Lithium chloride promotes diabetic corneal epithelial wound healing by activating the Wnt/β-catenin signaling pathway

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Abstract. Corneal epithelial abnormality is a common manifestation of diabetic keratopathy and leads to delayed epithelial wound healing. The Wnt/β-catenin signaling pathway participates in the development, differentiation and stratification of corneal epithelial cells. The present study compared the expression of Wnt/β-catenin signaling pathway related factors, including Wnt7a, β-catenin, cyclin D1 and phosphorylated (p-) glycogen synthase kinase 3 β (Gsk3b) between normal and diabetic mouse corneas, by reverse transcription-quantitative PCR, western blotting and immunofluorescence staining. It was found that the expression of the Wnt/β-catenin signaling pathway related factors was downregulated in diabetic corneas. Upon corneal epithelium scraping, the wound healing rate was significantly increased in diabetic mice after topical treatment with lithium chloride. After further investigation, significantly upregulated levels of Wnt7a, β-catenin, cyclin D1 and p-Gsk3b were found in the diabetic group 24 h after treatment, accompanied by β-catenin nuclear translocation observed by immunofluorescence staining. These results suggest that active Wnt/β-catenin pathway can promote diabetic corneal epithelial wound healing.

Introduction

Diabetic keratopathy, an ocular complication of diabetes mellitus, has attracted increasing attention in recent years and has been estimated to be present in 47-64% of patients with disease progression (1). Corneal epithelial abnormality is a common manifestation of diabetic keratopathy and is characterized by superficial punctate keratopathy, delayed epithelial wound healing and recurrent erosions (2). Epithelial fragility and poor wound healing will increase the susceptibility to pathogenic microorganisms, as well as the risk of post-surgical complications (3,4). A number of studies have been conducted to detect the pathogenesis of diabetic keratopathy, however, there are no clear and effective therapeutic targets for clinical use (5).

The canonical Wnt/β-catenin signaling pathway is an evolutionarily conserved set of signals with essential roles in embryonic and postnatal development, regeneration, stem-cell maintenance and fibrosis (6). β-catenin plays a critical role in the pathway. Without Wnt ligand, β-catenin is mainly expressed in the cytoplasm and constitutively phosphorylated and degraded through the interaction with the destruction complex formed by a scaffold protein (Axin), casein kinase Iα, glycogen synthase kinase 3 β (Gsk3b) and adenomatous polyposis coli. In the presence of Wnt, Wnt binds to the frizzled receptor and co-receptor low-density lipoprotein receptor-related protein 5 or 6 (LRP5/6); LPR5/6 is phosphorylated, which leads to the recruitment and phosphorylation of Dishevelled. Subsequently, Axin is recruited to the receptor complex and the destruction complex is disassembled, blocking the phosphorylation of soluble cytoplasmic β-catenin; β-catenin is then stabilized and translocates to the nucleus to activate the transcription of target genes (7,8).

Corneal epithelial wound healing is a complex physiological process involving cell death, proliferation, differentiation and migration and largely depends on limbal stem cells (LSCs) and remodeling of the basement membrane (9). The Wnt/β-catenin signaling pathway is actively expressed in ocular surface epithelial cells and promotes the nuclear localization of β-catenin and increases the proliferation and colony-forming efficiency of primary human LSCs (10). The Wnt/β-catenin pathway also participates in the development, differentiation and stratification of corneal epithelial cells (11-13). Delayed and decreased β-catenin expression in streptozocin (STZ)-induced type 1 diabetic rats during corneal epithelium healing has also been reported (14).

Lithium chloride (LiCl) is a well-established activator of the Wnt/β-catenin signaling pathway. Therefore, to provide a theoretical basis for the prevention and treatment of diabetic

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corneal epithelial lesions, in the present study, STZ-induced type 1 diabetic mice were used to investigate whether LiCl could play a role in the diabetic corneal epithelial wound healing process by activating the Wnt/β-catenin pathway.

