

Treatment of corneal epithelial wounds in dogs using basic fibroblast growth factor

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ABSTRACT: An experimental study examined the effect of basic fibroblast growth factor (bFGF) on the healing of corneal epithelial wounds in dogs. A corneal wound was made on one eye from each of 40 dogs with a corneal trephine (6 mm diameter). Four concentrations of bFGF (0, 0.1, 0.5, and 1.0 µg/ml) were applied to the affected eyes three times daily. Fluorescein staining was used to assess the closure of the corneal epithelial wounds. The morphological characteristics were determined on histological examination. The wound healing rate was significantly greater in the bFGF-treated group compared with controls 1, 3, 5, and 7 days ($P < 0.01$) after the topical administration of bFGF. Both 0.5 and 1.0 µg/ml bFGF increased the wound healing rate significantly (at Days 3 and 5, $P < 0.05$) compared to 0.1 µg/ml bFGF. Moreover, two control cases still showed poor healing 10 days after the corneal wound. None of the eyes developed corneal clouding or neovascularization during the experiment. The histological examination showed more epithelial layers, a more regular rearrangement, and fewer inflammatory cells in the epithelium of the bFGF-treated group; the epithelium was reconstructed more quickly in the bFGF-treated group compared with the control group. These results suggest that bFGF promotes canine corneal epithelial wound healing effectively, making bFGF suitable for curing canine corneal epithelial wounds.

Keywords: corneal epithelium; corneal epithelial wound healing; basic fibroblast growth factor; bFGF; dogs

The corneal epithelial cells on the surface layer of the cornea form a defensive barrier to noxious agents. Growth factor-mediated renewal of the corneal epithelium plays a functional role in maintaining the barrier function and corneal transparency (Li and Lu, 2005). Corneal wounds caused by trauma, surgery, or disease are very common in small animals (Ollivier, 2003). Inadequate healing of epithelial injuries can lead to corneal ulcers, corneal perforation, or even acroisa. The limited regenerative ability of corneal cells after injury or surgical intervention may necessitate transplantation of a functional donor cornea (Hoppenreijds et al., 1994). The potential regeneration of the cornea is a complex process involving enlargement, migration, coalescence, and mitosis (Waring et al., 1982). In recent years, it has been found that the action of growth factors can accelerate wound healing (Bennett and Schultz, 1993; Steed, 1995). The effects of fibroblast growth factor (FGF) on corneal tissues in stimulating and

maintaining corneal cell density and function have attracted considerable attention.

Originally, FGF was identified as the active factor in pituitary and brain extracts responsible for stimulating the growth of 3T3 cells (Rifkin and Moscatelli, 1989). Acidic and basic FGF (a/bFGF) were the first FGFs to be purified, sequenced, and cloned (Brazzell et al., 1991). Most of the activity of the FGFs in the eye can be attributed to bFGF, which is 10–100 times more potent than aFGF (Tripathi et al., 1992). bFGF is a well-known mitogen for various types of cells. It influences cell growth, migration, differentiation, regeneration, and neovascularization. Numerous studies have documented the ability of FGF to stimulate the proliferation of corneal epithelial cells (Hecquet et al., 1990), stromal fibroblasts, and endothelial cells (Hoppenreijds et al., 1994) *in vitro*, in cell and organ culture systems. FGF also has been reported to enhance the healing of the corneal epithelium

of rabbits (Fredj-Reygrobellet et al., 1987) and the corneal endothelium of cats (Rich et al., 1992) and rabbits (Rieck et al., 1992). However, few experimental studies have examined the effects of bFGF on canine corneal epithelial cells and wounds.

This study used a canine epithelial wound model to analyze the effect of bFGF on canine corneal epithelial wounds *in vivo*. The goal of this research is to determine the suitable therapeutic dose of bFGF for curing canine epithelial wounds, and to provide a theoretical basis for its clinical application.

MATERIAL AND METHODS

Corneal epithelial wound

The animals were maintained and handled according to the Association for Research in Vision and Ophthalmology (ARVO) Resolution on the Use of Animals in Research. Before inclusion in the study, all dogs underwent a complete ophthalmic examination, including indirect ophthalmoscopy, slit-lamp biomicroscopy, Schirmer's tear test, and fluorescein staining. The dogs had no corneal epithelial abnormalities, lid conformational defects, distichiasis, ectopic cilia, or any clinical evidence of systemic disease. All the animals were dewormed and allowed to become accustomed to being approached for a week. Feed was withheld for 24 h before the start of the experiment.

