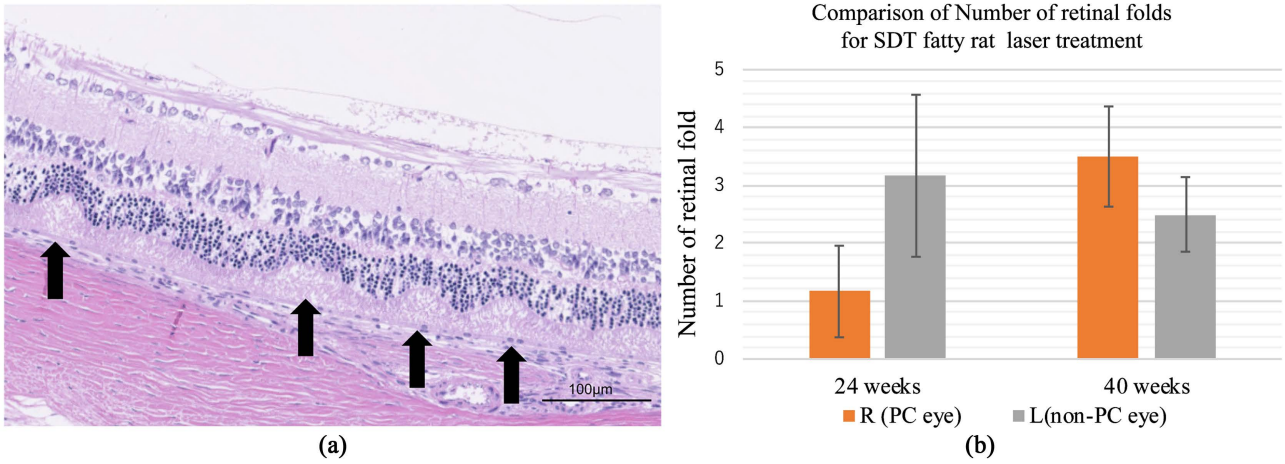
Thickened retinas and retinal folds are common pathological changes in DR in SDT fatty rats [9]. The average retinal thicknesses in 24-week and 40-week SDT fatty rats at distances of 500, 1000, and 1500 μm from the optic disc are reported.

In 24-week SDT fatty rats, at 500 μm, the mean retinal thickness was 165.67 ± 7.82 μm in treated eyes and 166.67 ± 32.53 μm in untreated eyes. At 1000 μm, the mean retinal thickness was 149.33 ± 25.82 μm in treated eyes and 155.67 ± 35.50 μm in untreated eyes. At 1500 μm, the mean retinal thickness was 147.5 ± 44.85 μm in treated eyes and 145 ± 28.42 μm in untreated eyes (**Figure 1(b)**).

In 40-week SDT fatty rats, at 500 μm, the mean retinal thickness was 181.25 ± 31.85 μm in treated eyes and 194.5 ± 30.49 μm in untreated eyes. At 1000 μm, it was 155.25 ± 32.43 μm in treated eyes and 158.5 ± 33.01 μm in untreated eyes. At 1500 μm, it was 147.75 ± 43.5 μm in treated eyes and 138.5 ± 20.5 μm in untreated eyes (**Figure 1(c)**).

No significant differences in retinal thickness were observed between the laser-treated eyes and untreated eyes at either age. For the 24-week SDT fatty rat, retinal thickness measurements were 500 μm from the optic disc ($p = 0.944$), 1000 μm from the optic disc ($p = 0.731$), and 1500 μm from the optic disc ($p = 0.910$). For the 40-week SDT fatty rat, measurements were 500 μm from the optic disc ($p = 0.569$), 1000 μm from the optic disc ($p = 0.892$), and 1500 μm from the optic disc ($p = 0.713$).