Materials and methods

Animals. A total of 120 male C57BL/6 mice (age, 6-8 weeks; weight, 20-24 g) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. The mice were kept in an environment of 45±5% relative humidity, 22-23˚C, and 12 h light and dark cycles. The type 1 diabetic mice were induced with intraperitoneal STZ (50 mg/kg; MilliporeSigma) injection for five consecutive days (15). At 12 weeks after the final STZ injection, the mice with blood glucose level >16.7 mmol/l were chosen. The blood samples were derived from the caudal vein. All animal experiments were approved by the Ethics Committee of Shandong Eye Institute (approval no. 20200018) and conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Mice were sacrificed once they reached a humane endpoint or the study endpoint. The humane endpoints for this study were >20% body weight loss, inactivity and moribundity. A total of four mice were found dead and the others were sacrificed by cervical dislocation.

Corneal epithelial wound healing. The mouse central corneal epithelium (diameter 2.5 mm) was scraped with an Algerbrush II corneal rust ring remover (Alger Equipment Co., Inc.). The experiment included four groups: Control, control + LiCl, diabetic and diabetic + LiCl. At 24 h before epithelium scraping, the control + LiCl and diabetic + LiCl groups were injected subconjunctivally with LiCl (5 μl, 0.15M/eye; MilliporeSigma) and the control and diabetic groups were injected with equal phosphate buffer saline (PBS) (16). As soon as the corneal epithelium was scraped, the second subconjunctival injection was administered and ofloxacin eye ointment was used to prevent infection. After 12, 24 and 36 h, corneal epithelial defects were observed by staining with fluorescein sodium and photographed under a slit-lamp microscope (BQ900; Haag-Streit AG). The stained area was analyzed using ImageJ (version 1.52; National Institutes of Health) and compared as the healing rate.

Reverse transcription-quantitative (RT-q) PCR. The expression of the Wnt/β-catenin signaling pathway-related genes was measured using RT-qPCR. Total RNA was extracted from the whole cornea (unwounded and 24 h after scraping) using the TransZol Up Plus RNA kit (TransGen Biotech Co., Ltd.) according to the manufacturer's instructions. Complementary DNA was synthesized using the HiScript III RT SuperMix for qPCR (+gDNA wiper; Vazyme Biotech Co., Ltd.). R-T PCR was performed using ChamQ Universal SYBR qPCR Master Mix (Vazyme Biotech Co., Ltd.) and the Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.). The qPCR cycling conditions include two stages: i) A predenaturation stage at 95˚C for 30 sec; and ii) A cycling stage (40 cycles) at 95˚C for 10 sec and 60˚C for 30 sec. The relative mRNA expression levels were calculated according to the $2^{-\Delta\Delta Ct}$ method (17). Gene-specific primer sequences were synthesized by Thermo Fisher Scientific and are shown in Table I.

Western blotting analysis. Total protein from corneas was extracted by using RIPA lysis buffer. The BCA protein assay kit (Beyotime Institute of Biotechnology) was used for determination of protein content. The extracted proteins (10-20 μg per lane) were separated using 12.5% SDS-PAGE gel and transferred to polyvinylidene difluoride membranes (PVDF; MilliporeSigma). The membrane was blocked with 5% bovine serum albumin (BSA) (Beijing Solarbio Science & Technology Co., Ltd.) for 1 h at room temperature and incubated overnight at 4˚C with primary antibodies against β-catenin (1:500; cat. no. 32572; Abcam), Wnt7a (dilution 1:1,000; cat. no. 183653; Abcam), cyclin D1 (dilution 1:200; cat. no. 16663; Abcam), p-Gsk3b (dilution 1:1,000; 9392, Cell Signaling Technology, Danvers, USA), Gsk3b (dilution 1:1,000; 12456, Cell Signaling Technology, Danvers, USA) and β-actin (dilution 1:1,000; cat. no. 8226; Abcam). On the second day, the membrane was incubated with a goat anti-rabbit IgG H&L HRP-coupled secondary antibody (dilution 1:2,000; cat. no. 6721; Abcam) for 1 h at room temperature. Finally, the bands were visualized by ChemiDoc Touch Imaging System (Bio-Rad Laboratories, Inc.) via enzyme-linked chemiluminescence using the Omni-ECL Femto Light Chemiluminescence kit (Shanghai Epizyme Biomedical Technology Co., Ltd.) and quantified using ImageJ (version 1.52; National Institutes of Health).

