Resolvin D1 promotes corneal epithelial wound healing and restoration of mechanical sensation in diabetic mice

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Purpose: To investigate the effect and mechanism of proresolving lipid mediator resolvin D1 (RvD1) on the corneal epithelium and the restoration of mechanical sensation in diabetic mice.

Methods: Type 1 diabetes was induced in mice with intraperitoneal streptozocin injections. The healthy and diabetic mice underwent removal of the central corneal epithelium, and then 100 ng/ml RvD1 or its formyl peptide receptor 2 (FPR2) antagonist WRW4 was used to treat the diabetic mice. Regeneration of the corneal epithelium and nerves was observed with sodium fluorescein staining and whole-mount anti-β3-tubulin fluorescence staining. The inflammatory response level was measured with hematoxylin and eosin staining (inflammatory cell infiltration), enzyme-linked immunosorbent assay (tumor necrosis factor alpha and interleukin-1 beta content), myeloperoxidase activity, and fluorescence staining (macrophage content). The reactive oxygen species (ROS) and glutathione (GSH) levels were examined with incubation with fluorescent probes, and oxidative stress–related protein expression levels were evaluated with fluorescence staining and western blotting.

Results: Topical application of RvD1 promoted regeneration of the corneal epithelium in diabetic mice, accompanied by the reactivation of signaling and inflammation resolution related to regeneration of the epithelium. Furthermore, RvD1 directly attenuated the accumulation of ROS and nicotinamide adenine dinucleotide phosphate oxidase 2/4 expression, while RvD1 enhanced GSH synthesis and reactivated the Nrf2-ARE signaling pathway that was impaired in the corneal epithelium in the diabetic mice. More interestingly, topical application of RvD1 promoted regeneration of corneal nerves and completely restored impaired mechanical sensitivity of the cornea in diabetic mice. In addition, the promotion of corneal epithelial wound healing by RvD1 in diabetic mice was abolished by its FPR2 antagonist WRW4.

Conclusions: Topical application of RvD1 promotes corneal epithelial wound healing and the restoration of mechanical sensation in diabetic mice, which may be related to the lipid mediator's regulation of inflammation resolution, the reactivation of regenerative signaling in the epithelium, and the attenuation of oxidative stress.

Diabetic mellitus represents a major chronic metabolic disease from which more than 400 million people suffer worldwide [1]. Among various diabetic disorders, ocular complications, predominantly including diabetic retinopathy and cataract, have become a leading cause of blindness [2]. In the cornea, diabetic complications include decreased sub-basal nerve fiber density, impaired corneal sensation, punctate keratitis, and even persistent epithelial defects [3-5]. Impaired regeneration of the corneal epithelium and nerves may further lead to corneal ulcers, microbial keratitis, and finally, perforation. Previous studies have confirmed that reduced innervation, increased oxidative stress, abnormal regeneration signaling pathways (such as phosphorylated epidermal growth factor recepter (EGFR) and silent information regulator 1 (Sirt1), and Akt) and impaired inflammation resolution (such as more neutrophil infiltration and

less alternative-activated M2 macrophage transition) play important roles in the delay of regeneration of the corneal epithelium and nerves [6-10].

Hyperglycemia-induced oxidative stress, resulting from the imbalance between reactive oxygen species (ROS) production and the antioxidant defense system, is one of the common pathological mechanisms in diabetic mellitus and its various complications [11-13], including diabetic keratopathy, neuropathy, and retinopathy [14-18]. Aggravated oxidative stress is reported to be involved in the persistence and progression of chronic diabetic complications via metabolic memory even when glycemic control is achieved [19]. The production of ROS mainly depends on mitochondrial oxidative phosphorylation and the nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase) system [20,21]. For the antioxidant response, the Nrf2-ARE signaling pathway plays an important role through regulating the expression of antioxidant genes, such as glutathione (GSH), manganese superoxide dismutase (MnSOD), NADPH-quinone oxidoreductase 1 (NQO-1), and heme oxygenase-1 (HO-1) [22,23].

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During healthy wound healing, acute inflammation must be resolved to prevent tissue damage and homeostasis, which is partially accomplished by the polyunsaturated fatty acid metabolites termed specialized proresolving mediators (SPMs) [24,25]. SPMs exhibit anti-inflammation, tissue protection, and healing activities. The decreased content of SPMs contributes to a wide range of diseases involving pathological inflammation [26,27]. As a bioactive lipid mediator derived from docosahexaenoic acid (DHA), resolvin D1 (RvD1) has been reported to promote inflammation resolution through regulating neutrophil infiltration, alternative-activated macrophage transition, and proinflammatory cytokine production [28-31]. A considerable body of previous evidence has indicated that the RvD1-dependent anti-inflammatory response is mediated by a receptor, the formyl peptide receptor 2 (FPR2), in multiple diseases, such as Sjögren's syndrome, pulmonary fibrosis, inflammatory arthritis, as well as RvD1-dependent rescue of macrophages from ROS-induced apoptosis during efferocytosis [32-35].

