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IGF-1 or ROS/Caspase 3/Apoptosis/EMPA-II/NET Signal Pathway, and Agptl2 Induce Aggravation of STZ-induced Type 1 Diabetes by Blue Light Irradiation

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Diabetes mellitus includes type 1 and type 2 diabetes. Type 1 diabetes is an autoimmune disease affecting young people. Although several factors that worsen type 1 diabetes are known, information on the effects of blue light remains obscure. In this study, we investigated the effects of blue light irradiation on diabetes using mice with streptozotocin (STZ)-induced type 1 diabetes. Furthermore, we investigated the potential of selected compounds in rescuing the blue light-induced aggravation of diabetes. Blue light irradiation exacerbated type 1 diabetes. It activated insulin-like growth factor-1 and reactive oxygen species/caspase 3/apoptosis/endothelial-monocyte activating polypeptide II/neutrophil/neutrophil extracellular trap-associated cell death (NETosis) system signaling and increased the expression of angiopoietin-like protein 2 (Agptl2). These results indicate that blue light worsens type 1 diabetes by increasing NETosis production and the expression of Agptl2. Administration of pantethine or tranexamic acid prevented the blue light-induced worsening of type 1 diabetes by suppressing neutrophil production and Agptl2 expression. Our results provide insights into the effects of blue light on type 1 diabetes and highlight the potential of compounds that can be used in ameliorating such effects.

Key words streptozotocin-induced type 1 diabetes, insulin-like growth factor-1, reactive oxygen species, neutrophil extracellular trap-associated cell death, angiopoietin-like protein 2

INTRODUCTION

The prevalence of diabetes in Japan continues to increase, with concomitant increase in the health damage caused by diabetic complications, such as nephropathy, retinopathy, and neurological disorders.[1)] Furthermore, the number of patients with vascular disorders, such as cerebral and myocardial infarction, due to the progression of arteriosclerosis, is increasing every year.[1)] Diabetes can be broadly classified into type 1, type 2, disease-related, and gestational diabetes.[2)] Type 1 diabetes accounts for approximately 5% of all diabetes cases, and it afflicts individuals over a wide age range, mainly the young people.[3,4)] Type 1 diabetes includes acute onset type 1 diabetes, fulminant type 1 diabetes, and slowly progressive type 1 diabetes. Type 1 diabetes occurs when insulin-producing beta cells in the pancreas break down, and their ability to produce insulin weakens or ceases, resulting in a state of chronic hyperglycemia.[5)] Typical symptoms of type 1 diabetes include dry mouth, polydipsia, polyuria, and weight loss. Ketone bodies are produced in the absence of insulin, leading to critical states, such as ketosis and ketoacidosis.[6)] The etiology of type 1 diabetes remains largely unknown. One of its causes is an abnormality in the immune system due to an autoimmune disease wherein antibodies that normally protect the body attack its own pancreas.[7)] Genetic background is involved in the onset of type 1 diabetes,[8)] and some environmental factors are also believed to be involved.[8)] Daylight hours (circadian rhythm) are considered an environmental factor,[9)] and light exposure may be a factor in the onset of type 1 diabetes.

Human body is exposed to blue light (380–495 nm visible light) from sources, such as light-emitting diodes (LED), fluorescent or incandescent lights, screens of computers and smartphones.[10,11)] Exposure to blue light disturbs the circadian rhythm and causes retinal damage owing to nonvisual physiological effects. Exposure to blue light at night suppresses melatonin secretion, increases the core body temperature and heart rate, and reduces sleepiness.[12-14)] We previously reported that blue light affects the ciliary muscles and causes eye strain.[15)] Furthermore, it affects not only the eyes but also the skin. In humans exposed to blue light, an increase in active oxygen and collagen-degrading enzymes (MMPs) was observed, suggesting that this may be the cause of photoaging.[16,17)] Additionally, an increase in melanin pigment and changes in skin color

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have been reported.[18,19] Thus, exposure to blue light is associated with many problems. However, the effects of blue light on type 1 diabetes have not been investigated.

In this study, we investigated the effects of blue light on type 1 diabetes. We irradiated streptozotocin (STZ)-administered mice, a model of type 1 diabetes, with blue light. Furthermore, we examined the effects of tranexamic acid (TA), which mitigates the harmful effects of UVB and blue light in living organisms,[15,20,21] in blue light-irradiated mice model.

MATERIALS AND METHODS

Animal Experiments We used 9-week-old male specific pathogen-free (SPF) Institute of Cancer Research (ICR) mice (SLC, Hamamatsu, Shizuoka, Japan) for experiments. Mice were individually housed in cages in an air-conditioned room at 23 ± 1°C under specific pathogen-free and stress-free conditions with a 12 h light/12 h dark cycle. The mice were divided into the following seven groups of five mice each: control, STZ treatment only, STZ treatment + blue light (STZ/blue light), STZ treatment + blue light + pantethine (STZ/blue light/pantethine), STZ treatment + blue light + carbazochrome (STZ/blue light/CC), STZ treatment + blue light + diisopropylamine dichloroacetate (STZ/blue light/DADA), and STZ treatment + blue light + tranexamic acid (STZ/blue light/TA). A fluorescent lamp with blue LED light (wavelength: 380–500 nm; peak emission: 479 nm; 40 kJ/m^2; ISLM-150X150-BB, CCS Inc., Kamikyo-ku, Kyoto, Japan) was used as the light source. The energy content of LED light was measured using a light analyzer LA-105 (Nippon Medical & Chemical Instruments Co., Ltd., Osaka, Japan). In addition, mice in the control and STZ treatment groups were irradiated with a fluorescent lamp that is normally used in breeding. The whole body of the mouse was exposed to LED blue light every day (10 min per day) for 28 days from the time of STZ injection to the final day of the study (Fig. 1). There was no difference in the effects on STZ between groups exposed to fluorescent light and LED light of wavelengths other than blue light, therefore this study was conducted using only blue light exposure (Suppl. Fig. 1). Our study showed that the most effective and minimum energy for blue light irradiation to affect living organisms is 40 kj/m (data not shown). Therefore, this test used an irradiation energy amount of 40 kJ/m. Our laboratory's irradiator can provide 40kJ/m of energy in 10 min of irradiation. Moreover, this amount of energy is enough to be exposed to in daily life. The study was approved by the Suzuka University of Medical Science Animal Experiment Ethics Committee on September 25, 2014, and was performed in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the Suzuka University of Medical Science (Approval number: 34). All surgeries were performed on mice under pentobarbital anesthesia and efforts were made to minimize animal suffering.