Immunofluorescence staining. The eyeballs were embedded in optimal cutting temperature compound (Sakura Finetek USA, Inc.) which was prefrozen at 4˚C and then immediately stored in a -80˚C refrigerator before being sectioned at 7 μm. The slides were heated to 37˚C for 10 min, fixed with 4% paraformaldehyde and sealed with 5% BSA for 1 h at room temperature. The samples were incubated with primary antibodies overnight at 4˚C and subsequently were incubated for 1 h at room temperature with the corresponding secondary antibody [rhodamine (TRITC) AffiniPure Goat Anti-Rabbit IgG (H + L); ZSGB-BIO]. All samples were counterstained with 4',6-diamidino-2-Phenylindole (DAPI; MilliporeSigma) and images were captured using an Eclipse TE2000-U microscope (Nikon Corporation).

Statistical analysis. Statistical analysis was performed using IBM SPSS Statistics (version 22.0; IBM Corp.). One-sample Kologorow-Smirnov test was used to test the normality of data distribution and the data in the present study conformed to the normal distribution. Student's t-test was used for between-group comparisons. One-way analysis of variance was used for multiple-group comparisons and the Tukey method was used for post hoc test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Expression of Wnt/β-catenin signaling pathway is downregulated in diabetic mouse cornea. The present study explored the expression of the Wnt/β-catenin signaling pathway in the control and diabetic mouse corneas using RT-qPCR, western

Table I. Primer sequences for reverse transcription-quantitative PCR.

Gene	Primers	Sequences
β-actin	Sense	ACGGCCAGGTCATCACTATTG
	Antisense	AGAGGTCTTTACGGATGTCAACGT
Wnt7a	Sense	GGAGTGTAAGTGCCATGGTGTGT
	Antisense	AAGGTGGGCCGCTTGTTT
β-catenin	Sense	CTGCAGAGGCCATTGAAGCT
	Antisense	CGACTGAAAGCCGCTTCTTG
cyclin D1	Sense	TGCTGCAAATGGAACTGCTT
	Antisense	CCACAAAGGTCTGTGCATGCT