For diabetic mellitus, previous reports have explained that hyperglycemia directly affects inflammation resolution, while RvD1 treatment promotes inflammation resolution and accelerates wound closure in type 2 diabetic mice [36]. RvD1 treatment reduces the expression of proinflammatory cytokines in cultured corneal epithelial cells [37]. Moreover, a previous study also indicated that daily injections of RvD1 improve motor and sensory nerve conduction velocities and improves thermal sensitivity in the corneal epithelium of diabetic mice [38]. Therefore, topical application of RvD1 may represent a potential approach for the treatment of corneal complications in diabetic mellitus. In the present study, we investigated the therapeutic potential of topical application of RvD1 on corneal epithelial wound healing and restoration of mechanical sensation in type 1 diabetic mice.

METHODS

Animals: Male C57BL/6 mice (6–8 weeks old) were purchased from the Institute of Laboratory Animal Sciences, Chinese Academy of Medical Sciences (Beijing, China). All animal experiments were conducted with the approval of the Ethics Committee of Shandong Eye Institute according to the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. Type 1 diabetes was induced in the mice with streptozocin (STZ; 50 mg/kg; Sigma-Aldrich, St. Louis, MO) injections and monitored based on our previous studies [39-41]. In the present study, only diabetic mice with a blood glucose level above 16.7 mmol/l 16 weeks after the final STZ injection were

used. For all experiments, only one eye of each mouse was wounded.

Corneal epithelial wound healing: The mouse central corneal epithelium (2 mm diameter) was removed by using the Alger-brush II remover (Alger Co, Lago Vista, TX) under systemic and local anesthetization. Systemic anesthetization with 40 µl Ketamine / 10 µl Chlorpromazine hydrochloride (in 0.5 ml 0.9% sodium chloride solution) intraperitoneal injection and local anesthetization with Proparacaine Hydrochloride eye drops (Alcon, Puurs, Belgium). The residual epithelial defect was visualized with fluorescein staining, and then we calculated the percentage of the original defect area with ImageJ software (NIH, Bethesda, MD) as in our previous studies [41-43]. For resolvin D1 treatment, diabetic mice were topically treated with 100 ng/ml RvD1 (5 µl/eye; Cayman, Ann Arbor, MI), which contained 0.1% ethanol, four times every day after the epithelium was removed, while age-matched control mice were topically treated with 0.1% ethanol as a vehicle control. For the block of the RvD1 FPR2 receptor, the diabetic mice were injected subconjunctively with 1 mg/ml FPR2 antagonist (WRW4, 5 µl/eye, one time per day; Tocris, Bristol, UK) at 24 h before and 0 and 24 h after the epithelium was removed. The treatment concentrations of RvD1 and WRW4 had been previously evaluated as having no statistically significant toxicity based on our preliminary experiments (data are not shown).

Measurement of corneal mechanical sensitivity: Corneal esthesiometry was performed according to our previous descriptions [40,43], with the Cochet-Bonnet esthesiometer (Luneau Ophtalmologie, Chartres Cedex, France) in unanesthetized control, diabetic, and RvD1-treated diabetic mice. Eye blink response was monitored after the touch of central cornea by nylon filament. The test began with the maximal length (6 cm) of nylon filament and shortened by every time by 0.5 cm until we found the corneal touch threshold. The longest filament length with a positive blink response was identified as the corneal sensitivity threshold.

Corneal reactive oxygen species and glutathione staining: For ROS staining, fresh corneal cryostat sections were incubated with 10 µM fluorescence probe 2,7-dichlorodihydrofluorescein diacetate, acetyl ester (DCHF-DA; Molecular Probes, Eugene, OR) for 30 min at room temperature. For intracellular GSH staining, the sections were incubated with monochlorobimane (MCB; Sigma-Aldrich) for 20 min at room temperature and visualized by using an Eclipse TE2000-U microscope (Nikon, Tokyo, Japan).

Corneal whole-mount and immunofluorescence staining: For nerve fiber staining, the whole-mount corneas were fixed in Zamboni stationary liquid (Solarbio, Beijing, China) and

TABLE 1. ANTIBODIES USED FOR IMMUNOFLUORESCENT STAINING AND WESTERN BLOTS.		
Antibodies	**Supplier**	**Code**
p-EGFR	Abcam	ab40815
EGFR	Abcam	ab52894
Sirt1	Abcam	ab12193
Ki67	Abcam	ab16667
NADPH oxidase 2	Abcam	ab129068
NADPH oxidase 4	Abcam	ab133303
Nrf2	Abcam	ab62352
MnSOD	Abcam	ab68155
NQO-1	Abcam	ab34173
HO-1	Abcam	ab13248
FITC anti-mouse F4/80	Biolegend	123,108
PE anti-mouse CD206 (MMR)	Biolegend	141,705
Anti-Beta lll-Tubulin	Millipore	AB15708A4
GAPDH	Kangchen	kc-5G5
β-actin	Abcam	ab8226
Alexa Fluor 488 donkey anti-rabbit IgG	Life technologies	A21206

incubated with the Alexa Fluor® 488 Conjugate anti-β-III tubulin antibody (Millipore, Billerica, MA). For immunofluorescence staining, the corneal sections were fixed in 4% paraformaldehyde and incubated with primary antibodies (Table 1) overnight at 4 °C. After three washings, the samples were further incubated with fluorescein-conjugated secondary antibodies (Table 1) and 4,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich) for nuclei staining. All staining was examined under an Eclipse TE2000-U microscope. ImageJ software was used to calculate the density of the corneal subbasal nerve fibers by gauging the percentage of the positive area.