STZ-induced Type 1 Diabetes Mice were quarantined for the first 7 days and then randomly divided into two groups according to body weight (10 mice per group). In the STZ group, mice were treated with STZ (250 mg/kg) (Sigma-Aldrich, Darmstadt, HE, Germany) administered via a single intraperitoneal injection. STZ is an antibiotic extracted from *Streptomyces achromogenes* and an N-nitroso derivative of glucose,[22] which causes rapid and irreversible necrosis of pancreatic B cell.[23] Four weeks after the injection, we measured body weight, daily water intake, daily urine output, and blood glucose levels. The control mice were untreated throughout the experiment.

Tranexamic Acid (TA) Treatment Approximately 12 mg kg^{-1} of TA (Daiichi Sankyo Healthcare Co., Ltd., Tokyo, Japan) dissolved in distilled water was administered orally three times a week for 4 weeks. Control animals were administered distilled water.[24] In addition, this dose was equivalent to administered to humans. Furthermore, when TA was administered without blue light irradiation, no effect on STZ was observed (Suppl. Fig. 2). Therefore, we reasoned that TA was potentially nontoxic to mice at the administered doses.

DADA Treatment Approximately 500 μg kg^{-1} of DADA (Daiichi Sankyo Healthcare Co., Ltd., Tokyo, Japan) dissolved in distilled water was administered orally three times a week for 4 weeks. Distilled water was administered to control ani-

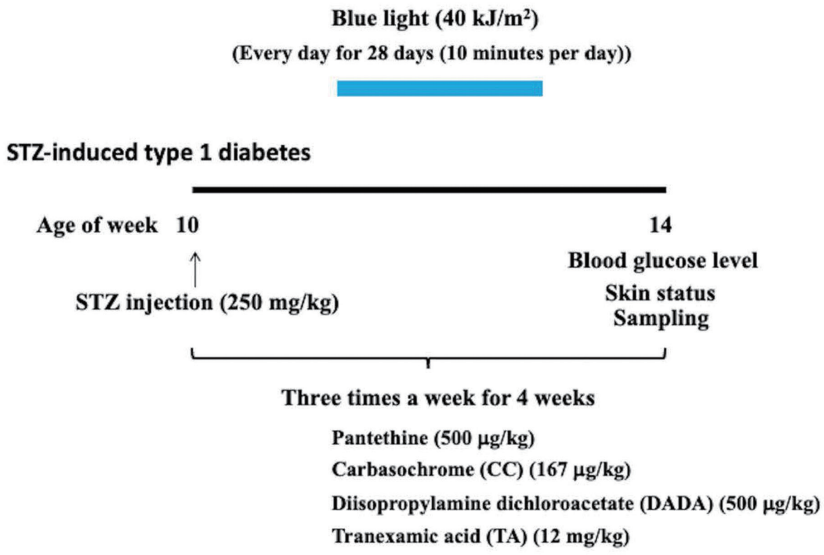


Fig. 1. Schematic of the Experimental Procedure.

mals. The DADA dose administered to mice is equivalent to that administered to humans. Furthermore, when DADA was administered without blue light irradiation, no effect on STZ was observed (Suppl. Fig. 2). Therefore, we reasoned that DADA was potentially nontoxic to mice at the administered doses.

Carbasochrome (CC) Treatment Approximately 167 μg kg^{-1} of CC (Daiichi Sankyo Healthcare Co., Ltd., Tokyo, Japan) dissolved in distilled water was administered orally three times a week for 4 weeks. Distilled water was administered to control animals. The CC dose administered to mice was equivalent to that administered to humans. Furthermore, when CC was administered without blue light irradiation, no effect on STZ was observed (Suppl. Fig. 2). Therefore, we reasoned that CC was potentially nontoxic to mice at the administered doses.

Pantethine Treatment Approximately 500 μg kg^{-1} of pantethine (Daiichi Sankyo Healthcare Co., Ltd., Tokyo, Japan) dissolved in distilled water was administered orally three times a week for 4 weeks. Distilled water was administered to control animals. The pantethine dose administered to mice was equivalent to that administered to humans. Furthermore, when pantethine was administered without blue light irradiation, no effect on STZ was observed (Suppl. Fig. 2). Therefore, we reasoned that pantethine was potentially nontoxic to mice at the administered doses.

