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Oral and ocular administration of crocetin prevents retinal edema in a murine retinal vein occlusion model

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Purpose: We investigated the effects of oral and ocular administration of crocetin in a murine retinal vein occlusion (RVO) model. Crocetin is a type of carotenoid contained in the fruit of gardenia (*Gardenia jasminoides* Ellis) and the stigma of saffron (*Crocus staruts* L).

Methods: This study was performed on a murine RVO model, which was created by laser irradiation of retinal veins. We evaluated the retinal thickness after the oral administration of crocetin (100 mg/kg) 1 and 6 h before laser irradiation, and immediately, 6 h, 12 h, and 18 h after laser irradiation in the murine RVO model. In addition, we measured the retinal layer thickness after administration of crocetin eye drops (0.03% or 0.10%) immediately, 6 h, and 12 h after laser irradiation. Western blotting of retinal tissue was used to determine the expression levels of matrix metalloproteinase (MMP-9), tumor nuclear factor (TNF-α), and occludin after oral administration of crocetin.

Results: Oral and ocular administration of crocetin improved retinal edema in the murine RVO model. Crocetin administration statistically significantly suppressed overexpression of MMP-9 and TNF-α, and reversed the reduction of occludin.

Conclusions: These findings indicate that crocetin can protect retinal tight junctions by suppressing retinal edema through an anti-inflammatory effect, which suggests that crocetin may be useful for RVO disease.

Retinal vein occlusion (RVO) is the second most common retinal vascular disorder. RVO can cause ocular morbidity, including macular edema, vitreous hemorrhage, and nonperfusion area, which leads to a reduction in visual acuity [1,2]. The number of patients with RVO is about 16 million, and the prevalence of RVO is increasing [3]. Some patients have an increased risk of cardiovascular and coagulation disorders [3]. The first choice of treatment for RVO is anti-vascular endothelial growth factor (VEGF) therapy. An intravitreal injection of anti-VEGF agents improves retinal edema in patients with RVO [4,5]. An intravitreal injection maintains the concentration of drugs in the vitreous body, and circumvents the blood–retinal barrier (BRB) by delivering the drug into the retina directly. However, there are several problems with intravitreal administration of anti-VEGF agents, such as poor vision from macular edema and nonperfused retinal areas [6,7]. Therefore, new drugs with mechanisms different from the VEGF signaling pathway are needed. Intravitreal administration of anti-VEGF antibody results in an increased physical and mental burden [8,9]. Thus, the development of non-invasive drug delivery is needed for patients with RVO. Drug delivery by oral administration to treat retinal diseases is preferred by patients compared to an injectable route [10,11]. In addition, administration of eye drops has few side effects, and is a simple administration method for the elderly [10,11]. Thus, we evaluated non-invasive routes of drug delivery to treat RVO.

Crocetin has a protective effect against retinal degeneration by peripheral administration [12-14]. Crocetin is a carotenoid contained in saffron crocus (*Crocus staruts* L) and gardenia fruit (*Gardenia jasminoides* Ellis). Saffron has been used in traditional herbal medicine, and crocetin has been used to maintain health. Crocetin is absorbed more rapidly than other carotenoids, because it is amphiphilic, has a low molecular weight, and has high transferability. Crocetin has an antioxidant effect by eliminating reactive oxygen species, and inducing antioxidant enzymes [15,16], and has an anti-inflammatory effect by suppressing the production of inflammatory factors in macrophages and endothelial cells [17,18]. Oral administration of crocetin protects against photoreceptor cell death in a light-induced retinal damage model and a retinal ischemic reperfusion injury model [12,13]. These data indicate that oral administration of crocetin for delivery into ocular tissues, such as the retina, is highly effective. Thus, the pathological symptoms of RVO may be improved by administration of crocetin.

Some patients with RVO had progression of retinal edema and the nonperfusion area, because of increased oxidative stress. In clinical studies, inflammatory cytokines, such

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as interleukin-6 (IL-6), monocyte chemoattractant protein-1 (MCP-1), and anti-matrix metalloproteinase (MMP-9), were increased in the eyes of patients with RVO [19,20]. An increase in inflammatory cytokines induces a breakdown of the vascular structure, and leakage of plasma components and water [21]. Therefore, excess inflammatory factors are associated with the formation of retinal edema in patients with RVO. A murine RVO model can be used to examine the effects of new agents and mechanisms on retinal edema from the increased expression of VEGF and inflammatory factors [22-24]. We investigated the effects and mechanisms of oral and ocular administration of crocetin on retinal edema in a murine RVO model.

