

Corneal Structure After Small-Incision Lenticule Extraction in Diabetic Rabbits

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Abstract

Background: To assess the corneal structure after femtosecond laser small-incision lenticule extraction (SMILE) in diabetic rabbits with different blood glucose levels.

Methods: Twelve diabetic rabbits were randomly distributed into three groups based on blood glucose levels: group A (G-A), 16-20 mmol/L (n=4); group B (G-B), 4.33-8.61 mmol/L (n=4); and group C (G-C), healthy controls (n=4). After 2 weeks, SMILE was performed. Corneas were evaluated using anterior segment optical coherence tomography (AS-OCT) and in vivo confocal microscopy (IVCM) preoperatively on day 1 and postoperatively at weeks 1, 4, and 12.

Results: Morphological results: Twelve weeks after surgery, a corneal interlamellar stromal scar in three eyes and corneal stroma edema in one eye were found in G-A; no obvious scar edema occurred in G-B and G-C. In G-A, the number of abnormal cells in each corneal layer increased, the cell arrangement was irregular, and nerve fibers decreased significantly; the morphology and arrangement in G-B and G-C were regular. Cell density results: Actual central corneal thickness (CCT) after SMILE was thicker than theoretical CCT in each group ($P<0.05$). The corneal thickness deviation (Δ CT) of G-A was thicker than that of G-B and G-C, and the pre-keratocyte density (pre-KD) of G-A was lower than that of G-B and G-C ($P<0.05$). Endothelial cell density (ECD) at 4 and 12 weeks after surgery only in G-A was lower than that before surgery.

Conclusions: Hyperglycemia is an adverse factor affecting corneal structure after SMILE. SMILE is safe and effective for diabetic rabbits with good blood glucose control.

Background

The number of people with diabetes worldwide has increased from 108 million in 1980 to 422 million in 2014, and there is a growing increase in the number of younger people suffering from diabetes¹. Diabetes patients are prone to complications such as corneal epithelial defects and delayed regeneration, stromal edema, decreased corneal nerve density and branches, decreased endothelial cell density, and a refractive state after eye surgery². Therefore, diabetes is listed as a relative contraindication of all corneal refractive surgery, which limits the acceptance of corneal refractive surgery in patients with diabetes.

With the improvement in equipment and surgical techniques in recent years, refractive surgery is becoming more and more adapted to the physiology of the cornea, and the complications are greatly reduced. As an advanced procedure, the small-incision lenticule extraction (SMILE) procedure has the advantages of no flap, micro-incision, and less nerve injury. However, at present, there is no clinical study on whether diabetes patients with different blood glucose levels can obtain a correction in refractive error by SMILE. Currently, the blood glucose level of most diabetes patients is effectively controlled by hypoglycemic drugs. Some case reports^{3, 4, 5} have shown that diabetes patients with no systemic and ocular complications and good blood glucose control do not have obvious corneal structural changes

and functional abnormalities after laser-assisted in-situ keratomileusis (LASIK), and that the refractive effect is satisfactory.

In this study, considering that the size and structure of cornea and normal blood glucose level of adult New Zealand white rabbit are similar to those of human, and the modeling technique is mature, diabetic rabbit models with different blood glucose levels were used to observe corneal structural changes after SMILE to provide a theoretical basis for feasible SMILE surgery in clinical diabetes patients.

Methods

Experimental animals

Thirty male New Zealand white rabbits (aged 6 months, weighing 2.5-3 kg) without any corneal haze were purchased from Wangdu Tonghui breeding Co. The animals were housed in conventional conditions, including room temperature (24°C), controlled humidity (60%), a 12-hour light/12-hour dark cycle, and free access to water. Before any experimental manipulations, the rabbits were allowed to acclimatize for 7 days.

All animals were treated according to the guidelines of the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. Ethical approval for this study protocol was obtained from the Ethics Committee of Second Hospital of Hebei Medical University, China.

Study design

The study flow diagram can be seen in Figure 1. Twelve rabbits were randomly distributed into three groups regarding blood glucose level: group A (G-A), 16-20 mmol/L (n = 4); group B (G-B), 4.33-8.61 mmol/L (n = 4); and group C (G-C), healthy controls (n = 4). After 2 weeks, SMILE was performed. All rabbits were administered sodium pentobarbital (Merck, Darmstadt, Germany, 60 mg/kg)⁶ intraperitoneally after 12-week postoperative examination.

Alloxan-induced diabetic rabbit model

In total, 26 rabbits were randomly administered a 5% solution of alloxan monohydrate (ALX; Sigma Aldrich Co., St. Louis, MO, USA) (150 mg/kg of body weight)^{7,8} intravenously to increase blood glucose levels. The four rabbits in G-C were given an equal amount of normal saline (0.9% w/v of NaCl). The fasting blood sugar (FBS) level was measured 2 weeks later, and rabbits with an FBS level more than 16 mmol/L were considered to be diabetic. Eight diabetic rabbits were then randomly distributed into G-A and G-B, depending on their blood glucose level. We controlled blood glucose levels by adjusting the dosage of insulin.

SMILE procedure

In total, 12 rabbits in the three groups underwent the SMILE procedure (Visumax®; Carl Zeiss Meditec, Jena, Germany) on one eye, which was randomly determined by flipping a coin, by an experienced surgeon 2 weeks after successful modeling. Three days before surgery, levofloxacin hydrochloride eye drops (Santen Pharmaceutical Co., Ltd., Osaka, Japan) were administered four times per day to prevent infection. Anesthesia was induced by intravenously injecting 3% pentobarbital sodium (Merck, Darmstadt, Germany, 0.8 mL/kg) and topical proparacaine hydrochloride application before the surgical procedure.