Preparation of Pancreas Samples and Staining On the last day of the experiment, pancreas samples were collected under anesthesia. The samples were fixed in 4% phosphate-buffered paraformaldehyde, embedded in Tissue Tek OCT compound (Sakura Finetek, Tokyo, Japan), and cryosectioned. The sections were stained with hematoxylin and eosin (HE) following established procedures for histological analysis of the skin. The sections were subjected to terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining using an *in situ* apoptosis detection kit (Takara, Shiga, Japan). The specimens were stained with antibodies for immunohistological analysis as previously described.[25] The pancreas specimens incubated with either mouse monoclonal anti-lymphocyte antigen 6 complex locus G6D (Ly6G: marker of neutrophil) (1:100; BD Biosciences, Franklin Lakes, NJ, USA), rabbit polyclonal anti-citrullinated histone H3 (citH3) (1:100; Abcam, Cambridge, MA, USA), rabbit polyclonal anti-protein arginine deiminase 4 (PAD4) (1:100; Abcam), or rabbit polyclonal anti-endothelial-monocyte activating polypeptide II (EMAP II) (1:100; Proteintech, Rosemont, IL, USA) primary antibodies. The samples were then washed and incubated with fluorescein isothiocyanate-conjugated anti-mouse or anti-rabbit secondary antibody(1:30; Dako Cytomation, Glostrup, Denmark). The expression levels of Ly6G, citH3, PAD4, and EMAPII were immunohistochemically evaluated via fluorescence microscopy. Additionally, the expression of Ly6G, citH3, PAD4, and EMAP II was quantitated by visualizing the staining in five random visual fields, with a constant area, using the ImageJ software ver. 1.53 (National Institutes of Health, Bethesda, MD, USA). Briefly, the original files were converted to monochrome 8-bit files. Next, the luminous intensity threshold was voluntarily established. Areas above the threshold were measured for each sample. These areas were defined as "intensity" in this study.

Measurement of the Levels of Insulin-like Growth Factor 1 (IGF-1), Transforming Growth Factor-β (TGF-β), Caspase 3, and Angiopoietin-like Protein 2 (Agptl2) in Plasma, and of Reactive Oxygen Species (ROS) and Hydrogen Peroxide (H_2O_2) in Pancreas Blood and pancreatic tissue samples were collected at the end of the experiment. Plasma was separated from blood samples by centrifugation at 3000 × *g* for 10 min at 4°C, and the supernatant was used for further analysis. The levels of IGF-1, TGF-β, caspase 3, and Agptl2 were measured using commercially available enzyme-linked immunosorbent assay kits (IGF-1: Proteintech, Rosemont, IL, USA; TGF-β: Promega, Madison, MI, USA; caspase 3 and Agptl2: Cusabio, Houston, TX, USA) as per the manufacturers' instructions. After homogenization, the samples were homogenized at 15000 × *g* for 15 min at 4°C, and the supernatant was collected for the assays. ROS and H_2O_2 levels in the pancreas were determined using an OxiSelect™ STA-347 *in vivo* ROS/RNS assay kit (Cell Biolabs, Inc., San Diego, CA, USA) in accordance with the manufacturer's instructions. The optical density was measured using a microplate reader (Mdecular Devices, Sunnyvale, CA, USA).

Statistical Analysis All data are presented as the mean ± standard deviation. Statistical differences between the different groups were determined using a two-way analysis of variance (ANOVA) followed by Tukey's posthoc test (SPSS version 20; IBM, Armonk, New York, USA). Values of $p < 0.05$ (*) and < 0.01 (**) were considered significant.

RESULTS

Effect of Pantethine, CC, DADA, and TA Treatment on Body Weight, Water Intake, Urine Production, and Blood Glucose Levels in Mice with STZ-induced Type 1 Diabetes The body weight (Fig. 2a), water intake (Fig. 2b), urine production (Fig. 2c), and blood glucose (Fig. 2d) levels in mice with STZ-induced type 1 diabetes. No changes in body weight were observed in any group. Water consumption, urine output, and blood glucose levels were increased in mice with STZ-induced type 1 diabetes, and blue light irradiation further increased these levels and worsened diabetes. Furthermore, the administration of TA or pantethine reduced these diabetic indicators and rescued the increase caused by blue light. The indicators remained high after the administration of CC or DADA.

Effects of Pantethine, CC, DADA, and TA Treatment on the Pancreatic Condition of Mice with STZ-induced Type 1 Diabetes The condition of the pancreas was observed under a microscope after blue light irradiation of mice for 4 weeks (Fig. 3). HE-stained images of the pancreas showed damage to β cells in mice with STZ-induced type 1 diabetes. Blue light irradiation further increased the damage. A reduction in damage was observed following the administration of pantethine and TA.

Effects of Pantethine, CC, DADA, and TA Treatment on the Expression of Neutrophils, cisH3, and PAD4 in Pancreas of Mice with STZ-induced Type 1 Diabetes We investigated the effects of neutrophil extracellular trap-associated cell death (NETosis) in mice with STZ-induced type 1 diabetes exposed to blue light. The expression of Ly6G (a neutrophil marker; Fig. 4a), cisH3 (Fig. 4b), and PAD4 (Fig. 4c), which are indicators of NETosis, was significantly increased in diabetic mice, and their expression was further increased upon blue light irradiation. Upon administration of each of the compounds, the expression of neutrophils, cisH3, and PAD4 was suppressed. Among these, TA had the most remarkable suppressive effect.