METHODS

Animals: Male Deutschland, Denken, and Yoken (ddY) mice (8 weeks old) were purchased from Japan SLC (Shizuoka, Japan). These animals were maintained at 23±3.0 °C under a 12 h:12 h light-dark cycle (lights on from 08:00 to 20:00), and ad libitum access to food and water. All experiments were performed in accordance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research, and the experimental protocols were approved by the Institutional Animal Care and Use Committee of Gifu Pharmaceutical University.

Murine RVO model: The murine RVO model was performed as described [24]. Mice were anesthetized with a mixture of ketamine (120 mg/kg; Daiichi-Sankyo, Tokyo, Japan) and xylazine (6 mg/kg; Bayer, Health Care, Osaka, Japan) with an intramuscular injection. After it was confirmed that the anesthesia was adequate, the mice were injected with rose bengal (8 mg/ml; Wako, Osaka, Japan) into the tail vein. The pupils were dilated with 0.5% tropicamide and 0.5% phenylephrine (Santen Pharmaceuticals Co., Ltd., Osaka, Japan). Then, 0.1% purified sodium hyaluronate was applied, and ten to 15 laser spots were performed to three branch veins (3 disc diameters from the optic nerve centers) in the right eye of each animal. The image-guided laser system was attached to the Micron IV Retinal Imaging Microscope (Phoenix Research laboratories, Pleasanton, CA), and laser irradiation was performed with a 532 nm laser light applied at 50 mW, 5 s, and 50 µm (Meridian AG, Bierigustrasse, Switzerland).

When laser irradiation was performed except the major blood vessel, formation of edema or upregulation of inflammatory cytokines did not occur. These data indicate that in a RVO model of the albino mouse, laser damage and laser-induced inflammation did not influence the formation of edema, and the formation of edema is caused by vascular occlusion and ischemia. This model is optimal as an RVO pathological model, because this murine RVO model has some features similar to the clinical condition, such as retinal edema and hard exudates by vascular occlusion.

Oral administration of crocetin: Crocetin was suspended in 0.5% carboxymethylcellulose sodium aqueous solution (CMC). Crocetin was orally administered at a dose of 100 mg/kg 1 and 6 h before laser occlusion and immediately, 6 h, 12 h, and 18 h after laser irradiation. The vehicle-treated group was administered 10 ml/kg CMC.

Ocular administration of crocetin by eye drops: Crocetin was suspended in 1% PBS (12.68 mM KCl, 1.47 mM KH_2PO_4, 137 mM NaCl, 8.10 mM Na_2HPO_4) containing 4% polyethylene glycol (PEG), 0.1% Tween-80, and 0.01% dimethyl sulfoxide (DMSO). Crocetin was administered by eye drops at doses of 0.10% and 0.03% (5 µl) immediately, 6 h, and 12 h after laser irradiation every 5 min five times. The vehicle-treated group was administered a solvent of eye drops.

Histological analysis: The mice were euthanized with cervical dislocation, and the eyes were enucleated. The eyeball was fixed in a fixative solution containing 4% paraformaldehyde (PFA) for at least 48 h at 4 °C. Six paraffin-embedded sections (thickness: 5 µm) were cut at the point the optic nerve disc connects to the eyeball, and stained with hematoxylin and eosin (H&E). All images were taken using a fluorescence microscope (BZ-X710; Keyence, Osaka, Japan). The damage induced by the retinal vein occlusion was evaluated in six randomly selected areas from each eye for the morphometric analysis. The thickness of the inner nuclear layer (INL) was measured on photographs every 240 µm from the optic disc toward the periphery with Image J (National Institutes of Health, Bethesda, MD). Quantitative data from three sections (selected randomly from the six sections) were averaged for each eye.

Western blotting: Western blotting was performed as reported [25]. The primary antibodies were anti-MMP-9 antibody (Merck Millipore, Burlington, MA, AB19016, rabbit polyclonal antibody), anti-tumor necrosis factor-α (TNF-α) antibody (Santa Cruz Biotechnology, Dallas, TX, sc-52746, mouse monoclonal antibody), anti-occludin antibody (Abcam, Cambridge, England, ab64482, rabbit polyclonal antibody), and mouse anti-β-actin antibody (Sigma, Tokyo, Japan, A2228, mouse monoclonal antibody). The secondary antibodies were horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody (1:1,000) and HRP-conjugated goat anti-mouse immunoglobulin G (IgG; 1:2,000; Pierce Biotechnology, Inc., Waltham, MA). Immunoreactive bands were visualized with Immuno star® LD (Wako Pure Chemical Industries, Osaka, Japan), and chemiluminescence was

detected with the LAS-4000 Luminescent Image Analyzer (Fuji Film Co. Ltd., Tokyo, Japan).