Western blotting: The total proteins were extracted from the corneal epithelium by lysis in radio immunoprecipitation assay (RIPA) buffer containing a proteinase inhibitor cocktail. All samples (10–20 μg total proteins) were separated and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore). The membranes were incubated with primary antibodies (Table 1) overnight at 4 °C and then with horseradish peroxidase (HRP)-conjugated secondary antibody (Amersham Biosciences, Piscataway, NJ). The target proteins were visualized via enzyme-linked chemiluminescence (ECL) using an ECL kit (Pierce, Rockford, IL). The densitometry of the immunoreactive bands was quantified with ImageJ software. According to our comparison of the expression levels of the two housekeeping genes GAPDH and β-actin in the corneal epithelium, there was no statistically significant difference between the healthy and diabetic mice.

Therefore, all analysis of the western blotting results is shown with the comparative expression of GAPDH (Appendix 1).

Enzyme-linked immunosorbent assay: The total proteins were extracted from the corneas of healthy, diabetic, and RvD1-treated diabetic mice with PBS (1X; 145.3 mM NaCl, 2.7 mM KCl, 15.5 mM Na_2HPO_4, 1.7 mM NaH_2PO_4; pH 7.4) at 24 h after removal of the epithelium. The supernatants were subjected to quantitative sandwich immunoassay with enzyme-linked immunosorbent assay (ELISA) kits, including myeloperoxidase (MPO; USCN, Wuhan, China), interleukin-1 beta (IL-1β, eBioscience, San Diego, CA), and tumor necrosis factor alpha (TNF-α, R&D Systems, Minneapolis, MN) according to the manufacturers' procedures. Absorbance was read with a microplate reader (Molecular Devices, Sunnyvale, CA) at 450 nm with a reference wavelength of 570 nm.

Statistical analysis: All data in this study were obtained from at least three different experiments and are presented as the means ± standard deviation (SD). Comparisons between two groups were performed using the Student *t* test, and a p value of less than 0.05 indicated statistical significance.

RESULTS

Resolvin D1 promotes regeneration of the corneal epithelium in diabetic mice: To assess the effect of resolvin D1 on regeneration of the corneal epithelium, diabetic mice were topically administered 100 ng/ml RvD1 after the epithelium was removed. According to the fluorescein staining, regeneration

of the corneal epithelium was delayed in diabetic mice when compared with age-matched healthy mice, while the application of RvD1 promoted regeneration of the corneal epithelium in diabetic mice (Figure 1A). The analysis of residual epithelial defects revealed that RvD1 statistically significantly accelerated regeneration of the diabetic corneal epithelium at 24 and 48 h after removal of the epithelium (24 h: 25.03% ± 2.760% in healthy mice, 46.57% ± 7.830% in diabetic mice, 31.10% ± 4.160% in RvD1-treated diabetic mice; 48 h: 1.21% ± 1.94% in healthy mice, 9.76% ± 4.10% in diabetic mice, 1.77% ± 2.78% in RvD1-treated diabetic mice, Figure 1B; n=6).

Resolvin D1 reactivates epithelial regeneration-related signaling pathways: According to previous studies, impaired activation of EGFR and Sirt1 contributes to delayed regeneration of the corneal epithelium in diabetic mice, accompanied by decreased expression of proliferation marker Ki67 in the corneal epithelium [10,44,45]. To explore the mechanism of RvD1-mediated promotion of regeneration of the corneal epithelium in diabetic mice, the expression levels of phosphorylated EGFR, Sirt1, and Ki67 were examined

with immunofluorescence staining and western blotting at 48 h after removal of the epithelium. Representative results showed that there was stronger staining of *p*-EGFR and Sirt1, and an increased number of Ki67-positive cells were found in the RvD1-treated epithelium compared with the vehicle-treated diabetic corneal epithelium (Figure 2A). The quantitative results of the western blots further confirmed that RvD1 treatment efficiently rescued the expression levels of *p*-EGFR, Sirt1, and Ki67 in the diabetic corneal epithelium, even restoring them to the same levels as those of the age-matched healthy corneal epithelium (Figure 2B,C; n=4).