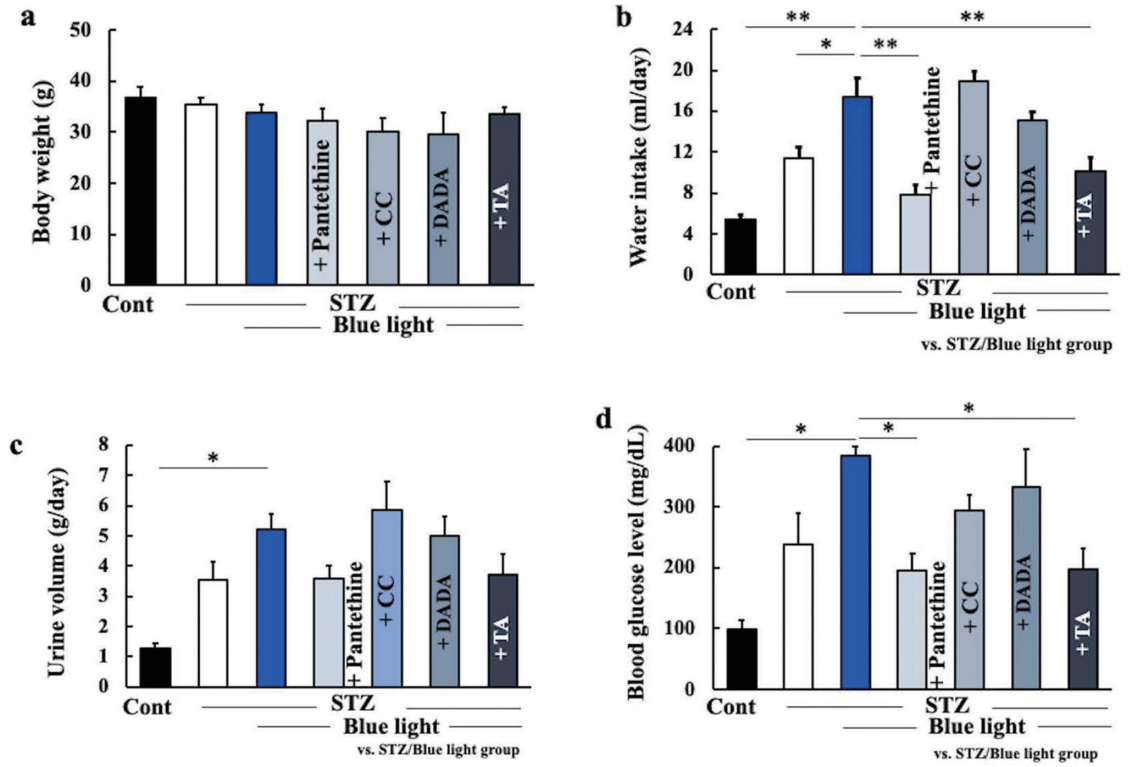


Fig. 2. Effect of Pantethine, Carbasochrome (CC), Diisopropylamine Dichloroacetate (DADA), and Tranexamic Acid (TA) Treatment on Body Weight (a), Water Intake (b), Urine Production (c), and Blood Glucose Levels (d) in Mice with Streptozotocin (STZ)-induced Type 1 Diabetes Irradiated with Blue Light.

The values are expressed as means ± SD for five animals. Statistical significance was evaluated by comparing with the STZ/blue light group. ** $p < 0.01$. * $p < 0.05$.

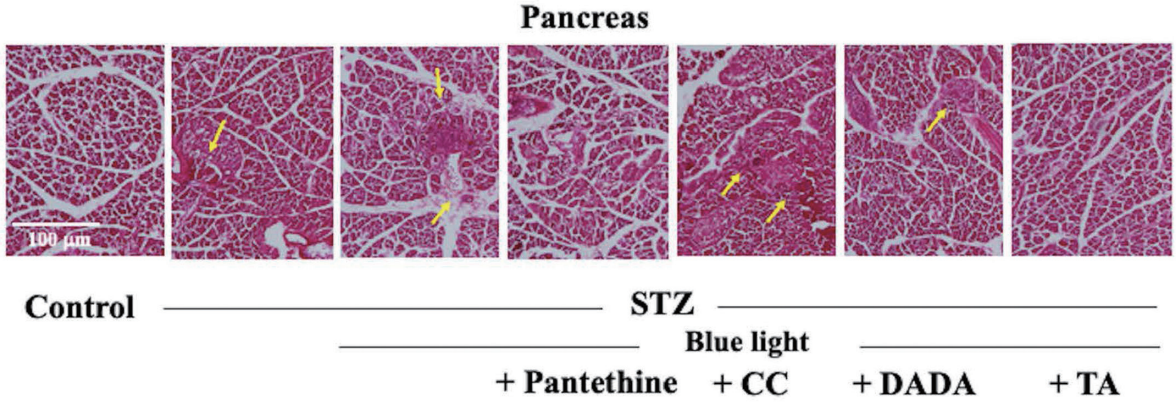


Fig. 3. Effect of Pantethine, Carbasochrome (CC), Diisopropylamine Dichloroacetate (DADA), and Tranexamic Acid (TA) Treatment on the Histology of Pancreas from Mice with Streptozotocin (STZ)-induced Type 1 Diabetes Irradiated with Blue Light.

Scale bar = 100 μm. Arrows indicate damaged β cells.

Effects of CC, DADA, and TA Treatment on the Levels of ROS, H_2O_2, IGF-1, Caspase 3 and on Apoptosis and EMPA-II in Pancreas of Mice with STZ-induced Type 1 Diabetes Next, we investigated the mechanisms underlying neutrophilia in mice with STZ-induced type 1 diabetes exposed to blue light. In the diabetic mice, blue light irradiation increased the levels of pancreatic ROS (Fig. 5a), H_2O_2 (Fig. 5b), and caspase 3 (Fig. 5d) as well as apoptosis (Fig. 5e) and EMPA-II expression (Fig. 5f). In contrast, IGF-1 (Fig. 5c) levels were decreased. Administration of TA suppressed the increase in the levels of ROS, H_2O_2, caspase 3, and EMPA-II and suppressed the decrease in IGF-1 levels. The administration of pantethine suppressed the increase in the expression of caspase 3 and EMPA-II and the decrease in IGF-1 levels.