Statistical analysis: The data are presented as the mean ± standard error of the mean (SEM). The significance of the differences was determined with the Student *t* test. A p value of less than 0.05 was considered statistically significant. In each figure, we performed the suitable sample number that showed a statistically significant difference (p=0.05) with power analysis to confirm the adequacy of the N value in this study. The sample numbers were investigated for parameters that indicated the significance level, amount of change, standard deviation, and statistical power. The value of the significance level (sig.level) was p=0.05, the value of statistical power (power) was 0.8, and the value of the amount of change (delta) and the standard deviation (SD) were used from each sample datum. We used R software to calculate the suitable sample numbers.

RESULTS

Decrease in retinal edema with oral administration of crocetin: We investigated whether retinal edema was improved with oral administration of crocetin. The treatment protocol for this experiment is shown in Figure 1A. We measured changes in retinal thickness 1 day after oral administration of crocetin with H&E staining. Oral administration of crocetin statistically significantly improved the increase in the thickness of the INL (Figure 1B,C).

Decrease in retinal edema with crocetin eye drops: We investigated whether crocetin eye drops decreased retinal thickness in a murine RVO model. We analyzed the retinal thickness of the INL in the control group, vehicle-treated group, and crocetin-treated group (0.1% and 0.03%) with eye drops. The treatment protocol for this experiment is shown in Figure 2A. Eye drops of 0.10% crocetin statistically significantly normalized retinal pathological thickening (Figure 2B,C).

Decreased expression of MMP-9 and TNF-α with oral administration of crocetin: We elucidated the mechanism of action of crocetin on retinal edema in a murine RVO model with western blotting. TNF-α is a typical inflammatory mediator induced in ischemic conditions, and activates downstream inflammatory factors [25]. The level of MMP-9 is activated by an increase in TNF-α and collapsed cell–cell junctions in vascular endothelial cells [26]. We investigated the expression level of MMP-9 12 and 24 h after laser irradiation in a murine RVO model. The expression of MMP-9 was increased 12 and 24 h after laser irradiation (Figure 3A,B). We investigated changes in MMP-9 and TNF-α expression 24 h after the oral administration of crocetin with western blotting. The expression levels of MMP-9 and TNF-α were increased in the vehicle-treated group, and oral administration of crocetin statistically significantly reduced these expression levels (Figure 3C–F).

Increased tight junction expression with oral administration of crocetin: Tight junctions are situated between two adjacent endothelial cells, and provide structural integrity to retinal blood vessels [27-29]. Occludin stabilizes tight junctions, and improves barrier function [29]. Loss of tight junctions increases vascular permeability, and induces cerebral edema [30]. We investigated the expression level of occludin after oral administration of crocetin with western blotting. The expression level of occludin was decreased in the vehicle-treated group compared with that of the control group, and this reduction was normalized with oral administration of crocetin.

DISCUSSION

We found that oral and ocular administration of crocetin improved retinal edema. Furthermore, the expression levels of MMP-9, TNF-α, and occludin were normalized 24 h after oral administration of crocetin.

Macular edema is the most important symptom in RVO pathology, which leads to loss of visual function [31]. Intravitreal injection of anti-VEGF antibody is used as a treatment for patients with RVO. However, constant intravitreal injection has risk of complications from vitreous hemorrhage and retinal detachment [8,12,30]. Some drawbacks, such as recurrence of pathological symptoms, increased economic burden, and endophthalmitis, for patients with RVO can occur [8,9,23]. Therefore, the development of therapies involving peripheral administration for RVO is needed.