A central corneal epithelial wound was made as described previously (Stiles et al., 2003). Each dog was anesthetized with an intramuscular injection of ketamine hydrochloride (10 mg/kg) and xylazine (6 mg/kg) and with topical 1% lidocaine hydrochloride. The upper and lower eyelids were braced using an eye speculum. Excess moisture was absorbed from the surface of the cornea using sterile cotton swabs, and two stitches were sutured at the upper and lower corneoscleral limbus. A 6-mm-diameter corneal trephine was placed over the cornea centered on the pupil, and the surgical assistant placed tension on the retention suture to bring the corneal trephine into contact with the cornea. Fresh 20% alcohol solution (v/v) was added to the central hole of the corneal trephine and left there for 20 s; the ocular surfaces were prevented from drying by the topical administration of sterile saline. The canine epithelial wound model was created after removing the corneal epithelium layer with corneal scissors and an iris separator.

Topic application of bFGF

The 40 mongrel dogs (40 eyes) used in this study were one year old, weighed 13–15 kg, and were allocated randomly to four groups: 0.1 µg/ml of bFGF [BFGF0.1], 0.5 µg/ml of bFGF [BFGF0.5], 1.0 µg/ml of bFGF [BFGF1.0], and saline solution. Each dose was administered to the eyes three times daily.

Determination of healing rate

Fluorescein staining was used to assess epithelial wound closure of the eye according to Jean (Park and Kim, 1999). The wound margin was outlined directly with fluorescein solution, and the eyes were photographed before the first dose and 24, 72, 120, and 168 h after initiating the fluorescein solution. Then, the wound area was measured using the Image Measurement Analysis System software package (Axioskop MOT, Carl Zeiss, Jena, Germany). To evaluate the effect of bFGF on wound closure, the wound area was plotted versus time for each group of corneas. In addition, any discharge and inflammation of the eyes and neovascularization in the cornea and conjunctiva were observed and documented every day.

Histological examination of corneal wound healing

Three eyeballs in each group were enucleated at 3, 7, and 14 days after wounding, and the globes were fixed with 10% neutral formalin, as described previously (Park and Kim, 1999). The fixed corneas were dehydrated through a graded alcohol series, immersed in xylene, and embedded in paraffin. Then, 5-µm slices were cut and stained with hematoxylin and eosin. Light microscopy (80i, Nikon, Japan) was performed and histology photographs were obtained. The morphological characteristics and closure of the wound were compared between the test and control groups.

Statistics

The arithmetic mean and standard error of the mean were calculated for each treatment group. The data were analyzed using analysis of variance (ANOVA) using SAS software (SAS Institute, Cary,

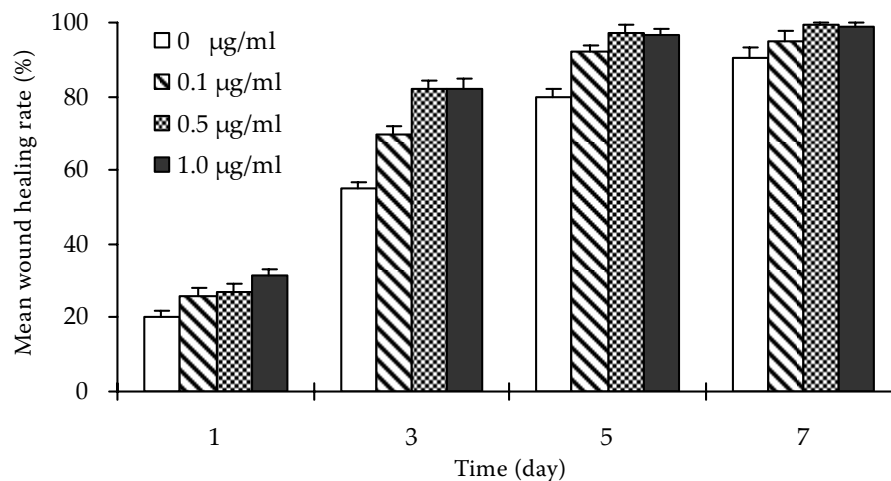


Figure 1. Wound healing rate of canine corneas (mean \pm SD). The mean wound healing rate is expressed as a percentage of the initial wound area. The mean healing rate in bFGF-treated corneas was significantly greater (at Days 1, 3, 5 and 7, $P < 0.01$) than that in control corneas after topical bFGF application. Both 0.5 and 1.0 $\mu\text{g/ml}$ bFGF increased the wound healing rate significantly (at Days 3 and 5, $P < 0.05$) compared to 0.1 $\mu\text{g/ml}$ bFGF