Effects of CC, DADA, and TA on the Level of Agptl2 in the Pancreas of Mice with STZ-induced Type 1 Diabetes We investigated the effect on the expression of Angptl2 (Fig. 6), a factor that exacerbates diabetes. Agptl2 expression was sig-

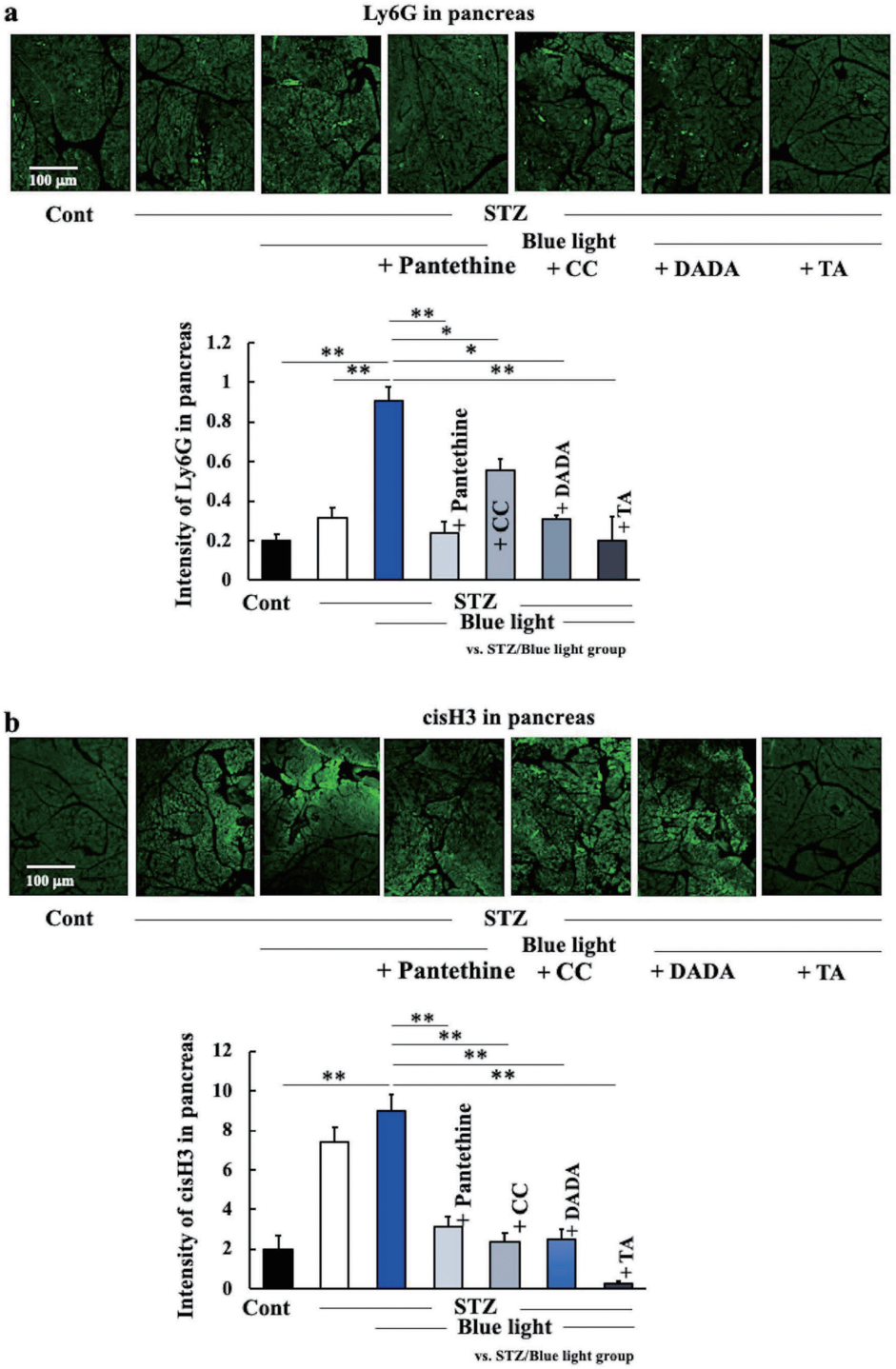


Fig. 4. Effect of Pantethine, Carbasochrome (CC), Diisopropylamine Dichloroacetate (DADA), and Tranexamic Acid (TA) Treatment on the Expression of Ly6G (Marker of Neutrophils) (a), cisH3 (b), and PAD4 (c) in Pancreas Sections from Mice with Streptozotocin (STZ)-induced Type 1 Diabetes Irradiated with Blue Light.

The values are expressed as means ± SD for five animals. Statistical significant was evaluated by comparing with the STZ/blue light group. ** $p < 0.01$. * $p < 0.05$.

nificantly increased in blue light-irradiated type 1 diabetic mice. However, the deterioration caused by blue light irradiation was inhibited by the administration of each of the compounds. This effect was the most pronounced for TA.