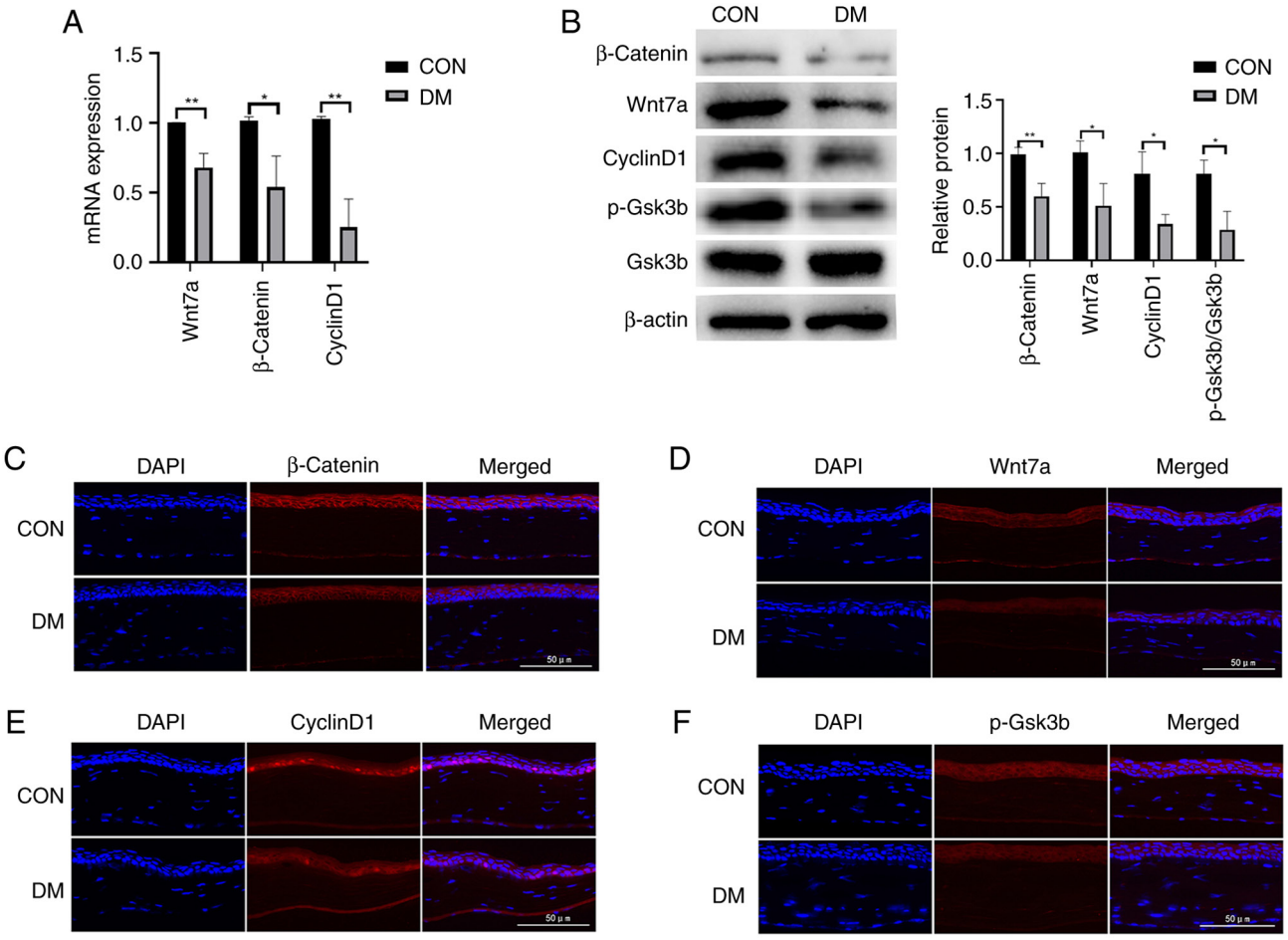

Figure 1. The expression of the Wnt/β-catenin signaling pathway in the control and diabetic mice. (A) The analysis of the mRNA expression of *β-catenin*, *Wnt7a* and *cyclin D1* in all groups (n=3 per group). (B) The western blotting bands and analysis of the protein expression of β-catenin, Wnt7a, cyclin D1, p-Gsk3b and Gsk3b in all groups (n=3 per group). Immunofluorescence staining showing the protein expression of (C) β-catenin, (D) Wnt7a, (E) cyclin D1 and (F) p-Gsk3b in all groups. *P<0.05. **P<0.01. Scale bar, 50 μm. p-, phosphorylated; Gsk3b, glycogen synthase kinase 3 β; Con, control; DM, diabetes mellitus.

blotting and immunofluorescence staining. RT-qPCR results revealed that the mRNA expression of *β-catenin* (P=0.021), *Wnt7a* (P=0.005) *and cyclin D1* (P=0.003) was significantly decreased in the diabetic cornea (Fig. 1A). Western blotting (Fig. 1B) results showed that the protein expression of β-catenin (P=0.007), Wnt7a (P=0.021), cyclin D1 (P=0.023) and p-Gsk3b (P=0.013) was significantly decreased in the diabetic cornea. Immunofluorescence staining (Fig. 1C-F) results further confirmed the downregulated expression of the target proteins in the diabetic cornea. These results suggested that the Wnt/β-catenin signaling pathway is downregulated in the diabetic cornea.