Oral administration of crocetin can protect vascular endothelial cells, and reduce hyperpermeability induced by ischemia and reperfusion [32-35]. In this study, we found that oral administration of crocetin reduced the degree of edema in a murine RVO model (Figure 1). Previously, we measured the concentrations of crocetin in the plasma and aqueous humor after oral administration at a dose of 50 mg/kg in the rat [12]. The aqueous humor concentration of crocetin was approximately 2 μM 1.5 h after oral administration, and protected against cell death in retinal ganglion cell culture [12]. Moreover, oral administration of crocetin at 100 mg/kg protected against retinal degeneration induced by *N*-methyl-D-aspartate in mice [14], which suggests that crocetin has protective efficacy in a murine RVO model. Oral administration of crocetin suppresses inflammatory factors, such as TNF-α and intercellular adhesion molecule 1 (ICAM-1), in a murine cerebral ischemic model [32]. In addition, crocetin reduces the production of inflammatory factors, such as

MCP-1 and IL-8, by inhibiting the nuclear factor-kappa beta (NF-kB) signaling pathway in human umbilical endothelial cells (HUVECs) [17]. The edema formation in pathological symptoms of RVO is associated with inflammatory response. The expression levels of inflammatory factors, such as IL-6, MCP-1, and ICAM-1, are increased in patients with RVO [36,37], and in a RVO murine model [24]. In a previous report, we performed laser irradiation except the major blood vessel to investigate laser damage and laser-induced inflammation in a sham group. As a result, the expression levels of inflammatory factors, such as IL-6, ICAM-1, and MCP-1, did not change in sham-operated group. These data indicate that laser damage and laser-induced inflammation do not influence the induction of inflammation, and the inflammatory response is caused by vascular occlusion and ischemia. In the typical images of histological analysis, the fluid leakage area and the