DISCUSSION

In this study, we found that STZ-induced type 1 diabetes is exacerbated by blue light irradiation. Furthermore, administration of TA and pantethine prevented the worsening of type 1 diabetes caused by blue light. Blue light irradiation increased

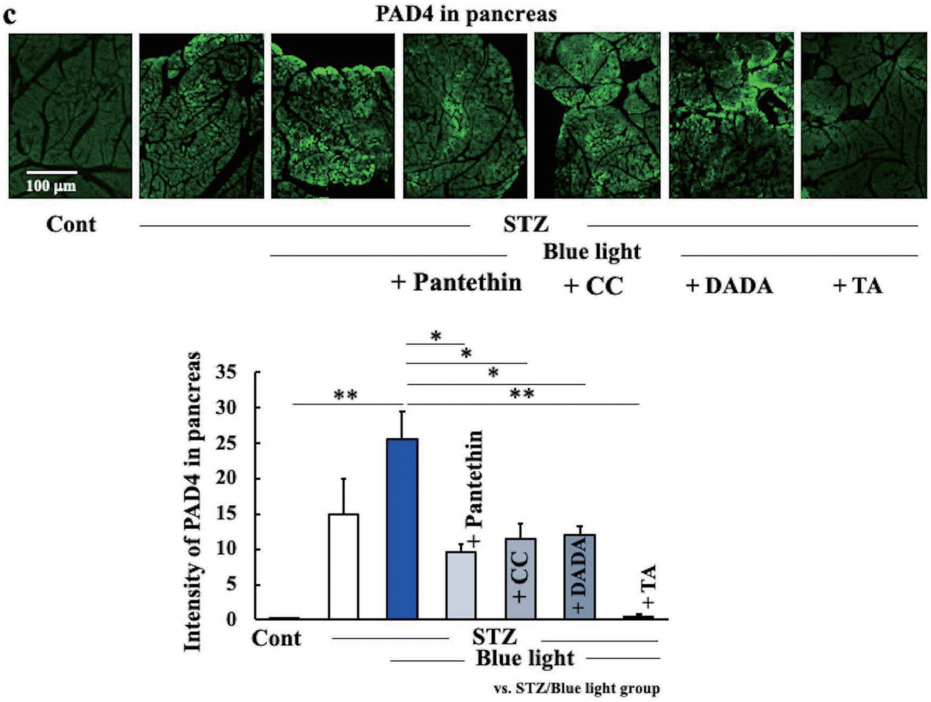


Fig. 4. (Continued)

the levels of Ly6G, cisH3, and PAD4 and induced NETosis. Additionally, ROS, H_2O_2, apoptosis, and Angptl2 levels increased. These effects were suppressed by TA administration. In addition, IGF-1 levels were decreased by blue light irradiation, but were increased by the administration of TA or pantethine.

NETosis increases after irradiation of neutrophils with blue light.[26] NETosis is composed of decondensed chromatin produced by neutrophilic granulocytes and is modified with antimicrobial peptides. NETosis is a biological defense reaction; however, it is also involved in autoimmune diseases. Type 1 diabetes is an autoimmune disease, and it is believed that increased NETosis in the pancreas induces apoptosis and increases the destruction of pancreatic β cells. The mechanism by which blue light stimulation affects pancreatic neutrophils involves the induction of oxidative stress. Mitochondrial oxidative stress is a type of oxidative stress induced by blue light irradiation.[27-29] Blue light also promotes ROS uptake into mitochondria.[30] Mitochondria trigger the activation of caspase-3, a cell death-inducing factor.[31] Caspase-3 increases the expression of EMAP-II, a chemotactic factor in neutrophils,[32] leading to the accumulation of neutrophils. Furthermore, we observed that blue light irradiation reduced the IGF-1 levels. IGF-1 is essential for cell differentiation, growth, and survival. It also suppresses apoptosis by inhibiting the activation of caspase-3, and suppresses the accumulation of neutrophils by inhibiting EMAP-II expression.[33] Blue light is believed to increase the accumulation of neutrophils by suppressing the expression of IGF-1 and increasing the induction of apoptosis, leading to the worsening of diabetes. Various studies have reported a relationship between blue light and IGF-1 levels. Xia *et al*. (2021) reported that blue light exposure significantly reduced the serum IGF-1 levels.[34] In contrast, Harada (2009) reported that blue light stimulates sensory nerves via the clock gene cry, thereby, promoting IGF-1 production throughout the body.[35] As described above, much remains unknown regarding the relationship between blue light and IGF-1 expression, warranting further research.

We also found that Agptl2 levels were significantly increased by blue light irradiation. Agptl2 is strongly expressed in visceral adipose tissue.[36] The secretion of Agptl2 by adipocytes induces degradation of the extracellular matrix by MMPs. Therefore, Agptl2 overexpression induces chronic inflammation in adipose tissue through macrophage infiltration and activation of inflammatory pathways in vascular endothelial cells, resulting in reversible tissue remodeling.[37, 38] Consequently, it is thought to be involved in the promotion of pathological conditions associated with metabolic abnormalities, such as obesity, systemic insulin resistance, and the development of diabetes.[36] Therefore, it is possible that the increase in Agptl2 levels caused by blue light irradiation induces tissue remodeling and influences the onset and worsening of diabetes. The role of clock genes in mediating the relationship between blue light and Agptl2 expression has been reported. [39,40] In recent years, attention has been focused on the fact that lifestyle-related diseases disrupt the circadian clock, causing metabolic syndromes and arteriosclerosis. Many knockout mice lacking the major constituent genes of the circadian clock mechanism exhibit symptoms of lifestyle-related diseases.[41,42] The expression rhythm of Agptl2 was reported to be abolished in double knockout mice of cryptochrome (Cry) 1 and 2, which are major clock genes.[43,44] When the rhythm of Agptl2 disappears and its expression continues to increase, chronic inflammation occurs, which leads to the onset and development of diabetes. Blue light affects clock genes by targeting the brain and muscle arnt-like 1 (Bmal1)/Clock.[45,46] Bmal1/Clock is closely associated with Cry1,2; therefore, blue light may affect the periodicity of Agptl2. However, the details