LiCl promotes corneal epithelial wound healing in both control and diabetic mice. The present study observed corneal epithelial wound healing by fluorescein staining at 12, 24 and 36 h after cornea epithelium scraping in the control, control + LiCl, diabetic and diabetic + LiCl groups. Corneal epithelial healing

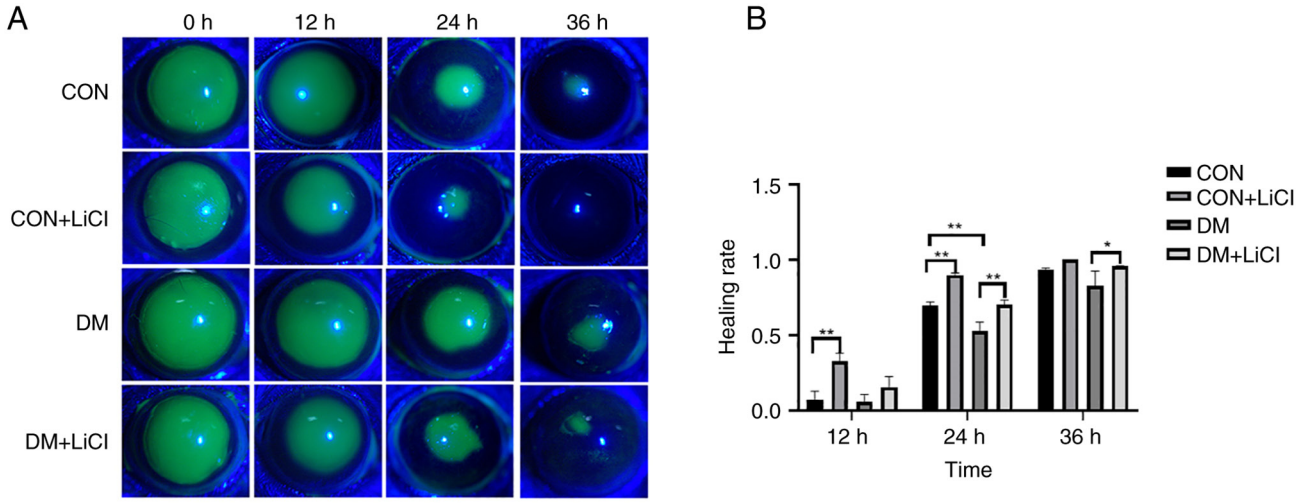

Figure 2. LiCl promotes corneal epithelial wound healing in both control and diabetic mice. (A) Fluorescein staining of the corneal epithelium at 0, 12, 24 and 36 h after scraping. (B) The analysis of the wound healing rates (n=8 per group). *P<0.05, **P<0.01. LiCl, lithium chloride; Con, control; DM, diabetes mellitus.

was delayed in the diabetic group. At 12 and 24 h after scraping, the healing rate in the control + LiCl group was significantly higher compared with the control group (P=0.003 and P=0.001, respectively). At 24 and 36 h after scraping, the healing rate in the diabetic + LiCl group was significantly higher compared with the diabetic group (P=0.001 and P=0.048, respectively). It was also found that the healing rate of diabetic + LiCl group was higher than the control group at 12 h and the healing rate of diabetic + LiCl group was nearly the same compared with the control group at 24 and 36 h (Fig. 2). This suggested that LiCl can promote the corneal epithelial wound healing in both the control and diabetic mice.

LiCl promotes corneal epithelial wound healing through activating Wnt/β-catenin signaling pathway. At 24 h after corneal epithelium scraping, the mRNA and protein expression levels of the Wnt/β-catenin pathway in the four groups was explored by using RT-qPCR and western blotting. The mRNA expression levels of *β-catenin*, *Wnt7a* and *cyclin D1* were significantly increased in the LiCl treatment groups compared with those in the matched control groups (Fig. 3A). Western blotting analysis showed that the protein expression levels of β-catenin, Wnt7a, cyclin D1 and p-Gsk3b were all significantly increased in the LiCl treatment groups compared with those in the matched control groups (Fig. 3B). These results suggested that LiCl may activate the Wnt/β-catenin signaling pathway to promote corneal epithelial wound healing.