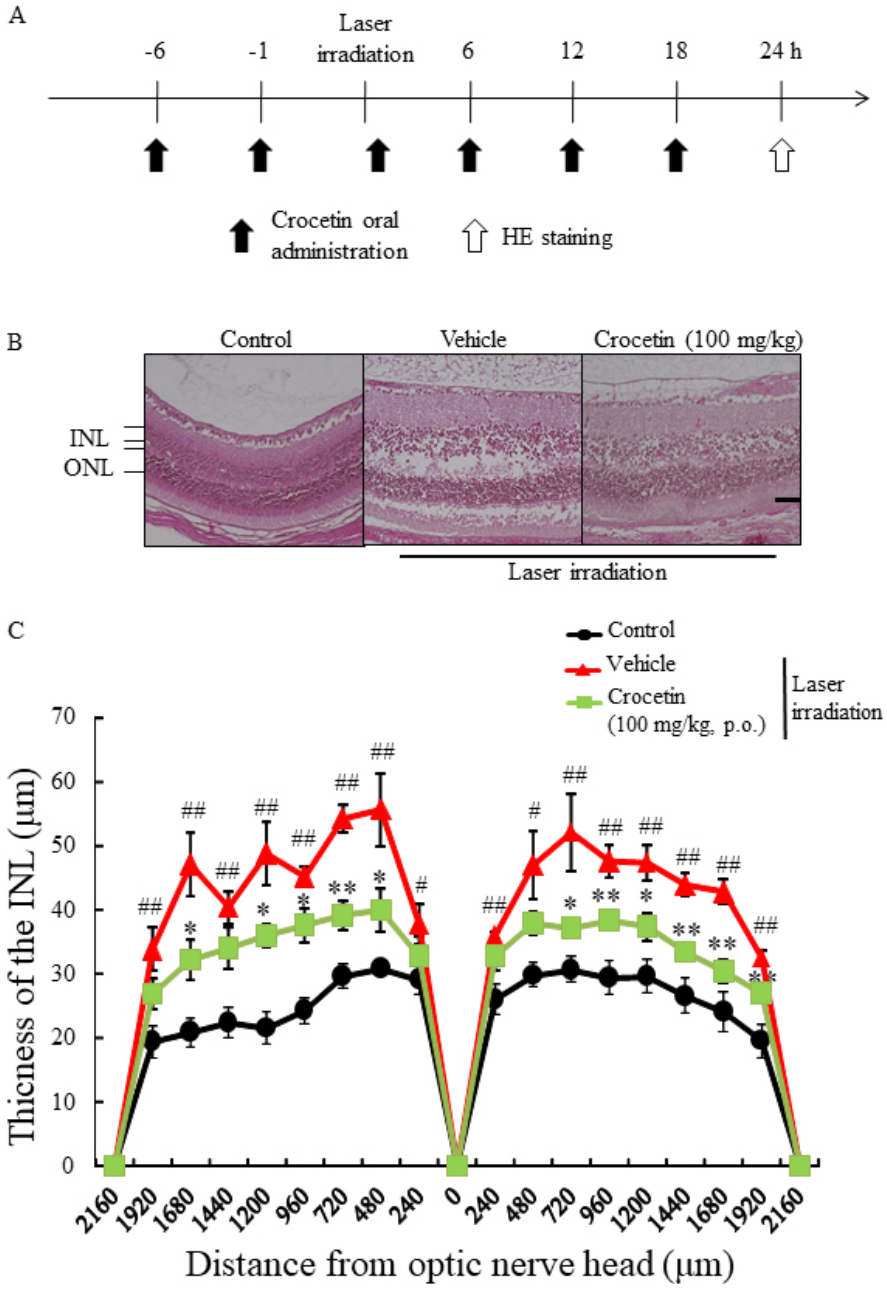

Figure 1. Effects of oral administration of crocetin on retinal thickness in the murine RVO model. **A**: Experimental protocol in vivo with oral administration of crocetin. Retinal vein occlusion (RVO) mice were orally administered crocetin 1 and 6 h before laser occlusion and immediately, 6 h, 12 h, and 18 h after laser irradiation. Retinal thickness was examined 24 h after laser irradiation. **B**: Representative images of hematoxylin and eosin (H&E) staining in the control, vehicle-treated, and crocetin-treated groups 24 h after laser irradiation. Scale bar indicates 50 μm. **C**: Quantification of the thickness of the inner nuclear layer (INL). The thickness of the INL was statistically significantly increased 1 day after laser irradiation, and this increase was improved with the crocetin treatment. Data are shown as mean ± standard error of the mean (SEM; n=5 or 6). $^{\#}p<0.05$, $^{\#\#}p<0.01$ (versus control group; Student *t* test). $^{*}p<0.05$, $^{**}p<0.01$ (versus vehicle group; Student *t* test). INL, inner nuclear layer; ONL, outer nuclear layer.

cystoid area were decreased in the crocetin-treated group, but a change in the nuclei of the cells existed (Figure 1B). In a previous report, the nuclei of the cells also changed in a murine RVO model group. The intravitreal injection of anti-VEGF antibody ameliorated the increase in the INL thickness, and improved the change in the nuclei of the cells [24]. These data suggested that the change in the nuclei of the cells is not led by laser damage and laser-induced inflammation. Crocetin did not ameliorate the increase in edema formation compared with the anti-VEGF antibody. The rate of suppression of edema formation was associated with the decrease in the abnormality of the nuclei. Namely, the change in the nuclei of the cells might be secondarily induced by the RVO effect due to edema formation caused by vascular occlusion and ischemic condition, and there is no artifact. Therefore, it is suggested that the oral administration of crocetin may