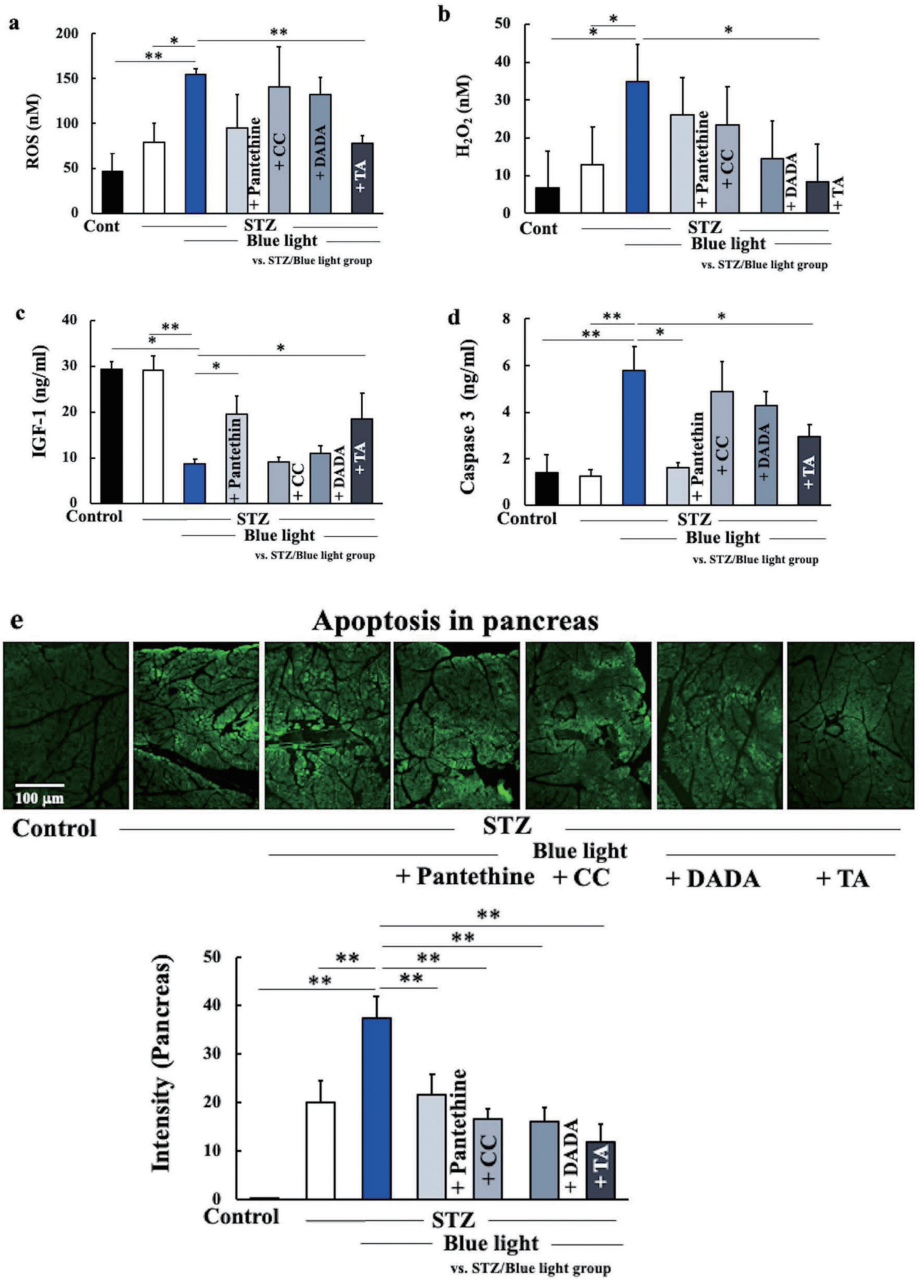
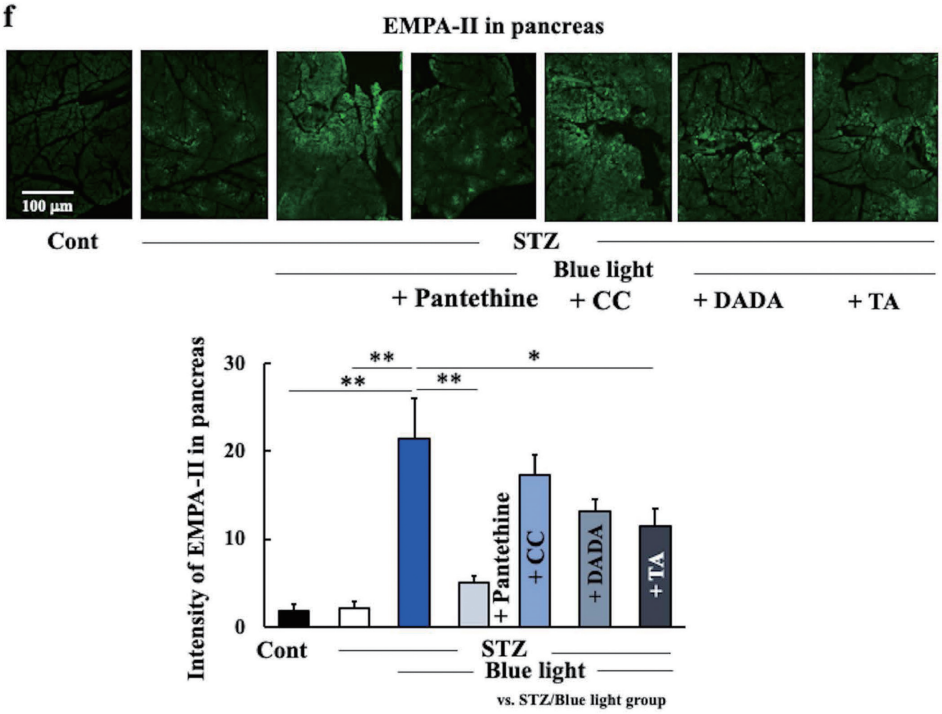