The SMILE procedure was performed using the VisuMax system. The surgery parameters were as follows⁹: 100- μ m cap thickness, 7.5-mm cap diameter, 6.5-mm intrastromal lenticule diameter, a refractive spherical correction of -6 diopters (D), and a 107- μ m thickness. The laser energy delivered was 150 nJ. The line and spot distances were set at 4.5 mm for the lenticule and cap. The width and position of the side-cut incision were 3.0 mm and 120 degrees, respectively.

Flunomil eye drops (Santen Pharmaceutical Co., Ltd., Osaka, Japan) and prapofen eye drops (Senju Pharmaceutical Co., Ltd., Osaka, Japan) were administered to all eyes after surgery for 4 weeks.

Examinations

AS-OCT (RS-3000 advance; NIDEK Co., Ltd., Gamagōri, Aichi Prefecture, Japan) and IVCM (HRT 3; Heidelberg Engineering GmbH, Heidelberg, Germany) were performed in all groups on 1 day before surgery and at 1 week, 4 weeks, and 12 weeks after surgery.

Line, cross, and radial scanning were performed with AS-OCT, and the trusted scanning results were automatically superimposed after 50 rapid scans of the same site by the program. CCT was measured and the interlamellar healing was observed by AS-OCT.

Preoperative CCT - Thickness of Lenticule = Theoretical CCT

Postoperative actual CCT - Theoretical CCT = Deviation of Corneal Thickness (Δ CCT)

IVCM was performed in the center of the cornea. At least 40 pictures were captured at each time point. The scope of observation ranged from the corneal epithelium to the endothelium. We obtained a clear morphology of corneal nerves and cells in each layer as far as possible and took photos. The anterior keratocyte density (pre-KD) was obtained by counting the number of stromal cells closest to the Bowman layer. The central range of 200 μ m \times 200 μ m of each scanning field section was selected to count endothelial cells.

Statistical analysis

The grouping and inspection time of all scans were blinded and stored in a random order; evaluations and measurements were performed by an observer who was blinded to both the patient and the time of the scan. Data were analyzed using SPSS (version 25.0; SPSS Inc, Chicago, IL). Data were expressed as mean \pm SD. The CCT, pre-KD, and endothelial cell density (ECD) at different time points were compared by

multivariate analysis of variance (MANOVA). When comparing the indexes of the three groups at the same time point, the homogeneity of variance was tested first. If the variance was homogeneous, a one-way ANOVA was used, and if it was not consistent with the homogeneity of variance, the Welch test was used. A paired t-test was used to determine differences between before and after values. All statistical tests were conducted at an alpha level of 0.05.

Results

Morphological results

Corneal epithelial cells: In G-A, during the time after surgery, the atypia of the corneal epithelial cells, intercellular space, and number of exfoliated cells gradually increased. However, in G-B and G-C, at each time point, the corneal epithelial cells were uniform in size and neatly arranged, and no obvious exfoliated cells were found.

Corneal endothelial cells: In G-A, endothelial cells were regular, uniform, and neatly arranged before surgery. At 1 week postoperatively, there were no obvious abnormalities; at 4 weeks postoperatively, a few irregular endothelial cells appeared; and at 12 weeks postoperatively, endothelial interface folds increased, there was an increase in the scatter of irregular cells, and the cell area increased (Fig. 2 A1-A4). In G-B and G-C, the endothelial cells were closely arranged and uniform in size (Fig. 2 B1-B4, C1-C4) at each time point.

Corneal nerve fibers: The course of the subepithelial nerve fibers in the three groups before surgery was normal. In G-A, the subepithelial nerve fibers were rare at 1 week postoperatively and could not be observed clearly at 4 and 12 weeks postoperatively (Fig. 3 A1, A2). In G-B and G-C, the course of the subepithelial nerve fibers was normal after surgery, and there was no obvious bifurcation; however, the curvature of the subepithelial nerves increased compared with that before operation (Fig. 3 B1, B2, C1, C2).

Stromal nerve fibers: Before SMILE, the morphology and course of the stromal nerve fibers in the three groups were normal (Fig. 4 C1, B1). In G-A, before and 1 week after the operation, the thickness of the nerve fibers was uniform, and the branches and branching connections were normal (Fig. 4 A2). The endings of the stromal nerve fibers showed that the single branches, decreased branches, and nerve fiber branching connections were rare with an uneven thickness of nerve fibers at 4 weeks postoperatively in G-A (Fig. 4 A3). Furthermore, only part of the residual corneal stromal nerves could be observed at 12 weeks postoperatively (Fig. 4 A4). In G-B, a single stromal nerve fiber was uneven in thickness, but no obvious abnormalities were found in the rest at 4 weeks postoperatively (Fig. 4 B2). In G-C, a single nerve fiber contained a high reflective cord at 1 week postoperatively (Fig. 4 C2), but no obvious abnormalities were found in the rest.

Cell density results

There were no significant differences in the CCT, pre-KD, and ECD among the three groups one day before SMILE.

CCT and Δ CT: The actual CCT of the three groups at each time point after surgery was thicker than the theoretical CCT of each group ($P < 0.05$). Δ CT in G-A was higher than that in G-B and G-C ($P_{A-B}=0.005$, $P_{A-C}=0.002$). There was no significant difference in Δ CT between G-B and G-C ($P_{B-C} > 0.05$). Δ CT in G-A was the smallest at 4 weeks after surgery and increased significantly at 12 weeks after surgery, while Δ CT in G-B and G-C at 12 weeks after surgery was significantly higher than that at 1 week and 4 weeks after surgery (Fig. 5).