Resolvin D1 restores the resolution of corneal inflammation: To investigate the effect of RvD1 on inflammation resolution during regeneration of the corneal epithelium, we examined the changes in proinflammatory cell infiltration after the removal of the corneal epithelium. Hematoxylin and eosin (H&E) staining showed that hyperglycemia was associated with more cells infiltrating the corneal stroma in diabetic mice than in age-matched healthy mice, while RvD1 treatment reduced the number of infiltrating cells in the diabetic corneal stroma 24 h after the injury (Figure 3A). Consistently,
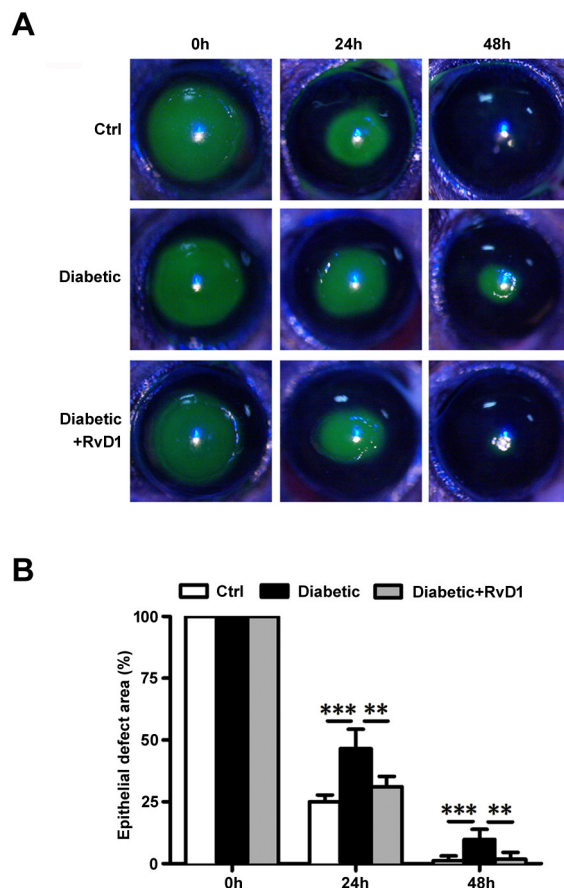

Figure 1. Resolvin D1 promotes regeneration of the corneal epithelium in diabetic mice. **A**: Diabetic mice were topically treated with 100 ng/ml RvD1 (5 µl/eye, four times per day) after the removal of the corneal epithelium. Meanwhile, healthy and diabetic control mice were topically treated with 0.1% ethanol. The residual epithelial defect was examined at 0, 24, and 48 h after the removal of the corneal epithelium with fluorescein staining as described previously. **B**: The histogram of the residual epithelial defect is presented as the percentage of the original wound area (n=6). Data are given as the mean ± standard deviation (SD); **p<0.01, ***p<0.001.
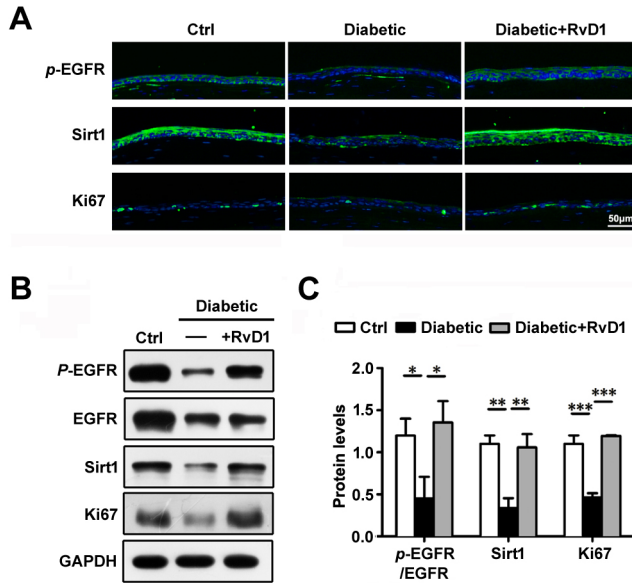
Figure 2. Resolvin D1 reactivates epithelial regeneration-related signaling pathways. (**A**) Immunofluorescence staining and (**B**) western blotting show the activation of the epithelial regeneration-related signaling pathways (including *p*-EGFR and Sirt1) and the expression of proliferation marker Ki67 in renewing the corneal epithelium 48 h after the removal of the corneal epithelium. **C**: The histogram shows the quantified results of the western blots (n=4). Data are given as the mean ± standard deviation (SD); *p<0.05, **p<0.01, ***p<0.001.

the ELISA results showed that MPO activity and TNF-α and IL-1β expression were reduced in the diabetic corneas after RvD1 treatment 24 h after injury (Figure 3B; n=6). Moreover, the process of macrophage transformation was later than the response of inflammatory cells and cytokines; the results revealed that the staining for anti-F4/80 (a macrophages marker) and anti-CD206 (an M2 macrophages marker)