NC, USA). Differences between treatment means were evaluated by Dunnett's post hoc test, after a significant F -test. $P < 0.05$ was considered to be statistically significant.

RESULTS

Clinical observation

None of the 40 bFGF-treated eyes developed any unusual corneal clouding or neovascularization and all remained clear throughout the experiment. Tears appeared after the corneas were wounded

and decreased gradually, and no episcleral vascular congestion occurred in any of the wounded eyes.

Corneal epithelial wound healing rate

To evaluate the effects of bFGF treatment on wound healing, the wound healing rate was plotted versus time for each treatment (Figure 1). On all of the days investigated, the mean healing rate of the bFGF-treated corneas was significantly greater (at Days 1, 3, 5 and 7, $P < 0.01$) than that of the control corneas after topical bFGF application. Both 0.5 and 1.0 $\mu\text{g/ml}$ bFGF increased the wound heal-

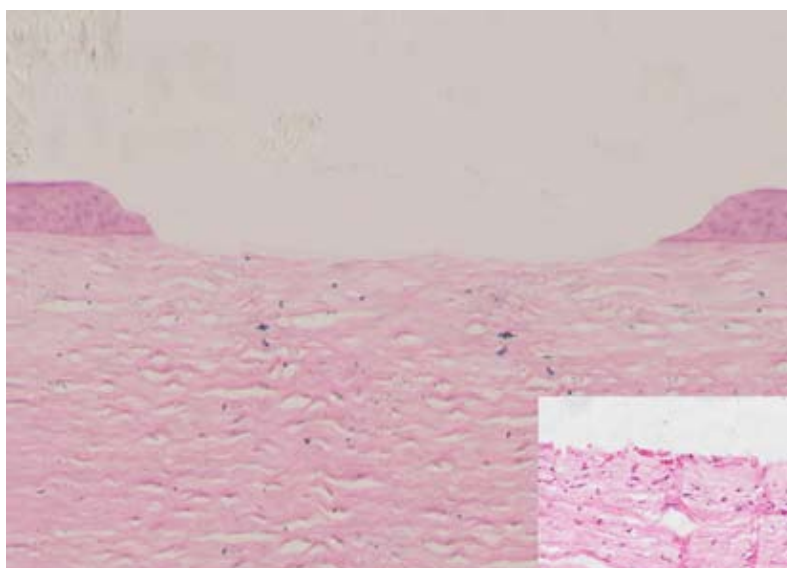


Figure 2. Light photomicrograph of a 6-mm-diameter excisional trephine wound in a canine cornea; magnification $\times 100$ (inset $\times 400$)

Table 1. Summary of the morphological features seen in the process of corneal reconstruction

Morphological features	No treatment (control)	bFGF-treatment		
		0.1 µg/ml	0.5 µg/ml	1.0 µg/ml
Epithelial thickness (cell layers)	4–5 cells	5–6 cells	5–6 cells	5–6 cells
Stromal layer	disorderly	orderly	orderly	orderly
Inflammatory reaction	+++	++	+	–
Vascularization	–	–	–	–

+++ = strong; ++ = intermediate; + = weak; – = negative

The thickness of the epithelial layer is represented by the number of individual cells that contribute to its thickness. The arrangement of the stromal layer was described as disorderly or orderly. The infiltration of inflammatory cells and vascularization were described

ing rate significantly (at Days 3 and 5, $P < 0.05$) compared to 0.1 µg/ml bFGF. All corneal wounds in the bFGF-treated groups closed within seven days, whereas two control wounds showed poor healing for 10 days.

Histopathological study

A central corneal epithelial wound was made on an eye (Figure 2). Some morphological features in

the process of corneal reconstruction are summarized in Table 1. Corneal epithelial cells migrate into the wounds and cover the stromal layer, then ultimately construct new normal-functioning corneal epithelium (Figure 3).