LiCl activates the Wnt/β-catenin signaling pathway and promoted β-catenin nuclear translocation. At 24 h after corneal epithelium scraping, the Wnt/β-catenin signaling pathway expression levels in all groups was also explored using immunofluorescence staining. Prior to the Wnt/β-catenin signaling pathway being activated, β-catenin was mostly located in the cytoplasm and after the application of LiCl it was observed that β-catenin was located in the cytoplasm and nucleus of the corneal epithelium and the total expression level had increased (Fig. 4A). The expression levels of Wnt7a, cyclin D1 and p-Gsk3b also increased (Fig. 4B-D). These results suggested that LiCl activates the Wnt/β-catenin signaling pathway and promotes the nuclear translocation of β-catenin.

Discussion

Diabetic keratopathy is a refractive disease characterized by superficial punctate keratopathy, delayed epithelial wound healing and recurrent erosions. As the number of diabetic patients increases, the morbidity of diabetic keratopathy also increases; however, there are still no really effective drugs for its treatment. Therefore, the present study explored the role of the Wnt/β-catenin signaling pathway in corneal epithelial wound healing in diabetic mice. First, the expression of the Wnt/β-catenin signaling pathway in control and diabetic mouse cornea was compared and it was found that the expression of β-catenin, Wnt7a, cyclin D1 and the phosphorylation of Gsk3b decreased in the diabetic group. To further explore the function of the pathway, an epithelial wound model was constructed and it was found that topical application of the Wnt/β-catenin pathway activator LiCl could promote control and diabetic mouse epithelial wound healing, accompanied by the upregulation of Wnt/β-catenin pathway-related factors, suggesting that the active Wnt/β-catenin pathway promotes diabetic corneal epithelial wound healing.

Wnt signaling is actively expressed in the corneal epithelium and contributes to the corneal wound healing process (10,18). Lyu *et al* (18) reported that Wnt5b and 7a were rapidly induced in wounded rat cornea and Wnt7a promoted proliferation of human corneal epithelial cells. Liang *et al* (19) found that diabetes-induced overexpression of kallistatin contributes to delayed corneal wound healing by inhibiting the canonical Wnt signaling. The present study found that the expression of Wnt7a was decreased in diabetic mouse cornea, accompanied by the delayed epithelial wound healing and after topical treatment of a Wnt activator, the expression of Wnt7a increased and the healing process was accelerated.

The maintenance of appropriate cell adhesion is crucial for cell migration and wound healing. A previous study reported that the E-cadherin/β-catenin complex is involved