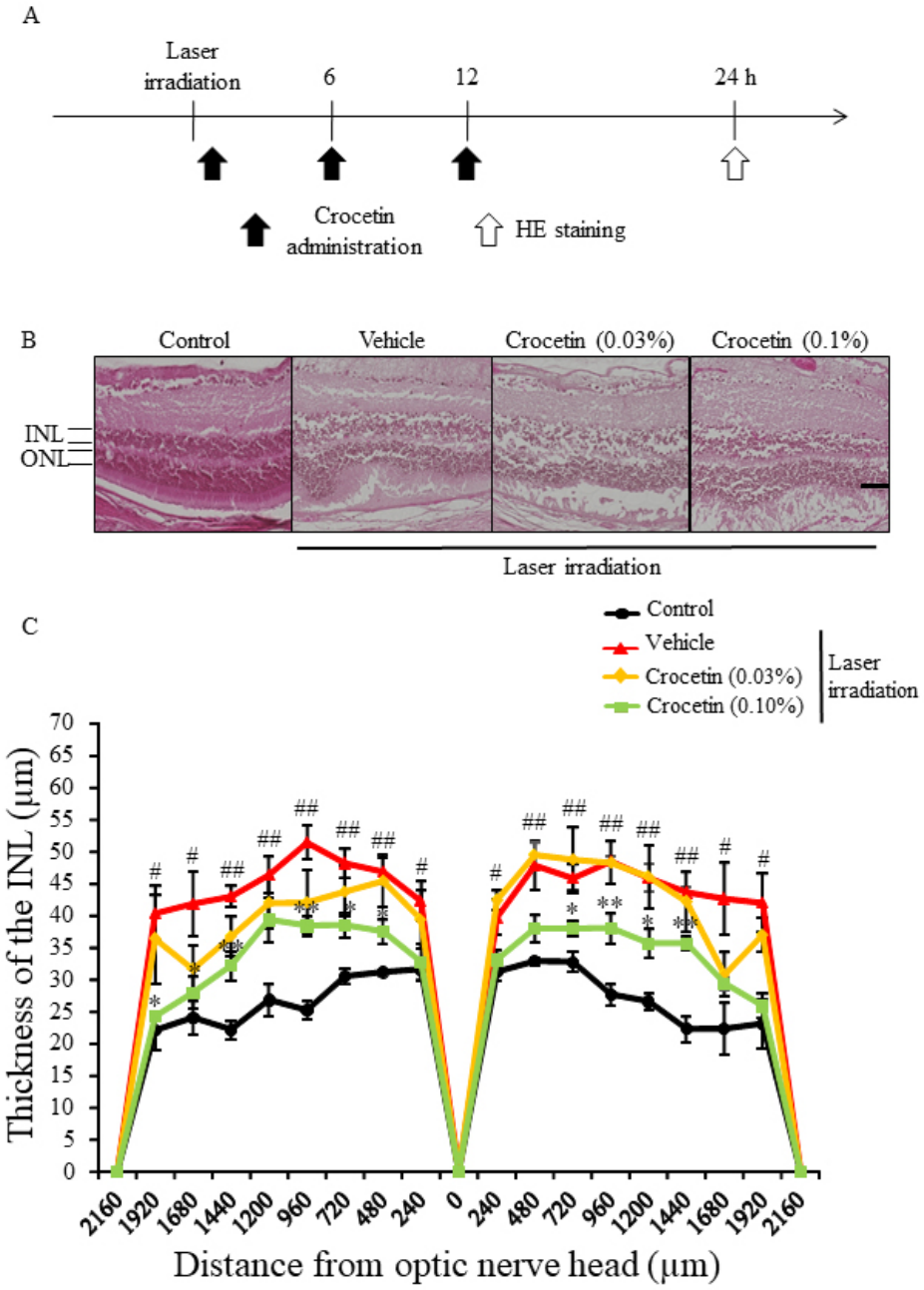

Figure 2. Effects of crocetin by eye drops on retinal thickness in the murine RVO model. **A**: Experimental protocol in vivo with eye drops of crocetin. Crocetin was administered immediately, 6 h, and 12 h after laser irradiation by eye drops. The retina was evaluated 24 h after laser irradiation. **B**: Representative images of hematoxylin and eosin (H&E) staining in control, vehicle-treated, and crocetin-treated groups are shown 1 day after laser irradiation. Scale bar represents 50 μm. **C**: Graphs show the thickness of the inner nuclear layer (INL). The increase in the thickness of the INL was suppressed with the administration of crocetin. Data are shown as mean ± standard error of the mean (SEM; n=3 or 4). $^{\#}p<0.05$, $^{\#\#}p<0.01$ (versus control group; Student *t* test). $^{*}p<0.05$, $^{**}p<0.01$ (versus vehicle group; Student *t* test). INL, inner nuclear layer; ONL, outer nuclear layer.