Pre-KD: Pre-KD in G-A was lower than that in G-B and G-C, and there was no significant difference between G-B and G-C ($P_{A-B} < 0.001$, $P_{A-C} < 0.001$, $P_{B-C}=0.597$, respectively). The pre-KD at each time point after surgery in the three groups was lower than that before surgery, and the pre-KD in G-A decreased significantly over time postoperatively; however, there was no significant difference in the pre-KD at each time point between G-B and G-C (Fig. 6).

ECD: ECD in G-A at 4 and 12 weeks after surgery was lower than that before the surgery ($P_{4w}= 0.016$, $P_{12w}= 0.039$). There was no significant difference in ECD at each time point in G-B and G-C (Fig. 7).

Discussion

In this study, we evaluated the safety and efficacy of the SMILE procedure in diabetic rabbits with different blood glucose levels for the first time by observing the corneal structure of rabbits after SMILE.

1. Morphological changes of cells and nerve fibers in various layers of cornea

There were no obvious morphological changes in corneal cells in G-B and G-C when comparing before and after SMILE; however, in G-A, with the prolongation of the disease course, the number of cells in each layer of the cornea decreased, heteromorphism increased, and cells became more irregular in arrangement.

Pathological hyperglycemia can accelerate the glycosylation reaction, which causes the accumulation of advanced glycation end products in the basement membrane of the corneal epithelium, resulting in changes in the molecular structure of the components of the basement membrane; therefore, the adhesion between corneal epithelial cells and the basement membrane is weakened, and there is repeated exfoliation of epithelial cells^{10,11}. Adults mainly rely on enlargement, expansion, and migration of endothelial cell bodies to cover damaged corneal endothelial areas¹². The number of corneal endothelial atypical cells increased in G-A, but no abnormal cells were found in G-B, indicating that persistent hyperglycemia destroyed the morphology of endothelial cells.

Nerve changes in the cornea include a decrease in nerve fiber density, decrease in nerve branches, an increase in nerve curvature, and so on¹³. There was no significant decrease in subepithelial nerve fibers in

G-B and G-C after surgery; only individual nerve degeneration was observed. In G-A, the number of subepithelial nerve fibers decreased significantly, the thickness of stromal nerve fibers was uneven, and the branches and connections decreased. This shows that the SMILE procedure itself can lead to local corneal nerve degeneration, while persistent hyperglycemia aggravates corneal nerve degeneration after SMILE. Good control of blood glucose level in diabetes patients can reduce corneal nerve degeneration. There is a process of degeneration and regeneration of corneal nerve fibers after corneal refractive surgery. It has been previously shown that corneal nerve fibers begin to regenerate 4 weeks after SMILE in rabbits¹⁴.

The increase in the Aldose reductase pathway activity in diabetes patients with persistent hyperglycemia leads to the accumulation of sorbitol in Schwann cells, resulting in mechanical compression and toxicity to damaged nerve axons, while a decrease in protein kinase C and Na⁺-K⁺-ATP enzyme activity leads to cell edema and demyelination of nerve cells¹⁵. He et al.¹⁶ reported that the corneal nerve density decreased significantly in diabetes patients with a course of disease of more than 5 years. Dehghani et al.¹⁷ found that the higher the HbA1c value and age, the lower the corneal nerve fiber density in patients with diabetes. This is consistent with the results of this study.

2. CCT

The actual CCT after SMILE in the three experimental groups was thicker than the theoretical CCT, and the Δ CCT in G-B and G-C increased gradually with the extension of time after the operation. Corneal epithelium and stroma show significant thickening after small-incision lenticule extraction. However, this remodeling effect did not affect the postoperative diopter stability^{18, 19}.

The Δ CCT in G-A was higher than that in G-B and G-C, but there was no significant difference in Δ CCT between G-B and G-C. The CCT of diabetes patients with persistent hyperglycemia increased^{20, 21}, and the longer the course of disease, the higher the CCT value²².

3. Pre-KD and ECD

There was no significant difference in the corneal pre-KD and ECD between G-B and G-C at each time point after SMILE. However, with the prolongation of the disease course, the corneal pre-KD and ECD in G-A decreased significantly. It is suggested that continuous hyperglycemia leads to a decrease in corneal pre-KD and ECD after SMILE. Some studies showed that the decrease in ECD was positively correlated with the course of diabetes and that there was no significant change in ECD in patients with early diabetes²³. After SMILE, there was no significant difference in ECD between G-B and G-C, as well as in each of the three groups before surgery, indicating that the SMILE operation itself did not lead to the decrease of ECD.

In this study, after SMILE, the corneal pre-KD in G-B and G-C was lower than that of each group before surgery, but there was no significant difference in pre-KD between each time point after the operation. It is

suggested that the SMILE operation itself will lead to a decrease in the number of stromal cells; Li et al.²⁴ reported that the density of stromal cells decreased immediately after Femtosecond laser-assisted in situ keratomileusis and SMILE, and there was no sign of recovery at 6 months after surgery, which may be related to the necrosis and apoptosis of stromal cells. Some studies²⁵ suggest that the blasting effect of the femtosecond laser damages corneal stromal cells, leads to interlamellar inflammation, release of cytokines and chemokines, which activates corneal stromal cells and induces corneal healing, resulting in a decrease in the number of corneal stromal cells after SMILE. Linna et al.²⁶ suggested that the decrease in stromal cell density after SMILE may be related to the denervation caused by corneal nerve fiber injury during lenticules making.