showed an increased number of macrophages (especially M1 macrophages) in the diabetic cornea, while application of RvD1 efficiently reduced the number of M1 macrophages and enhanced the proportion of M2 macrophages in the diabetic cornea 48 h after injury (Figure 3C). These results suggest that RvD1 restores the resolution of corneal inflammation that is impaired in diabetic mice.
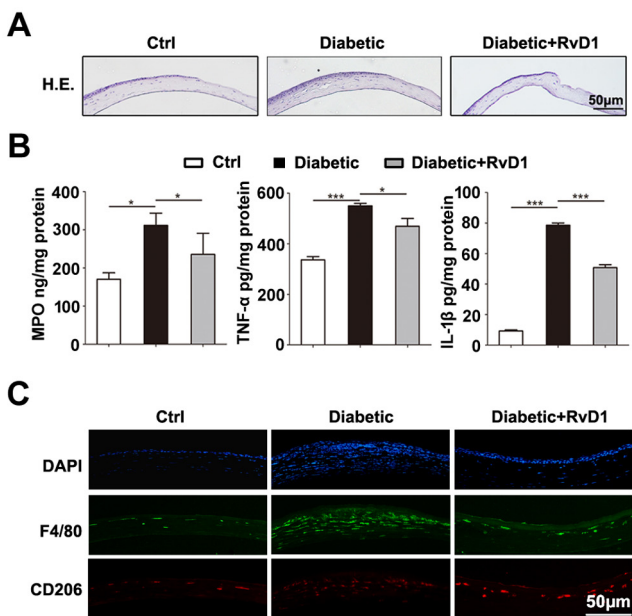

Figure 3. Resolvin D1 restores the resolution of corneal inflammation. **A**: Hematoxylin and eosin (H&E) staining showed the representative histologic appearance of the healing cornea 24 h after removal of the corneal epithelium in the control, diabetic, and resolvin D1 (RvD1)-treated diabetic mice. **B**: Corneas harvested 24 h after injury were homogenized and assayed for levels of myeloperoxidase (MPO) activity and tumor necrosis factor alpha (TNF-α) and interleukin-1 beta (IL-1β) expression with enzyme-linked immunosorbent assay (ELISA; n=6). **C**: Immunofluorescence staining was performed with the macrophage marker anti-F4/80 (green fluorescence) and the M2 macrophage marker anti-CD206 (red fluorescence) 48 h after removal of the corneal epithelium. Data are given as the mean ± standard deviation (SD); *p<0.05, ***p<0.001.
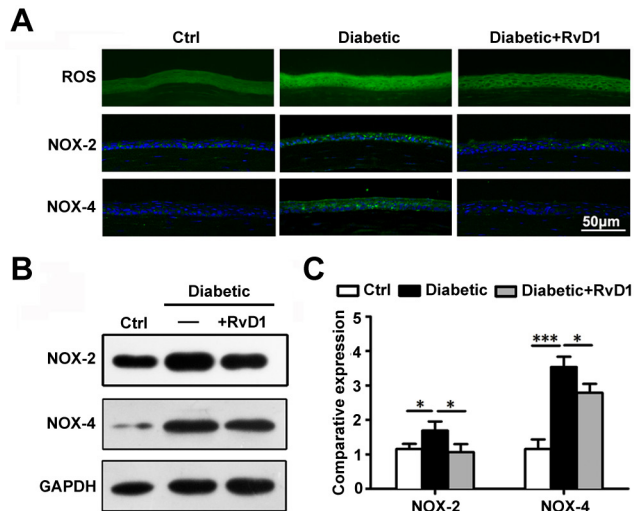
Figure 4. Resolvin D1 attenuates ROS accumulation and NADPH oxidase 2/4 expression. **A**: Generation of reactive oxygen species (ROS) and expression of NADPH oxidase 2/4 were examined with immunofluorescence staining in healthy, diabetic, and 4 day resolvin D1 (RvD1)-treated diabetic corneal epithelia. **B**: The corneal epithelia were harvested from healthy mice and diabetic mice with or without 4 day RvD1 treatment and then evaluated with western blotting to examine the protein levels of NADPH oxidase 2/4. **C**: The quantified data of the western blotting results are shown (n=4). Data are given as the mean ± standard deviation (SD); *p<0.05, ***p<0.001.

Resolvin D1 attenuates ROS accumulation and NADPH oxidase 2/4 expression: Representative immunofluorescence staining showed that hyperglycemia increased ROS accumulation and NADPH oxidase 2/4 expression in the corneal epithelium, while topical application of RvD1 for 4 days statistically significantly decreased ROS accumulation and NADPH oxidase 2/4 overexpression in the diabetic corneal epithelium (Figure 4A). The quantitative analysis of the western blots showed that NADPH oxidase 2 expression in the diabetic corneal epithelium was almost fully recovered to the healthy level accompanied by the decreased NADPH oxidase 4 expression level in the diabetic corneal epithelium after RvD1 treatment for 4 days (Figure 4B,C; n=4).

Resolvin D1 elevated glutathione levels and antioxidant gene expression: To explore the recovery effect of RvD1 on antioxidant responses in the diabetic corneal epithelium, we examined the expression of GSH, Nrf2, and Nrf2-ARE signaling downstream genes, including *MnSOD, NQO-1*, and *HO-1*. Representative immunofluorescence staining showed that the expression of GSH, Nrf2, MnSOD, NQO-1, and HO-1 decreased statistically significantly in diabetic mice compared with age-matched control mice, while 4 days of treatment with RvD1 restored the expression levels (Figure 5A); these results were further confirmed by the western blotting results for Nrf2, MnSOD, NQO-1, and HO-1 after 4 days of treatment with RvD1 (Figure 5B, C; n=4).