Fig. 5. Effect of Pantethine, Carbasochrome (CC), Diisopropylamine Dichloroacetate (DADA), and Tranexamic Acid (TA) Treatment on the Levels of ROS (a), H_2O_2 (b), IGF-1 (c), and Caspase 3 (d), and on Apoptosis (e) and EMPA-II Expression (f) in Pancreas Sections from Mice with Streptozotocin (STZ)-Induced Type 1 Diabetes Irradiated with Blue Light.

Scale bar = 100 µm. The values are expressed as means ± SD for five animals. Statistical significance was evaluated by comparing with the STZ/blue light group. ** $p < 0.01$. * $p < 0.05$.

of the mechanism by which blue light increases Agptl2 expression are unknown, and further investigation is required.

In this study, we investigated the effects of TA, CC, DADA, and pantethine, agents that affect blood vessels, in alleviating the worsening of blue light irradiation-induced diabetes. Administration of TA and pantethine had an excellent effect mitigating the worsening of diabetes caused by blue light. TA significantly suppressed blue-light-induced NETosis in neutrophils, and the diabetes-improving effect of TA was believed to be mainly due to the suppression of NETosis. Furthermore, TA and pantethine suppressed the blue light-induced induction of IGF-1/caspase 3/Apoptosis/EMPA-II/neutrophil signaling. This suggests that the administration of TA and pantethine suppressed the accumulation of neutrophils, which forms the basis of NETosis. In addition, TA and pantethine suppressed the blue light-induced increase in Agptl2 expression, which is a cause of worsening of diabetes, and is also considered to be one of manifestations of improvement in the worsening of diabetes. Thus, TA was found to have an ameliorating effect via suppression of IGF-1/caspase 3/Apoptosis/EMPA-II/neutrophil system signaling, increasing Agptl2 expression and NETosis in diabetic mice exposed to blue light. In contrast, pantethine suppressed NETosis to a lesser extent than did TA, but suppressed IGF-1/caspase 3/Apoptosis/EMPA-II/



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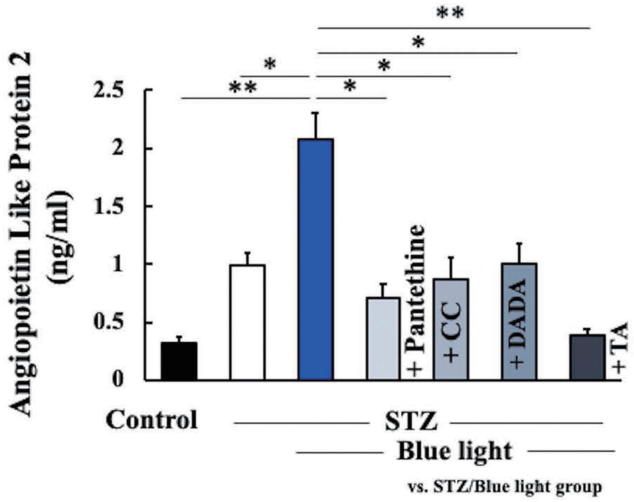

Fig. 6. Effect of Pantethine, Carbasochrome (CC), Diisopropylamine Dichloroacetate (DADA), and Tranexamic Acid (TA) Treatment on the Levels of Agptl2 in Pancreas Sections from Mice with Streptozotocin (STZ)-Induced Type 1 Diabetes Mice in Irradiated with Blue Light.

The values are expressed as means ± SD for five animals. Statistical significance was evaluated by comparing with the STZ/blue light group. ** $p < 0.01$. * $p < 0.05$.

neutrophil system signaling and increased the expression of Agptl2, suggesting that blue light irradiation can rescue diabetes aggravation. This is considered a contributing factor for the observed effects. However, the molecular mechanism used by TA and pantethine in influencing the IGF-1/caspase 3/Apoptosis/EMPA-II/neutrophil system signaling, increase in Agptl2 expression and NETosis remains unknown, warranting further research.

CONCLUSION

In this study, we show that blue light irradiation worsens STZ-induced type 1 diabetes. However, this result was obtained in mice. In humans, NETosis formation is infrequent at moderate doses. Additionally, NETosis are rapidly cleared by DNase, followed by their phagocytosis.[47,48] Exposure to moderate amounts of sunlight is unlikely to cause serious reactions. However, the mechanism demonstrated in this study would be extremely beneficial under conditions of exposure to large amounts of blue light. In addition, intake of TA and pantethine may improve type 1 diabetes.

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Conflict of Interest Authors S. Kubo, K. Tsuji, D. Sugiyama and H. Hamano were employed by the company Daiichi Sankyo Healthcare Co., Ltd. The authors declare that this study received funding from Daiichi Sankyo Healthcare Co., Ltd. The funder was not involved in the study design, collection, analysis, or interpretation of data; the writing of this article; or the decision to submit it for publication.

Institutional Review Board Statement This study strictly followed the recommendations and guidelines for the Care and Use of Laboratory Animals of Suzuka University of Medical Science (approval number: 34). All surgical procedures were performed under pentobarbital anesthesia, and every effect was adjusted to minimize animal suffering.

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