Conclusion

In conclusion, persistent hyperglycemia destroyed the structure of corneal cells and nerve fibers after SMILE in diabetic rabbits; however, there were no obvious morphological changes in corneal cells and nerve fibers after SMILE in diabetic rabbits with good blood glucose control, as well as no significant difference in cell density and central corneal thickness between the diabetic rabbits and the normal control group. This study provides evidence for the safety and effectiveness of SMILE in diabetic rabbits with good blood glucose control and provides a theoretical basis for diabetic patients to undergo SMILE surgery.

Abbreviations

SMILE: small-incision lenticule extraction; AS-OCT: anterior segment optical coherence tomography; IVCM: in vivo confocal microscopy; CCT: central corneal thickness; Δ CT: Deviation of Corneal Thickness; pre-KD: pre-keratocyte density; ECD: Endothelial cell density; LASIK: laser-assisted in-situ keratomileusis; ALX: alloxan; FBS: fasting blood sugar; MANOVA: multivariate analysis of variance.

Declarations

Ethics approval and consent to participate

This study was approved by the ethics committees at Second Hospital of Hebei Medical University (NO.2019-P059, Hebei, China).

Consent for publication

Not applicable.

Availability of data and materials

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

Competing interests

The authors have no financial or proprietary interest in the materials presented herein.

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Authors' contributions

All authors have read and approved the manuscript. Study concept and design (XG, WG, CG); data collection (WG, BW, MG); analysis and interpretation of data (XG, WG, CG, ZS); writing the manuscript (XG, WG, CG, BW, MG); critical revision of the manuscript (XG, WG, CG, ZS); statistical expertise (XG, WG, ZS); administrative, technical, or material support (XG, WG, ZS); supervision (XG, CG)

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Figures

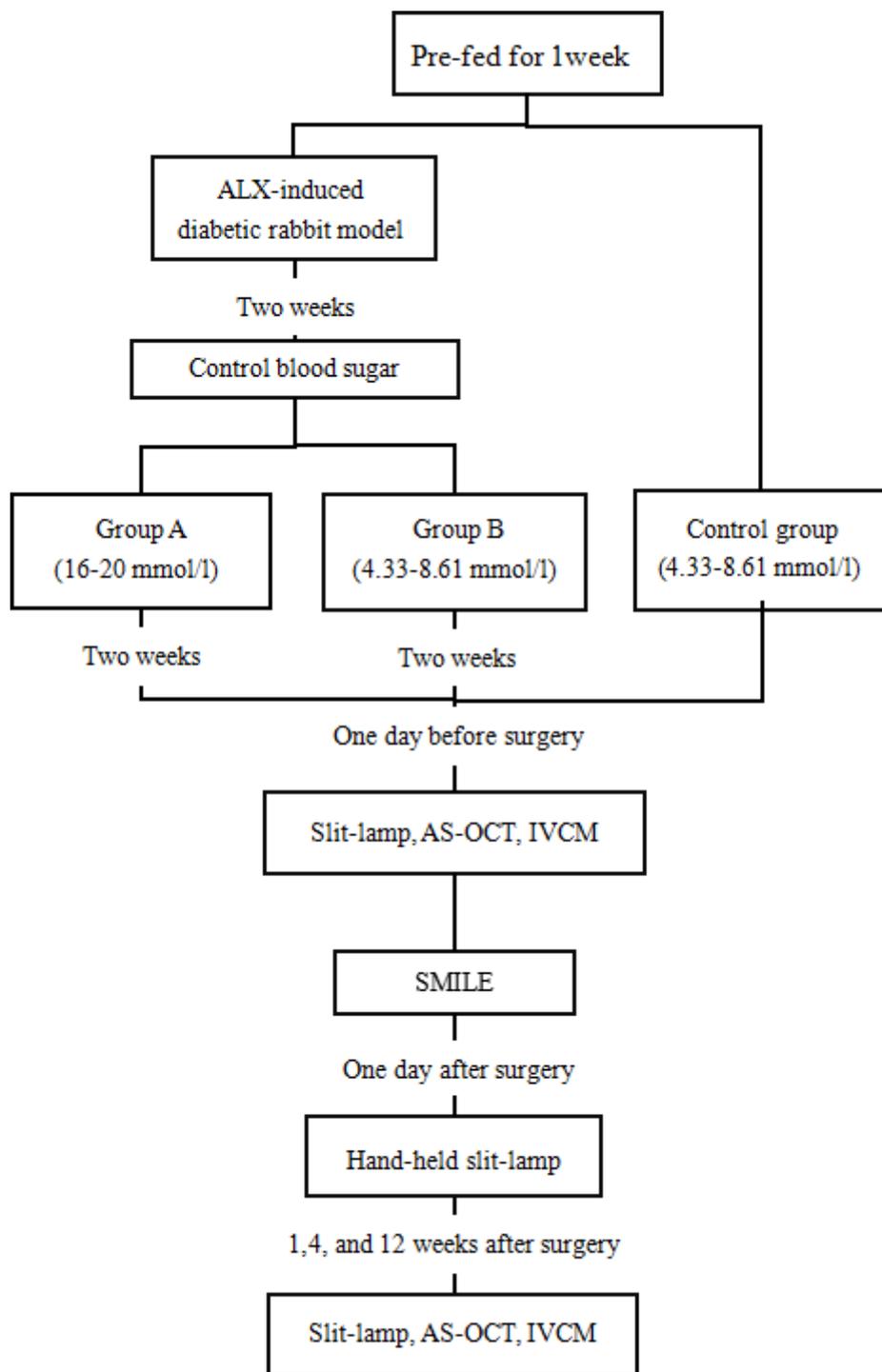


Figure 1

Study flow diagram.