Resolvin D1 promotes regeneration of diabetic corneal nerves and restoration of mechanical sensation: To investigate the effect of topical application of RvD1 on restoration

of impaired corneal sensation in diabetic mice, corneal mechanical sensitivity without epithelial injury was first measured after 4 days of application of RvD1 or vehicle control (four times per day). These results showed that the application of topical RvD1 statistically significantly restored corneal sensation in diabetic mice (healthy mice: 5.63±0.35 cm, diabetic mice: 4.69±0.46 cm, 4 day RvD1-treated diabetic mice: 5.50±0.27 cm, Figure 6A; n=8). Subsequently, corneal nerve regeneration and sensation recovery in diabetic mice with epithelial injury were quantified at 3, 7, 10, and 14 days after application of RvD1 for the measurement of corneal mechanical sensitivity and at 14 days after application of RvD1 for the measurement of the regeneration of the corneal nerves. The results for the whole-mount corneal nerve staining and analysis showed that 14 days of treatment with RvD1 statistically significantly improved the regeneration of corneal nerves in diabetic mice, almost reaching the same level as that of the age-matched healthy mice (Figure 6B, C; n=4). Similarly, corneal mechanical sensitivity in diabetic mice was also completely restored to similar levels as in healthy mice after 7 days of RvD1 treatment after removal of the epithelium (Figure 6D; n=6).

FPR2 mediated the RvD1-induced promotion of regeneration of the corneal epithelium in diabetic mice: To further investigate whether FPR2 mediated the RvD1-induced promotion of regeneration of the corneal epithelium in diabetic mice, the FPR2 antagonist WRW4 was injected subconjunctivally at 24 h before and 0 and 24 h after the removal of the epithelium in diabetic mice. Fluorescence staining results showed that the FPR2 antagonist completely abolished the promotion
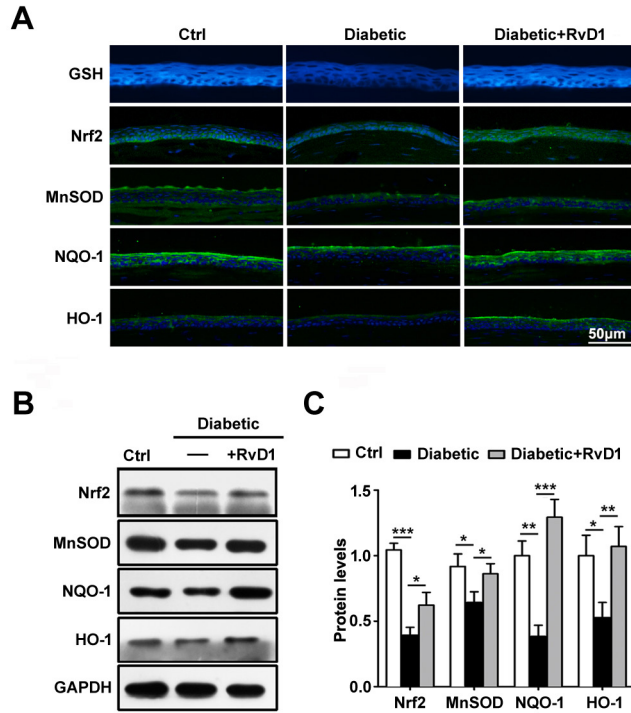
Figure 5. Resolvin D1 elevated the glutathione level and increased antioxidant gene expression. **A**: Immunofluorescence staining showed the expression of anti-ROS proteins, including Nrf2, GSH, MnSOD, NQO-1, and HO-1, in healthy, diabetic, and 4 day resolvin D1 (RvD1)-treated diabetic corneal epithelia. **B**: Western blotting showed the expression of anti-reactive oxygen species (ROS) proteins, including Nrf2, MnSOD, NQO-1, and HO-1, in healthy, diabetic, and 4 day RvD1-treated diabetic corneal epithelia. **C**: The quantified data of the western blotting results are shown (n=4). Data are given as the mean ± standard deviation (SD); *p<0.05, **p<0.01, ***p<0.001.
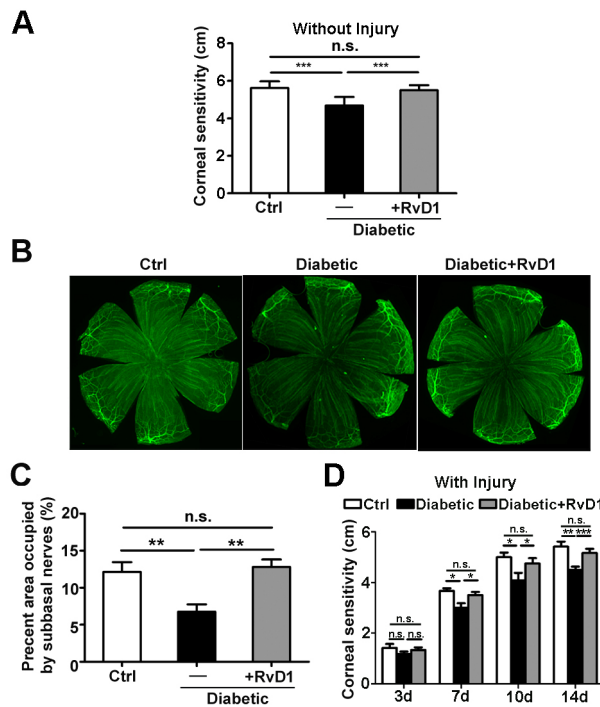