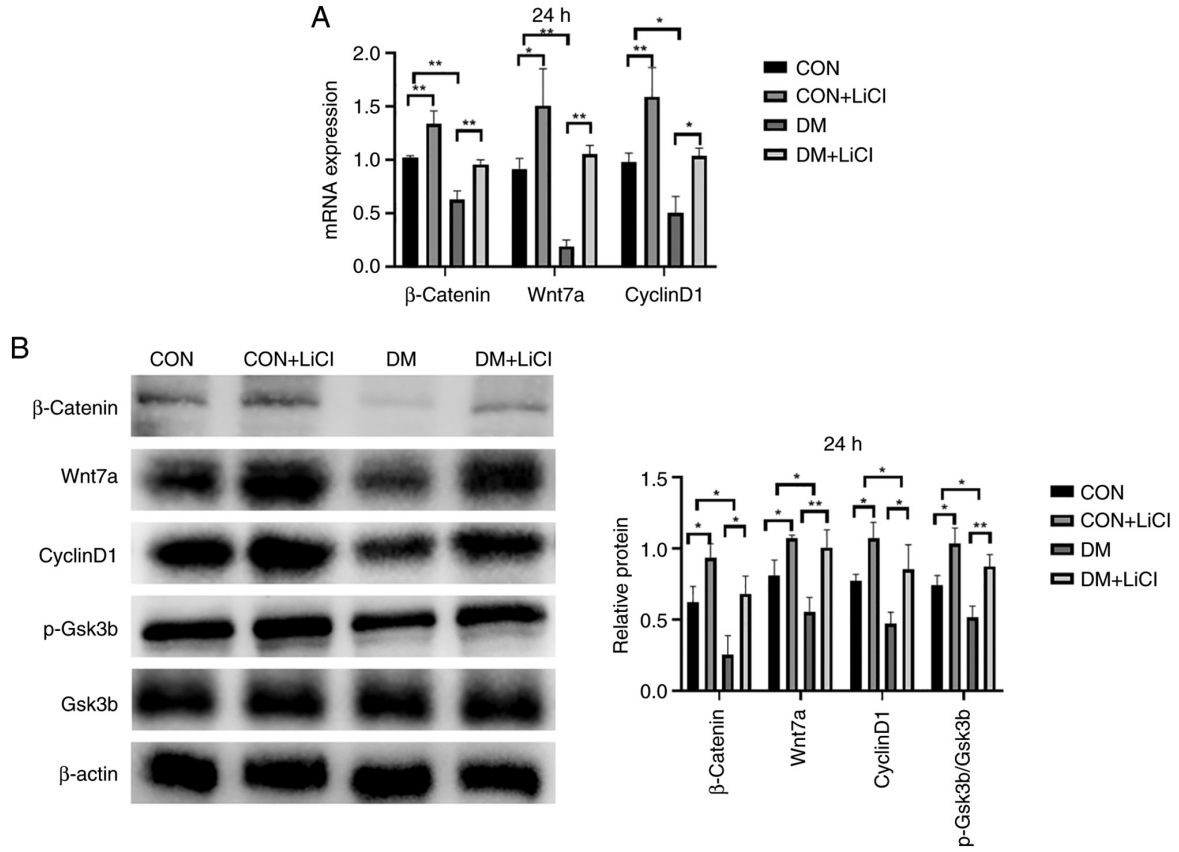

Figure 3. The expression of Wnt/β-catenin signaling pathway at 24 h after the local treatment with LiCl post epithelium scraping. (A) Analysis of the mRNA expression of *β-catenin*, *Wnt7a* and *cyclin D1* in all groups (n=3 per group). (B) Western blotting bands and analysis of the protein expression of β-catenin, Wnt7a, cyclin D1 and p-Gsk3b in all groups (n=3 per group). *P<0.05, **P<0.01. LiCl, lithium chloride; p-, phosphorylated; Gsk3b, glycogen synthase kinase 3 β; Con, control; DM, diabetes mellitus.