Regarding retinal folds, **Figure 3(a)** illustrates their presence, indicating the development of diabetic retinopathy (DR) in SDT fatty rats. Retinal folds were observed in all cases as evidence of DR. In 24-week-old SDT fatty rats, the average number of retinal folds was 1.17 ± 1.94 in laser-treated eyes and 3.17 ± 3.43 in untreated eyes. In 40-week-old SDT fatty rats, the average numbers of retinal folds were 3.5 ± 1.73 in treated eyes and 2.5 ± 1.29 in untreated eyes (**Figure 3(b)**).



(a) (b)

Figure 3. (a) Retinal folds, indicated by arrows and defined as deformed tissue extending from the photoreceptor layer to the outer retinal granule layer, are counted from the optic disc outward to 1500 μm. (b) Comparison of the numbers of retinal folds in treated and untreated SDT fatty rats is shown. R stands for right, L for left, and PC for photocoagulation.

No significant differences were observed in the number of retinal folds between laser-treated and untreated eyes of SDT fatty rats at either age (24-week SDT fatty rat number of retinal folds, $p = 0.242$; 40-week SDT fatty rat number of retinal folds, $p = 0.390$).

3.3. Immunohistochemistry

In DR, ganglion cells exhibit increased levels of VEGF and GFAP, resulting in a higher percentage of area positive for VEGF and GFAP immunostaining in SDT fatty rats [9]. Suppressing DR should decrease the positive area ratio of both VEGF and GFAP. **Figure 4(a)** and **Figure 5(a)** illustrate immunostaining for VEGF and GFAP on tissue sections for immunohistochemical studies, compared to the original images.

The average area ratios for each group are as follows: in 24-week SDT fatty rats, the VEGF-positive area ratios averaged 14.4% ± 4.87% in treated eyes and 11.5% ± 2.53% in untreated eyes. In 40-week SDT fatty rats, these ratios were 12.4% ± 1.99% in treated eyes and 17.7% ± 5.87% in untreated eyes (**Figure 4(b)**).

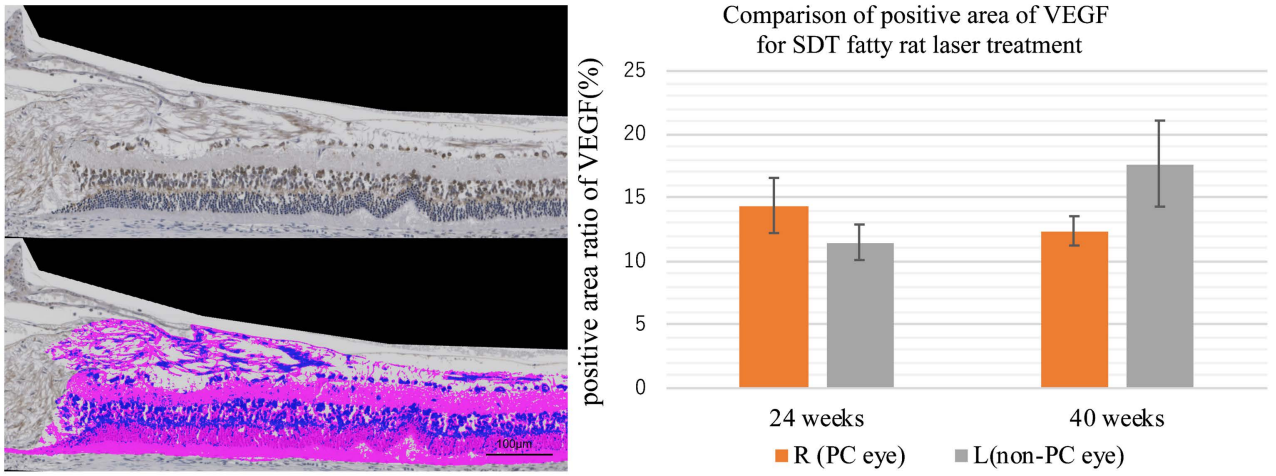


Figure 4. (a) Quantitative analysis of VEGF-positive areas. The original image is at the top, and the processed image is at the bottom. Positive areas were quantitatively analyzed, and area ratios were calculated using the Hybrid Cell Count Module/BZ-H3C software (Keyence, Chicago, IL, USA) within 1500 μm from the optic disc. Blue indicates positive areas; magenta indicates negative areas. (b) Comparison of VEGF-positive areas in treated and untreated SDT fatty rats. R, right; L, left; PC, photocoagulation.