Figure 6. Resolvin D1 promotes diabetic regeneration of corneal nerves and restoration of mechanical sensation. **A**: A Cochet-Bonnet esthesiometer was used to test the mechanical sensitivity of the cornea in healthy, diabetic and 4 day resolvin D1 (RvD1)-treated diabetic mice without removal of the corneal epithelium (n=8). **B**: Two weeks after the removal of the corneal epithelium, the renewing corneas were harvested, and the regenerated corneal nerve fibers were examined with corneal whole-mount staining. **C**: The immunofluorescence intensity of nerve fibers 14 days after injury was calculated with ImageJ (n=4). **D**: The restored corneal mechanical sensitivity was examined 3, 7, 10 and 14 days after the removal of the corneal epithelium in healthy mice, diabetic mice, and diabetic mice treated with 4 day RvD1 (n=6). Data are given as the mean ± standard deviation (SD); *p<0.05, **p<0.01, ***p<0.001, n.s: not significant.
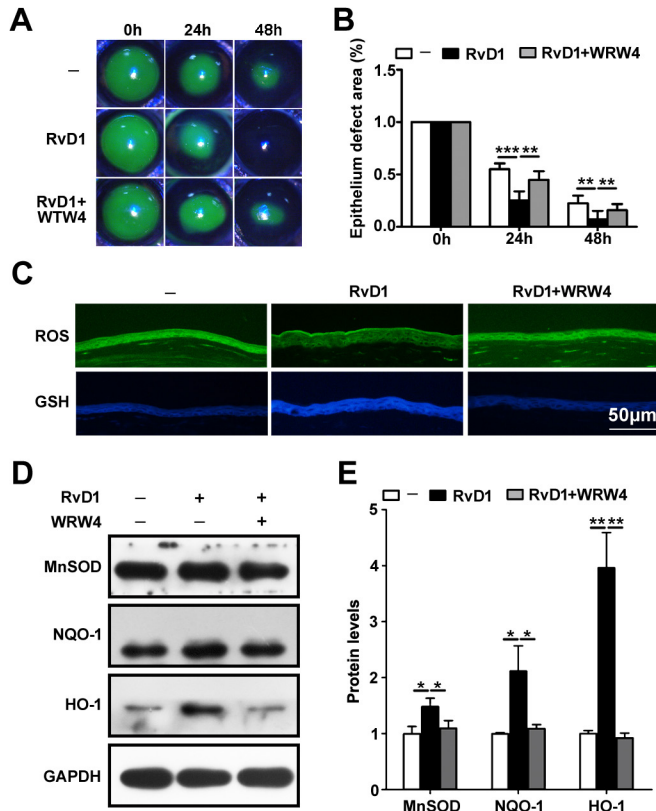
Figure 7. FPR2 mediated the RvD1-induced promotion of regeneration of the corneal epithelium in diabetic mice. These experiments were performed in diabetic, resolvin D1 (RvD1) treated, and RvD1 plus WRW4-treated diabetic mice. **A**: The residual epithelial defect was examined at 0, 24, and 48 h after the removal of the corneal epithelium with fluorescein staining as described previously. **B**: The histogram of the residual epithelium defect is presented as the percentage of the original wound area (n=6). **C**: The accumulation of reactive oxygen species (ROS) and the synthesis of glutathione (GSH) were tested with immunofluorescence staining at 48 h after removal of the epithelium. **D**: The western blotting results show the expression level of antioxidants, including MnSOD, NQO-1, and HO-1, 48 h after the removal of the epithelium. **E**: The quantified data of western blotting results are shown (n=4). Data are given as the mean ± standard deviation (SD); *p<0.05, **p<0.01, ***p<0.001.

of regeneration of the corneal epithelium in diabetic mice with the application of RvD1 (Figure 7A,B; n=6). Moreover, blocking FPR2 statistically significantly abolished the function of RvD1 in accelerating the elimination of ROS and GSH synthesis during regeneration of the corneal epithelium 48 h after the removal of the epithelium (Figure 7C). Similarly, the western blots also showed that the promotion of antioxidant expression (including MnSOD, NQO-1, and HO-1) with topical application of RvD1 was almost fully reversed by the delivery of WRW4 at 48 h after the removal of the epithelium (Figure 7D,E; n=4).