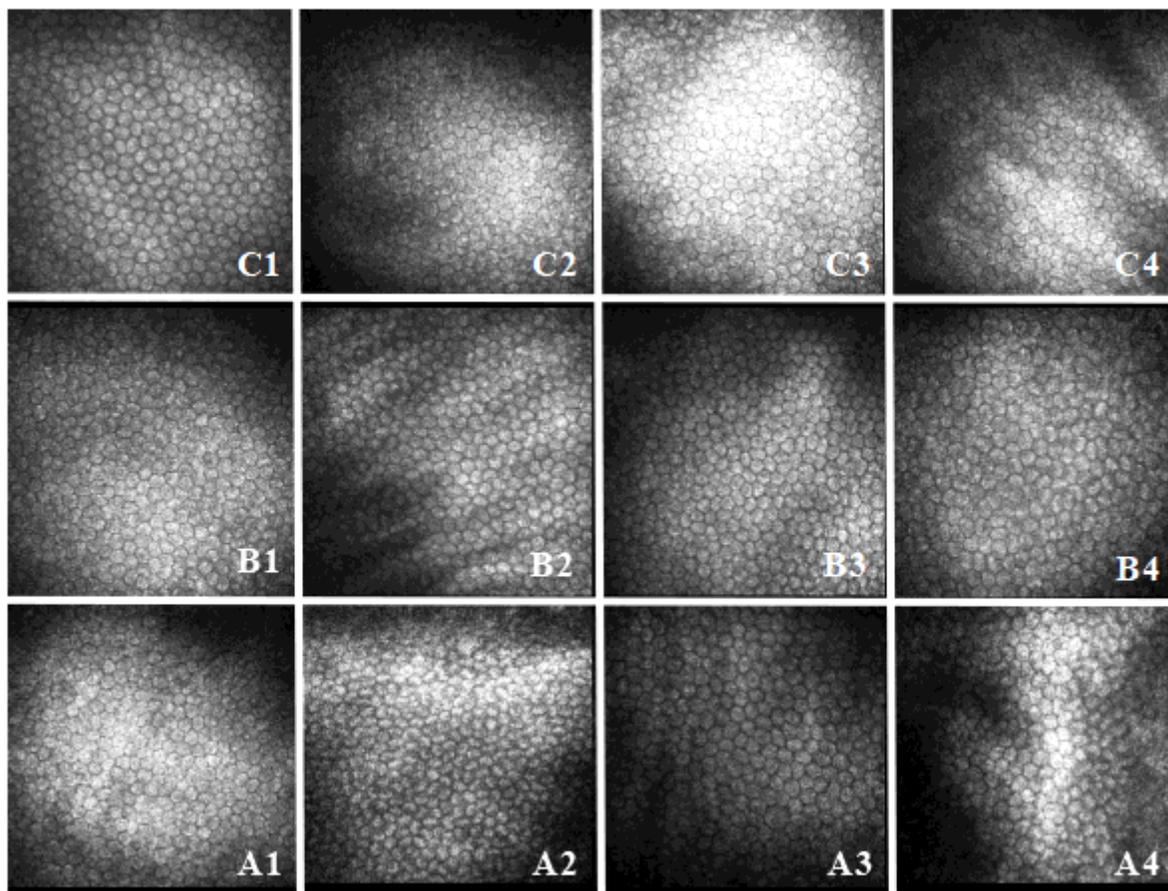


Figure 2

The IVCM images of corneal endothelial cells in the three groups at each time point ($\times 800$). Note: "1" means 1st day before surgery, "2" means 1st week after surgery, "3" means 4th week after surgery. "4" means 12th week after surgery. G-C1-G-C4 and G-B1-G-B4: Atypical corneal endothelial cells were not found at any time points in G-C and G-B. G-A1-G-A4: Atypical endothelial cells gradually appeared, and the cell area was significantly larger than that of normal cells.