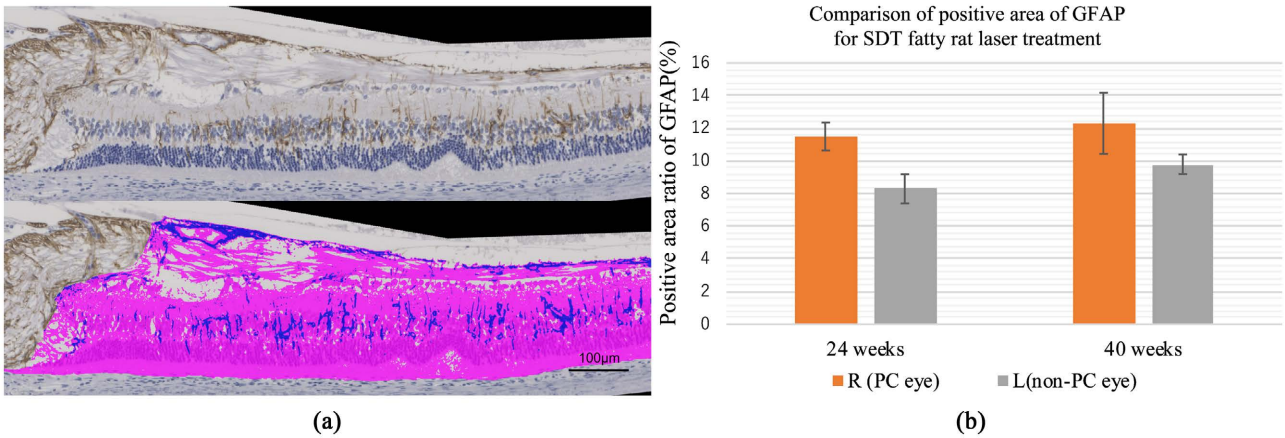


Figure 5. (a) Quantitative analysis of GFAP-positive areas. The original image is at the top, and the processed image is at the bottom. Positive areas were quantitatively analyzed, and area ratios were calculated using the Hybrid Cell Count Module/BZ-H3C software (Keyence, Chicago, IL, USA) within 1500 μm from the optic disc. Blue indicates positive areas, while magenta indicates negative areas. (b) Comparison of GFAP-positive areas in treated and untreated SDT fatty rats. R, right; L, left; PC, photocoagulation.

In 24-week SDT fatty rats, the mean GFAP positive area ratios were 11.5% ± 1.93% in treated eyes and 9.0% ± 6.0% in untreated eyes. In 40-week SDT fatty rats, these ratios were 12.3% ± 3.24% in laser-treated eyes and 9.8% ± 4% in untreated eyes (**Figure 5(b)**).

No significant differences in VEGF immunostaining were observed between untreated and treated eyes of SDT fatty rats at either age (24-week SDT fatty rat area positive for VEGF, $p = 0.264$; 40-week SDT fatty rat area positive for VEGF, $p = 0.193$). Similarly, no significant differences in GFAP immunostaining were noted between untreated and treated eyes of SDT fatty rats at both ages (24-week SDT fatty rat area positive for GFAP, $p = 0.078$; 40-week SDT fatty rat area positive for GFAP, $p = 0.260$).

4. Discussion

The purpose of this study was to investigate whether DR can be prevented by applying pattern scanning laser treatment to the retina without avascular areas before the onset of DR. The SDT fatty rats used in this study were treated with laser at 8 weeks of age, considered a pre-retinopathy stage [7] [8], so we believe that there were no avascular areas present at that time.