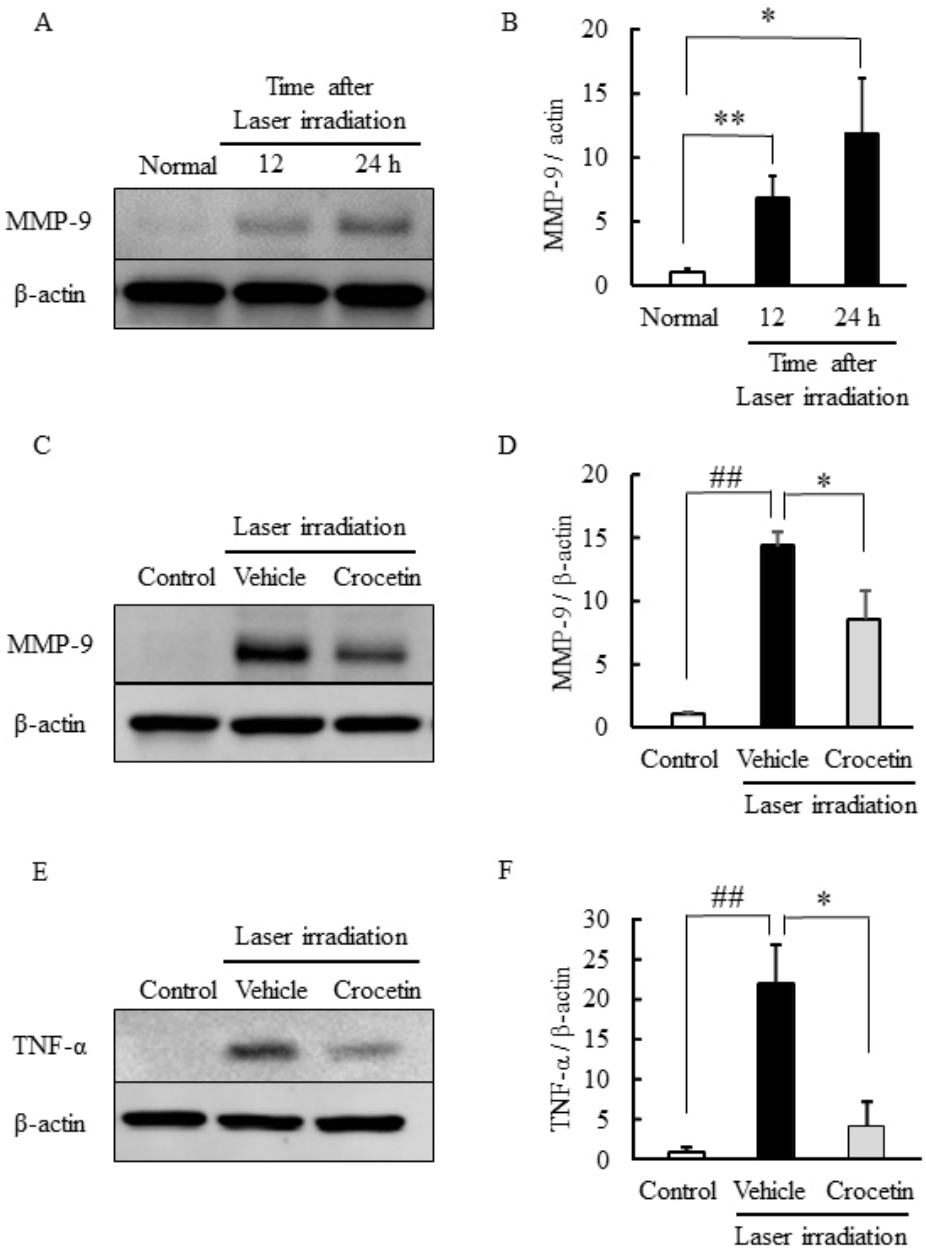

Figure 3. Effects of crocetin on inflammation factors in the murine RVO model. **A**: Representative images of western blotting showing the expression level of matrix metalloproteinase (MMP-9) 12 and 24 h after laser irradiation. **B**: Quantitative analysis of MMP-9 normalized to β-actin. The expression level of MMP-9 was statistically significantly increased 12 to 24 h after laser occlusion. Data are shown as mean ± standard error of the mean (SEM; n=5). *$p<0.05$, **$p<0.01$ (versus normal group; Student *t* test; **C**, **E**: Representative images of western blotting showing the expression level of MMP-9 and tumor nuclear factor (TNF-α) 24 h after laser irradiation. **D**, **F**: Oral administration of crocetin suppressed the expression levels of MMP-9 and TNF-α compared with that of the vehicle-treated group. Data are shown as mean ± standard error of the mean (SEM' n=4 to 8). $^{\#}p<0.05$, $^{\#\#}p<0.01$ (versus control group; Student *t* test). *$p<0.05$, **$p<0.01$ (versus vehicle group; Student *t* test).

regulate the formation and recurrence of edema through the regulation of inflammatory factors in a murine RVO model.

Eye drops are non-invasive, have few side effects, and are a simple administration method for the elderly. We found that crocetin eye drops improved the degree of retinal edema in a murine RVO model (Figure 2). Crocetin is amphiphilic, has a low molecular weight, and has high tissue migration compared with other carotenoids [38]. An eye drop drug delivery route can be used for systemic, corneal, and non-corneal pathways [10]. A portion of an ophthalmic drug enters the systemic pathway and partly reaches the retina [10]. The corneal pathway is unlikely to be the main route, because the fluorescence of liposomes after eye drop administration was observed only at the corneal surface, and not in the vitreous body [10]. The cornea is composed of three layers, and this barrier function prevents drugs from entering the interior of the eye [10]. The conjunctiva is a possible route, because it is not a strong barrier [10]. However, additional investigation of delivery routes to the retina with crocetin eye drops in the RVO model is needed.