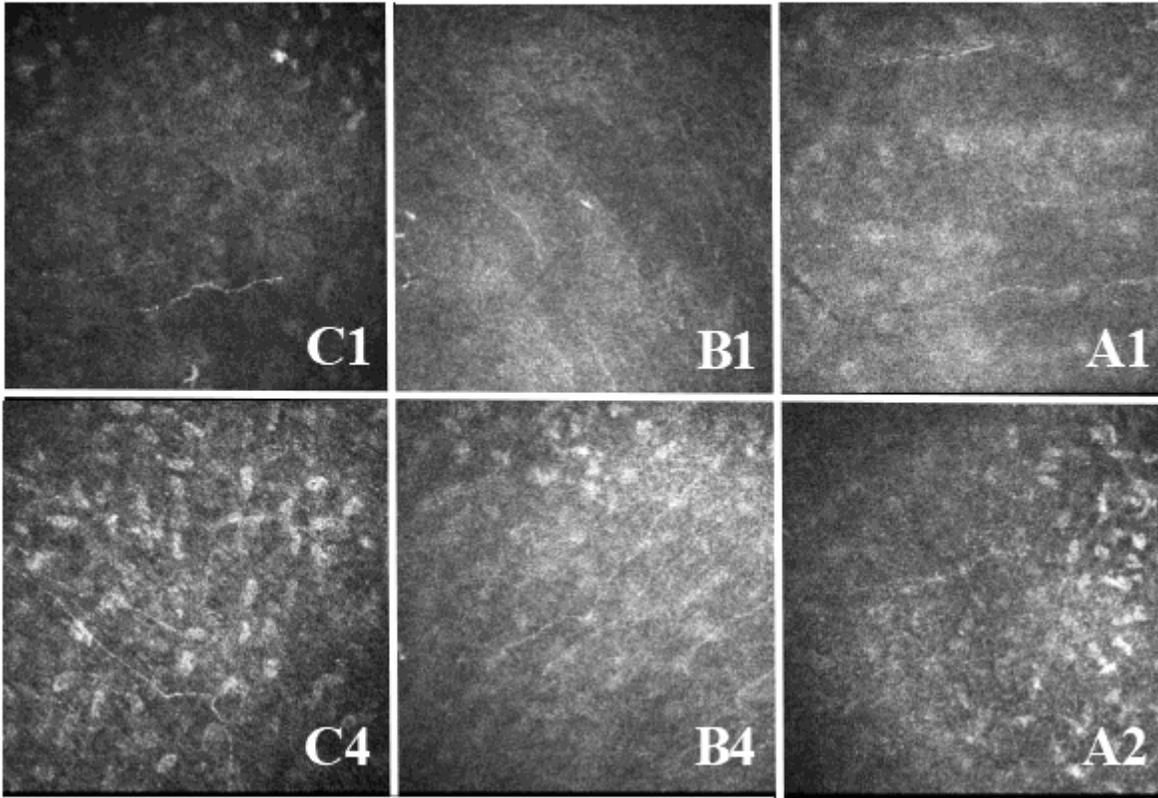


Figure 3

IVCM images of subepithelial nerve fibers in the three groups ($\times 800$). Note: G-C1, G-B1, and G-A1: On the 1st day before surgery, corneal subepithelial nerve fibers in the three groups are straight and evenly distributed. G-C4 and G-B4: On the 4th week after surgery, the course of the corneal subepithelial nerves in G-B and G-C was normal, but the curvature of the nerves increased. G-A2: On the 1st week after surgery, the corneal subepithelial nerve fibers of G-A were rarely seen.

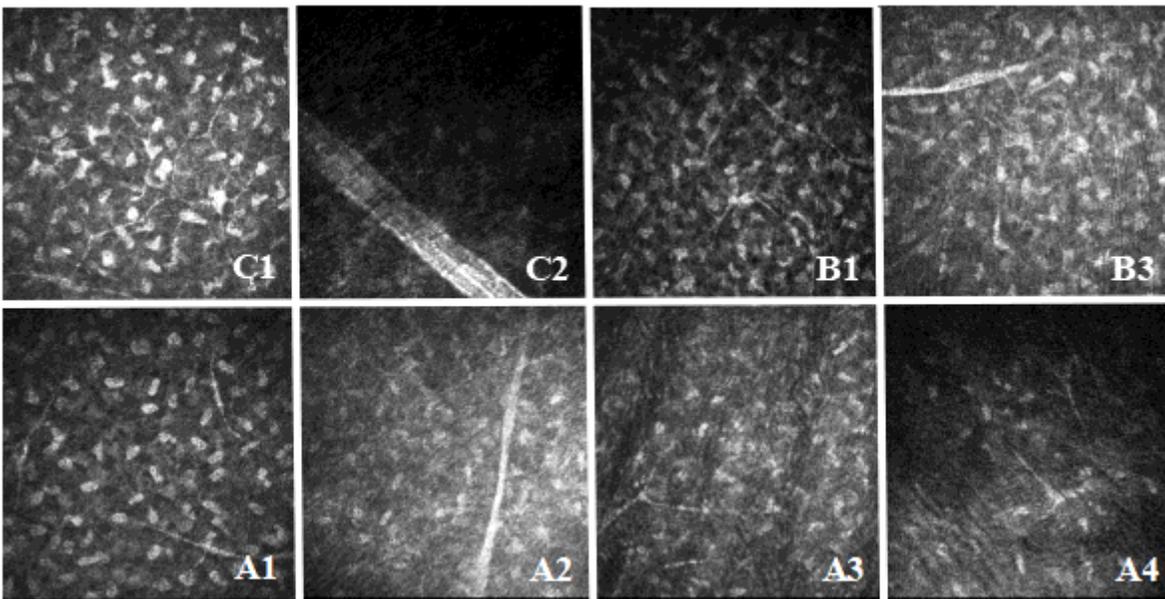


Figure 4

IVCM images of stromal nerve fibers in the three groups ($\times 800$). Note: C1 and B1: In G-C and G-B, preoperative corneal superficial stromal nerve fibers were uniform in thickness, rich in branches, and connected to each other. C2: On the 1st week after surgery, there were high reflective bands in the nerve fibers in G-C. B3: On the 4th week after surgery, the thickness of the stromal nerve fibers was uneven in G-B. A1: In G-A, preoperative nerve fibers and branch connections decreased. A2: On the 1st week after surgery, the thickness of nerve fibers was uneven in G-A. A3: On the 4th week after surgery, terminal branches of superficial matrix nerve fibers appeared, and the branch connections decreased. A4: On the 12th week after surgery, only part of the residual corneal stromal nerves can be observed in the superficial stromal layer.