This study focused on the reduction of oxygen demand during retinal photocoagulation, the primary treatment to halt the progression of retinopathy. Photocoagulation on an ischemic retina leads to degenerative necrosis of photoreceptor cells and retinal pigment epithelial (RPE) cells, both of which consume significant amounts of oxygen. Correcting ischemia by reducing metabolic demand in the outer retinal layer decreases oxygen consumption, maintaining the oxygen partial pressure in the inner retinal layer. This supports the function and metabolism of glial cells and vascular endothelial cells. Consequently, the production of angiogenesis-promoting factors such as VEGF is suppressed, preventing the development and regression of new blood vessels. Therefore, performing photocoagulation on an ischemic retina suppresses the progression of diabetic retinopathy (DR). The goal was to use this mechanism to prevent the onset of DR by performing photocoagulation before retinal ischemia can develop.

We also explored how retinal photocoagulation suppresses VEGF production. The theory was that stimulating the RPE with a pattern scanning laser would increase PEDF secretion from the RPE, subsequently inhibiting VEGF production and preventing diabetic retinopathy (DR). However, we did not observe a preventative effect of prophylactic pattern scanning laser retinal photocoagulation on DR development in SDT fatty rats.

In this study, we calibrated the laser based on previous research [23] [24]. If the laser's output is too strong or there are too many coagulation spots, it can cause complete necrosis of the RPE cells and decrease the release of PEDF expression. Conversely, a too weak laser output will not reduce the oxygen consumption by the RPE cells, nor will it adequately induce the expression and release of PEDF. Testing at 400 and 450 mW led to over-coagulation, while 280 to

300 mW produced faint coagulation spots. Setting the laser to 360 mW resulted in consistent solid coagulation spots in all rats, without significant retinal and choroidal damage. However, this setting might still be considered weak, indicating that careful adjustment of laser power and coagulation number is crucial.

Normalization of oxygen partial pressure through laser application has been shown to significantly prevent exacerbation of DR. However, the complex molecular mechanisms involved in DR are not yet fully understood. Factors implicated in the onset and progression of DR include increases in the polyol pathway, protein kinase C, expression of growth factors such as insulin-like growth factor-1, hemodynamic changes, oxidative stress, activation of the renin-angiotensin-aldosterone system, subclinical inflammation, capillary occlusion, and the previously mentioned VEGF and AGEs [25]. There is a high possibility that various complex mechanisms, yet to be defined, occur in SDT fatty rats whose high blood sugar persists for a long period. Normalization of oxygen partial pressure through photocoagulation may not suffice to prevent the progression of DR.

We attempted to use pattern scanning laser on SDT fatty rats, focusing on stabilizing the oxygen partial pressure in the inner retinal layer and promoting PEDF expression in the RPE, but did not obtain any results. PEDF is a crucial factor in suppressing VEGF. However, in this experiment, changes in PEDF were not quantified. It was deemed necessary to quantify changes in PEDF after retinal photocoagulation at different laser outputs using an enzyme-linked immunosorbent assay. Additionally, evaluating other diabetic rat models with milder hyperglycemia than SDT fatty rats was necessary. These are considered our future research topics.

5. Conclusion

Prophylactic pattern scanning laser retinal photocoagulation for diabetic retinopathy (DR) in spontaneously diabetic Torii (SDT) fatty rats was unsuccessful. However, preventing DR remains an urgent issue. Further research is necessary to find simple and clinically applicable methods to prevent the onset of DR.

Author Contributions

Conceptualization, R.T. and Y.T. and A.K.; Methodology, R.T. and Y.T. and A.K.; Validation, R.T. and Y.T. and T.H.; Formal Analysis, R.T. and T.H.; Writing—Original Draft Preparation, R.T. and A.K.; Writing—Review & Editing, R.T. and A.K. and M.S. and T.S. and M.S. and Y.K.; Supervision, A.K.; Project Administration, T.K. and A.K.; Funding Acquisition, R.T. and A.K.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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