DISCUSSION

In the present study, we found that topical application of RvD1 statistically significantly promoted corneal epithelial wound healing and restored corneal mechanical sensation in type 1 diabetic mice. Similar to previous descriptions, RvD1 not only accelerated the resolution of abnormal inflammation but also reactivated the EGFR and Sirt1 signaling pathways related to regeneration of the corneal epithelium in diabetic mice. More interestingly, this study is the first to suggest a

novel regulatory function for RvD1 in oxidative stress during diabetic corneal epithelial wound healing. Mechanistically, RvD1 treatment alleviated ROS accumulation and NADPH oxidase 2/4 expression, while the treatment reactivated Nrf2-ARE signaling to increase glutathione synthesis and the expression of multiple antioxidant genes. In addition, we showed that the promotion of RvD1 on regeneration of the corneal epithelium was mediated by the FPR2 receptor.

As a proresolving mediator, RvD1 has been extensively investigated in many diseases, such as arthritis, pulmonary disease, and kidney injury [46-48]. The major mechanism of RvD1 is to accelerate inflammation resolution through the regulation of proinflammatory cell infiltration and the macrophage phenotype transition. Similarly, the beneficial effects of RvD1 have also been reported in several diabetic complications. In obese type 2 diabetic mice, RvD1 enhanced the resolution of peritonitis and accelerated cutaneous wound closure, accompanied by the decreased accumulation of apoptotic cells and macrophages [36]. In type 1 diabetic mice, RvD1 played a protective role against diabetic retinopathy by attenuating NLRP3 inflammasome activation and cytokine

production [49]. Moreover, RvD1 was confirmed as a potential target for experimental autoimmune neuritis and diabetic neuropathy [50,51]. Consistently, the present study demonstrated that application of RvD1 promoted corneal epithelial and nerve regeneration in type 1 diabetic mice. In addition, application of RvD1 decreased neutrophil infiltration and reduced the expression of IL-1β and TNF-α, accompanied by reducing the number of macrophages and enhancing the proportion of M2 macrophages in the diabetic cornea. Topical application of RvD1 at the 100 ng/ml concentration was sufficient to restore the impaired corneal mechanical sensation of diabetic mice to the same level as that of healthy mice, no matter with or without an epithelial wound. These results indicate the advantages of topical application of RvD1 in the recovery of regeneration of corneal nerves and mechanical sensation in diabetic mice, suggesting that topical application of RvD1 could achieve similar treatment outcomes as those for daily injections of RvD1 shown in a previous study [38].

Oxidative stress, as shown by the increased level of reactive oxygen species, has been identified as having a major effect on the development and progression of multiple diabetic complications [52]. ROS accumulation is due to increased ROS production and inadequate antioxidant function. In corneal epithelial cells or organ culture, high glucose and advanced glycation end products increase intracellular ROS generation through elevated activity of mitochondrial and NADPH oxidases, which has been further confirmed during the delayed regeneration of the corneal epithelium in diabetic animals [7,9]. Moreover, potential therapeutic treatments to promote regeneration of the corneal epithelium in diabetic animals, such as substance P, NGF, the antioxidant N-acetylcysteine (NAC), and anti-RAGE antibodies, are actually accompanied by the attenuation of ROS accumulation [9,39,53]. Coincidentally, the present study showed that application of RvD1 also attenuated ROS accumulation during the regeneration of the diabetic corneal epithelium by inhibiting NADPH oxidase expression, which may represent the common function of proresolving lipid mediators. In addition, we found that application of RvD1 reactivated the oxidative stress–responsive transcription factor Nrf2 and its downstream antioxidant gene expression, which was impaired by hyperglycemia in the diabetic corneal epithelium. Therefore, the attenuation of ROS generation and the elevation of antioxidant expression with the RvD1 treatment contributed to the regulation of hyperglycemia-induced oxidative stress in regeneration of the corneal epithelium in mice.

FPR2 and GPR32, two receptors of RvD1, have been identified through multiple screening systems [54]. However, as a classical chemoattractant receptor of G-protein-coupled receptors [55], FPR2 was reported to be involved in inflammation resolution and the metastasis of some cancers [33,35,56,57], which is consistent with the present findings showing that the FPR2 receptor antagonist completely abolished the promotion of regeneration of the corneal epithelium in diabetic mice by RvD1. In addition, FPR2 is also a receptor of proresolving lipid mediator LXA4, which has been confirmed to stimulate regeneration of the corneal epithelium [58-60]. Therefore, the use of FPR2 antagonists for abolishing RvD1 promotion of diabetic corneal epithelial wound healing cannot rule out a blockage of endogenous LXA4. Further studies should continue to discriminate between the different effects of LXA4 and RvD1. In conclusion, we provided evidence that topical application of RvD1 directly promotes corneal epithelial wound healing and restoration of mechanical sensation in diabetic mice, which may indicate that RvD1 can be used as a novel potential therapy for the treatment of diabetic corneal complications.

APPENDIX 1. EXPRESSION LEVELS OF HOUSEKEEPING GENES.

To access the data, click or select the words "Appendix 1"

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