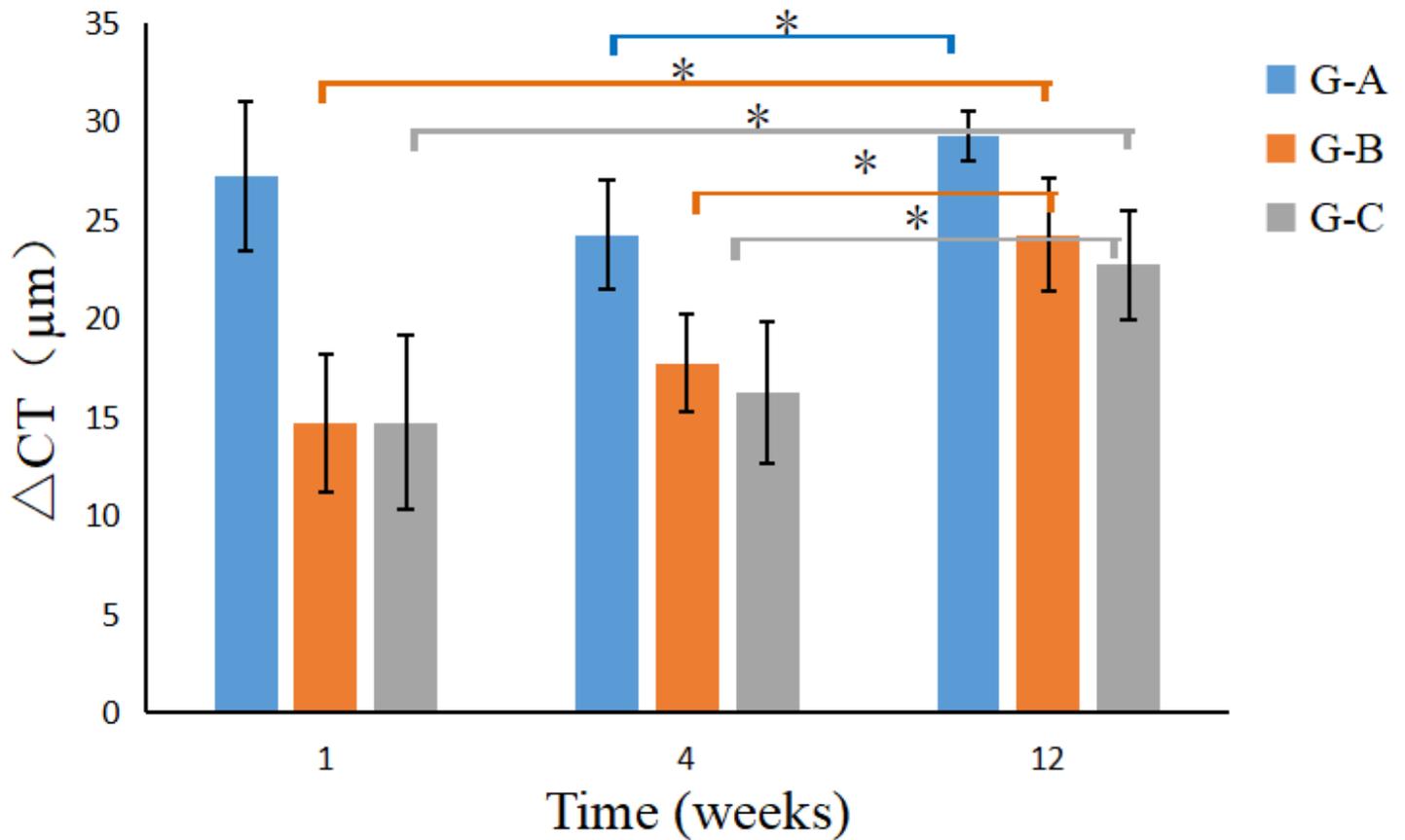


Figure 5

Δ CT (μm) at different time points in each group. Note: Data are expressed as mean \pm SD. MANOVA of repeated measures: $F_{\text{group}} = 14.173$ $P_{\text{group}} = 0.002$; $F_{\text{time}} = 33.117$ $P_{\text{time}} < 0.001$; $F_{\text{interaction}} = 3.691$ $P_{\text{interaction}} < 0.001$. Pairwise comparison among the three groups: PA-B = 0.005, PA-C = 0.002. Pairwise comparison of Δ CT in a group: * $P < 0.05$.