In the control group, the corneal epithelium layer was indistinct and the cell structure was disordered. The wound did not heal completely and there was still an inflammatory cell infiltrate seven days post-wounding. There was little fibrous tissue and were few fibroblasts in the stroma. Large gaps between

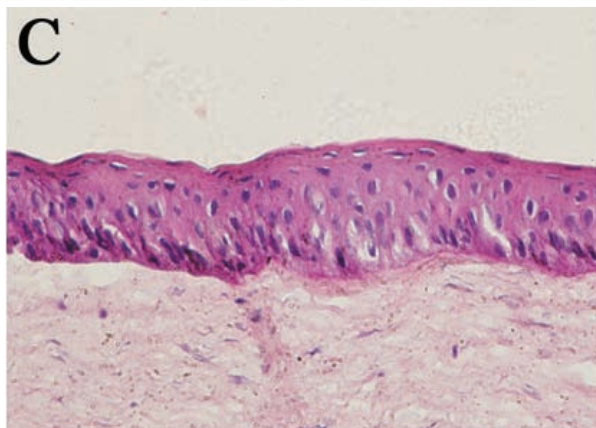
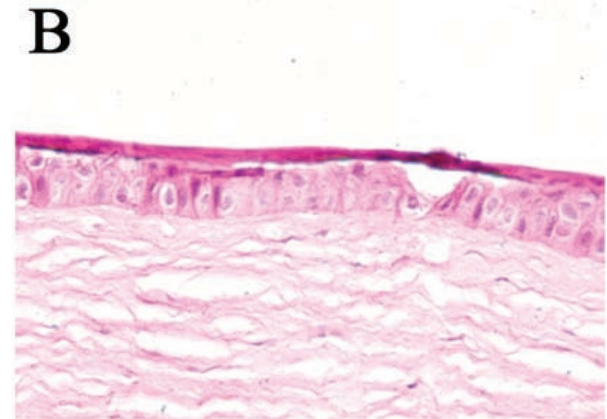
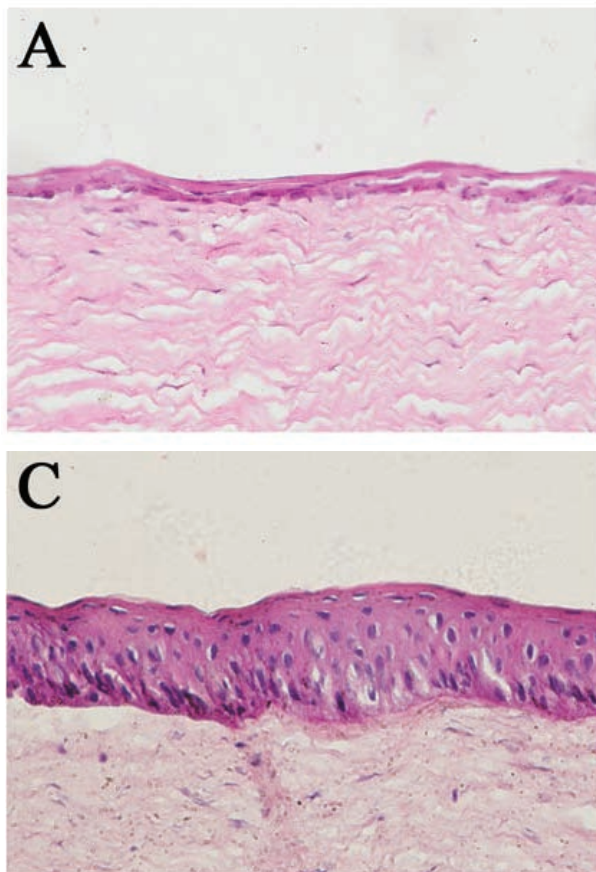