The expression of MMP-9, which has a role in degrading basement membrane and extracellular matrix, is induced by TNF-α in the NF-kB pathway [39-41]. In the RVO model, the levels of expression of MMP-9 statistically significantly increased after laser irradiation (Figure 3). In addition, oral administration of crocetin reduced the expression levels of MMP-9 and TNF-α in the murine RVO model (Figure 3). The results indicated that TNF-α and MMP-9 were produced by leukocytes in a murine RVO model. It has been reported that TNF-α and MMP-9 are produced by leukocytes in inflammatory response and vascular hyperpermeability [42,43]. The expression levels of MCP-1 and ICAM-1 are increased in patients with RVO [36], and in the murine RVO model [24]. The expression levels of MCP-1 and ICAM-1 were increased by binding to the VEGF receptor in vascular endothelial cells, and induced the inflammatory response, with the migration and adhesion of leukocytes in the retinal vascular [44,45]. Therefore, the partial mechanism of retinal hyperpermeability in a murine RVO model is considered to be associated with the production of TNF-α and MMP-9.

In addition, expression of MMP-9 degrades the levels of tight junction proteins, which are associated with vascular permeability and the formation of edema in diabetic retinopathy [46]. The disruption of tight junctions in vascular endothelial cells increases vascular permeability, angiogenesis, and the formation of edema [30]. In particular, the transmembrane protein occludin regulates tight junctions in endothelial cells [27-29]. We found that expression of occludin was decreased in the RVO model, and this reduction was inhibited with oral administration of crocetin (Figure 4). The expression level of occludin stabilizes the vascular structure by regulating adherens junctions and tight junctions in vascular endothelial cells [27-29]. Crocetin prevents VEGF-induced cell migration and advanced glycation end products (AGEs)-induced cell apoptosis in HUVECs and bovine aortic endothelial cells (BECs) [47,48]. In the present murine RVO model, the expression levels of VEGF and MMP-9 increased 1 day after vein occlusion in the retina (Figure 3A) [49]. It has been reported that VEGF and MMP-9 decrease the expression levels of occludin in the RPE [46,50,51]. Occludin is also associated with the formation of tight junction in the RPE, and we think that VEGF and MMP-9 could induce a reduction in the expression level of occludin in the subretinal RPE. Therefore, the retinal hyper-permeability of the present RVO model may be associated with disruption of the outer blood–retinal barrier. In conclusion, crocetin protected tight junctions through anti-inflammatory effects, and inhibited the formation of edema in a murine RVO model, which suggests that oral and ocular administration of crocetin may improve RVO pathology.

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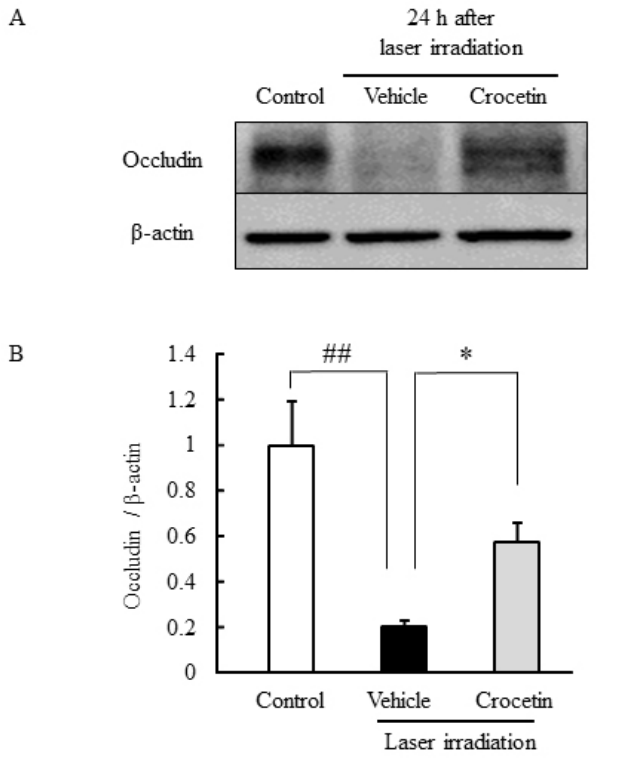

Figure 4. Effects of oral administration of crocetin on the expression of occludin in the murine RVO model. **A**: Representative images of western blotting showing the expression level of occludin 24 h after the administration of crocetin. **B**: The expression level of occludin was decreased in the vehicle-treated group. Oral administration of crocetin inhibited the reduction of expression of occludin. Data are shown as mean ± standard error of the mean (SEM; n=6 or 7). $^{\#}p<0.05$, $^{\#\#}p<0.01$ (versus control group; Student *t* test). $*p<0.05$, $**p<0.01$ (versus vehicle group; Student *t* test).

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