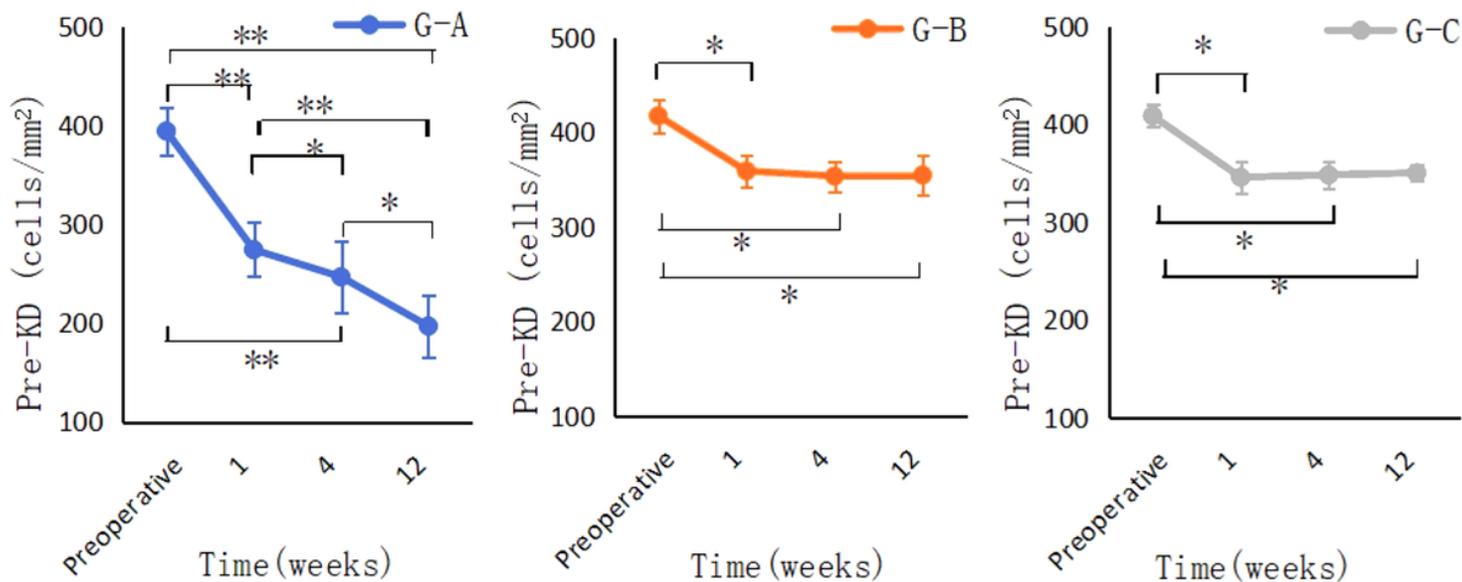


Figure 6

Pairwise comparison of pre-keratocyte density at each time point in each group. Note: Data are expressed as mean \pm SD. MANOVA of repeated measures: $F_{\text{group}} = 25.821$ $P_{\text{group}} < 0.001$; $F_{\text{time}} = 413.618$ $P_{\text{time}} < 0.001$; $F_{\text{interaction}} = 66.491$ $P_{\text{interaction}} < 0.001$. Pairwise comparison among the three groups: $PA-B < 0.001$, $PA-C < 0.001$, $PB-C = 0.597$. Pairwise comparison in a group: * $P < 0.05$. ** $P < 0.001$.

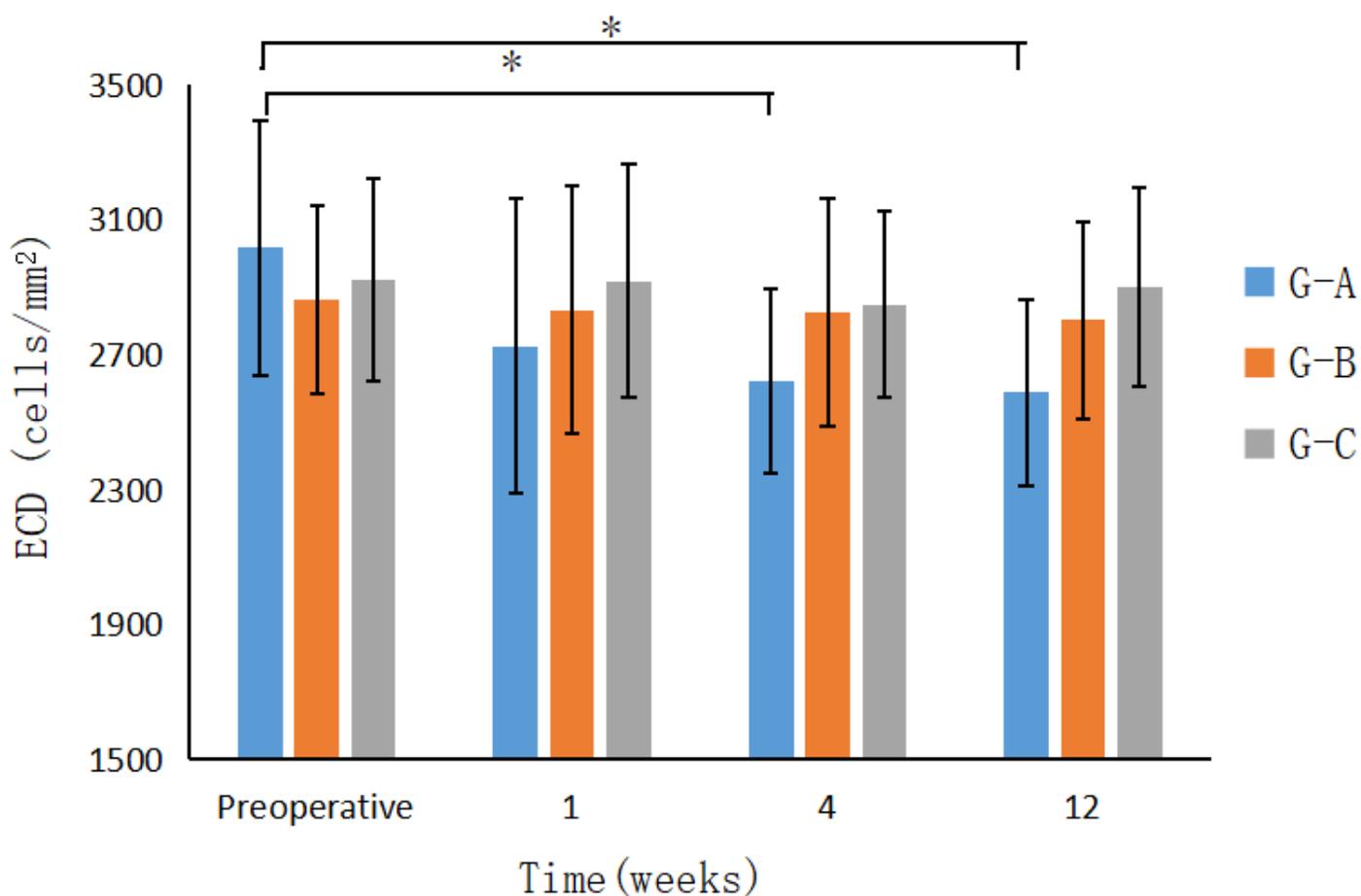


Figure 7

ECD at different time points in each group (cells/mm²). Note: Data are expressed as mean \pm SD.
MANOVA of repeated measures: F_{group}= 0.248 P_{group}= 0.785; F_{time}= 8.438 P_{time}< 0.001; F_{interaction}= 4.444 P_{interaction}= 0.003. Pairwise comparison in a group: *P < 0.05.

Supplementary Files

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