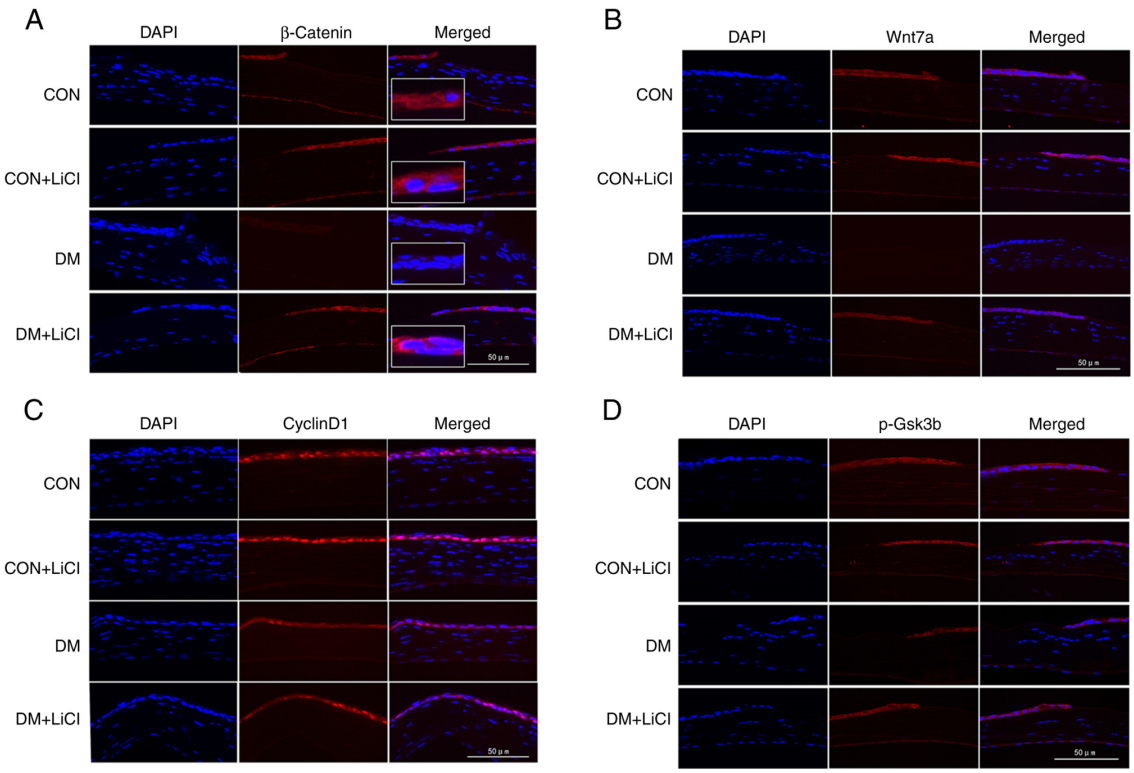

Figure 4. The immunofluorescence staining of Wnt/β-catenin signaling pathway at 24 h after the local treatment with LiCl post epithelium scraping. The expression of (A) β-catenin, (B) Wnt7a, (C) cyclin D1 and (D) p-Gsk3b. Scale bar, 50 μm. LiCl, lithium chloride; p-, phosphorylated; Gsk3b, glycogen synthase kinase 3 β; Con, control; DM, diabetes mellitus.

in the adhesion of corneal epithelial cells and high glucose induced decreased expression of E-cadherin and β-catenin in mouse corneal epithelial cells, as well as reducing the formation of β-catenin/E-cadherin complex (20). β-catenin plays an essential role in the Wnt/β-catenin pathway. Without Wnt ligand, β-catenin is mostly located in the cytoplasm and when the pathway is activated, cytoplasmic β-catenin accumulates and translocates to the nucleus, initiating the transcription of downstream target genes (7). The present study found that the expression of β-catenin decreased in the diabetic cornea and after treatment with the Wnt activator, the expression level increased. β-catenin nuclear translocation was detected by immunofluorescence staining.

β-catenin is phosphorylated and degraded with Gsk3b to form a degradation complex, which is finally degraded by ubiquitin modification. Therefore, Gsk3b inhibitors are widely used to investigate the Wnt/β-catenin signaling pathway. LiCl is a commonly used Wnt activator and has been shown to directly inhibit Gsk3b by triggering phosphorylation (21). In the present study, the ratio of p-Gsk3b/Gsk3b increased in the groups treated with LiCl, consistent with the increased expression of β-catenin.

Cyclin D1 is known as a regulator of cell cycle progression and modulates the transition from the G_1 to S phase; it is also a downstream target of the Wnt/β-catenin signaling pathway (22). In the present study, the expression of cyclin D1 decreased in the diabetic group but increased after activation of Wnt/β-catenin pathway.

The present study found that the expression of the Wnt/β-catenin pathway was downregulated in diabetic corneas, topical treatment with LiCl could promote diabetic corneal epithelial wound healing and the healing process was accompanied by the activation of the Wnt/β-catenin signaling pathway. The present study provides a new strategy for the clinical treatment of diabetic keratopathy.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

JD designed the experiments and was a major contribution in writing the manuscript. TT performed the animal experiments, RT-qPCR, western blot and participated in the statistical analysis. MW and JG performed the immunofluorescence staining. QY and LW also participated in the statistical analysis. PW guided the design of the experiments and the writing of the manuscript. All authors confirm the authenticity of all the raw data and read and approved the final manuscript.

Ethics approval and consent to participate

All animal experiments were approved by the Ethics Committee of Shandong Eye Institute (approval no. 20200018) and conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Patients consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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