Figure 3. Photomicrographs of healing epithelial wounds in canine corneas treated with bFGF. A = corneal epithelial cells migrated into the wound and covered the stromal layer; B = the defect was re-epithelialized; this epithelium is 1~2 cell layers thick; C = the wound was covered by 5~7 layers of epithelial cells, forming new normal-functioning corneal epithelium; magnification $\times 400$

cells were observed 14 days post-wounding. The BFGF0.1 group did not differ from the control group; the stroma was covered by a single cell layer and some inflammatory cells infiltrated the wound. Reconstruction of the corneal epithelium was the same as in the control group after three days of treatment. Fourteen days post-wounding, a stratified layer of 4~5 cells had formed, and fibrous tissue and fibroblasts were well arranged. The canine corneal epithelial cells grew more rapidly in groups BFGF0.5 and BFGF1.0, and the stromal layer was covered by a stratified layer of 2~3 cells after three days of treatment. The stroma was arranged in an orderly manner and no inflammatory infiltrate was seen. The canine corneal epithelial cells formed clear layers. Fibrous tissue and fibroblasts proliferated in the stroma. Fourteen days post-wounding, a stratified layer of 5~7 cells had formed, and there was further proliferation of fibrous tissue and fibroblasts.

DISCUSSION

It has been shown that bFGF promotes the proliferation of corneal epithelial cells and improves the quality of corneal wound healing (Assouline et al., 1989; Rieck et al., 1992). bFGF facilitates the recovery of corneal epithelial cells, shortens the closure time for corneal epithelial wounds, increases the density of corneal epithelial cells, and accelerates the restoration of corneal epithelial cells (Rieck et al., 1993; Imanishi et al., 2000).

In our canine model of epithelial wound healing, the administration of bFGF significantly shortened the wound closure time compared to the control group. Histologically, the wounds in the bFGF-treated groups were significantly smaller from Day 1 onward; this accelerated wound closure started immediately after wounding. Sabatier (Sabatier et al., 1996) investigated the wound closure time in human models with the same results. Cell proliferation and stratification, causing the re-establishment of multicellular layers, are important continuous phases in the healing of epithelial wounds (Lu et al., 2001). Because cell division was stimulated marginally by bFGF, bFGF-induced cell migration must be the most important factor in wound healing in the bFGF-treated groups. The migrating cells appeared to move into the wound area as one group, a behavior also called "spreading" (Sabatier et al., 1996). A few cells moved individually into the

wound area. The migration process may include both faster migration and increased numbers of migrating cells. The shorter wound closure time in the bFGF-treated group strongly suggests that there is a faster migration rate of cells near the wound boundary.

After corneal wounding, bFGF enhances the repair of the stroma, reduces the degree of the inflammatory reaction, and maintains cornea transparency (Andreson and Banks, 1982). We observed that the extent of conjunctival congestion and corneal bedewing in the bFGF-treated groups was lower than in the control group. The local administration of bFGF appears to reduce the inflammatory reaction in the cornea. In the bFGF-treated groups, the leukocyte infiltrate was reduced on histological examination and there were fewer inflammatory cells than in the control group. The possible roles of bFGF in reducing the inflammatory cell infiltration include (1) protecting the antero-stroma via accelerated healing of the corneal wound (Rieck et al., 1994), and (2) altering the inflammatory response (Ohtani et al., 1993). bFGF inhibits the proliferation of lymphocytes in response to IL-1 and IL-2 and the production of cytokines, and reduces antigen II expression.

This study also clearly demonstrated that the topical application of bFGF accelerated epithelial healing when the concentration exceeded 0.1 µg/ml. Kitazawa (Kitazawa et al., 1990) reported that the effect of bFGF on promoting wound healing was dose-dependent. In our experiment, however, there were few differences between the BFGF0.5 and BFGF1.0 groups. The facts that (1) a concentration more than 50 µg/ml did not further accelerate healing and (2) Ho (1974) found that using 50–2000 µg/ml growth factor did not produce a dose-dependent response implies that downregulation occurs with *in vivo* topical application. We did not detect conspicuous vascularization in the cornea with the short-term use of bFGF. A further study should examine whether the long-term use of high bFGF doses leads to angiogenesis.

In summary, bFGF effectively accelerated the proliferation of canine corneal epithelial cells and wound healing. The cornea is vulnerable to damage. Although this study demonstrated the potential of bFGF as a therapeutic agent for traumatized corneal endothelium, the pharmacokinetics and toxicology of bFGF eyedrops in the canine eye requires further study. As bFGF is a basic peptide, it is sensitive to heat or acid. In addition, the optimal

therapeutic dose and treatment methods also must be investigated thoroughly.

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