·Basic Research·

Angiogenesis induced by micropocket assay on rat cornea

Wei Zhao[1], *Ai-Hua Jiang*[2], *Zu-Guo Liu*[2]

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[1]Department of Ophthalmology, Guangzhou Children's Hospital, Guangzhou 520120, Guangdong Province, China

[2]Eye Institute of Xiamen University, Xiamen 361005, Fujian Province, China

Correspondence to: Wei Zhao. Department of Ophthalmology, Guangzhou Children's Hospital, Guangzhou 520120, Guangdong Province, China.zhaowei7163@yahoo.com.cn

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Abstract

• AIM: To explore the skills and characteristics of corneal neovascular model in rat induced by micropocket assay.

• METHODS: Nine eyes of nine Sprague-Dawley rats were studied. Pellets made of vascular endothelial growth factor (VEGF), poly-2-hydroxylethylmethacrylate and sucralfate were implanted into the corneal stroma no closer than 1mm from the limbus. Biomicroscopic features of corneal neovascular were observed on 1, 3, 5, 7th day after the implantation.

• RESULTS: On day 1 after operation, the limbal vessels were dilated, with no angiogenesis appeared. On day 3, angiogenesis began to invade pericornea with a brush shape, the area of CNV was (2.23±0.59)mm^2. On day 5, new vessels reached the lower margin of pellet densely, and the area of CNV was (6.81±1.35)mm^2. On day 7, new vessels continued to elongate, parts of them extended as loops toward the pellet, and the area of CNV was (8.92 ±1.79)mm^2. Neither hyphema or other complications occurred.

• CONCLUSION: Corneal neovascularization induced by micropocket assay in rat grows steadily without complication, and is suitable for quantitative researches.

• KEYWORDS: angiogenesis; micropocket assay; vascular endothelial growth factor

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INTRODUCTION

Corneal neovascularization (CNV), abnormal formation of blood vessels in the cornea, is a common and serious complication of many corneal diseases. The condition is associated with severe visual impairment and is a high risk factor for graft rejection after allograft corneal transplantation [1,2]. Corneal angiogenesis model is the most important means for researching the CNV mechanism and evaluating the treatment. There are several CNV models at present that have been described, including intrastromal suturing [3], chemical or thermal injury [4], and micropocket assay [5]. Among these models, the micropocket assay is unique because it is dependent on direct stimulation of blood vessels rather than on indirect stimulation by the induction of inflammation. The aim of this study was to evaluate whether the model is superior for angiogenesis study, and explore the skills and characteristics of corneal neovascular model in rat induced by micropocket assay.

MATERIALS AND METHODS

Animals Nine SD rats(female; 8-10 weeks of age; approximate weight, 160g) were used for the study. All animals were maintained and treated in accordance with the Association for Research in Vision and Ophthalmology Statement for the use of Animals in Ophthalmic and Vision Research. The animals were examined and found to have no ophthalmic diseases before pellet implantation.

Corneal Neovascularization Model

Pellet preparation Pellets were prepared according to the method previously described [5-7]. Sterile casting solution was prepared by dissolving the Hydron polymer (Hydron, Sigma Co. USA) powder in absolute ethanol (12% w/v) at 37℃ with continuous stirring overnight. Sucralfate (Sigma Co, USA) stock solution was prepared by suspending Sucralfate in sterile PBS at 100mg/mL. An equal volume of Hydron and sucralfate were combined. Each pellet for the corneal pocket assay contained 160ng of vascular endothelial growth factor (VEGF) (recombinant rat VEGF, R&D systems) in 3μL of casting gel. This solution was pipetted on a sterile

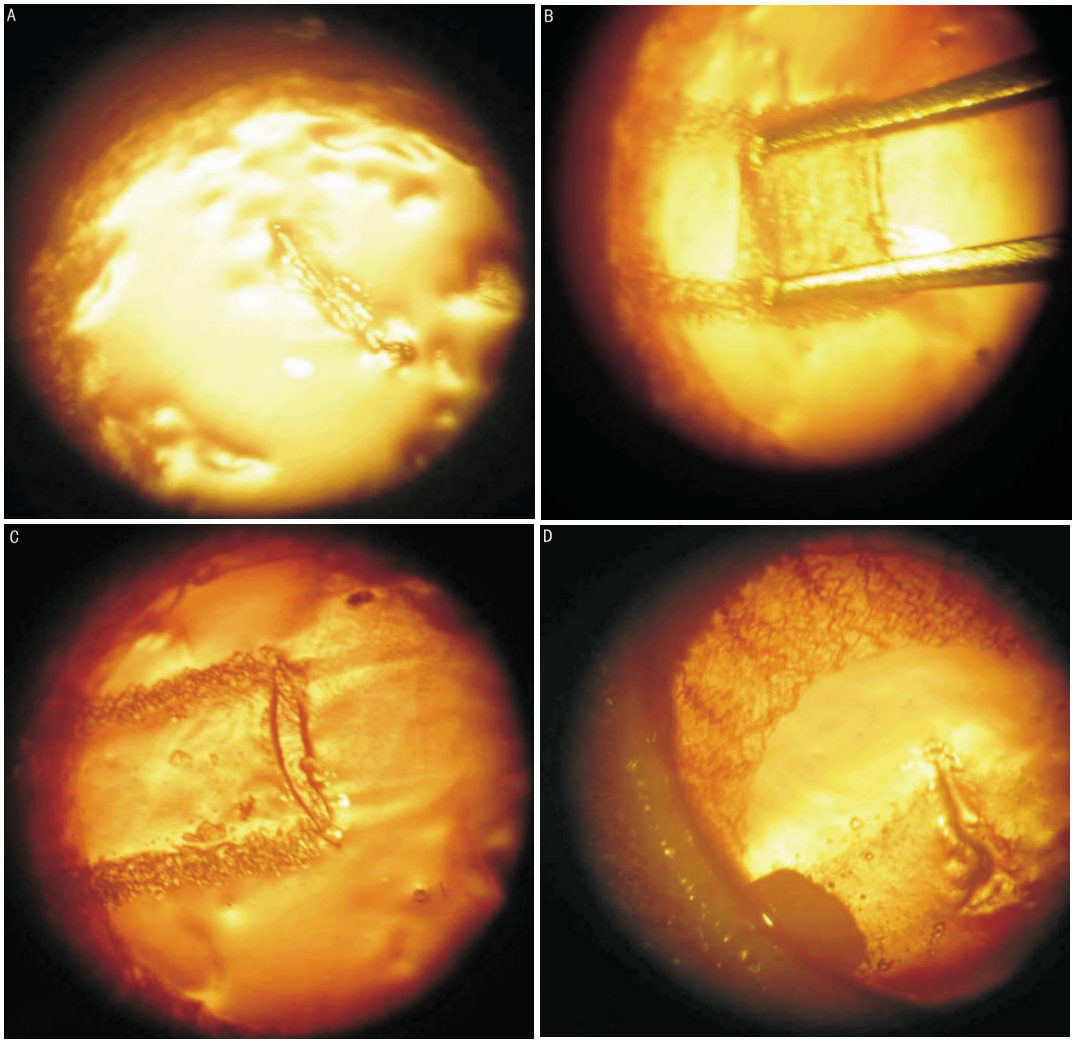

Figure 1 Surgical procedure for pellet implantation into the rat corneal stroma A: A1.5mm incision was made at the center of the cornea;B:A microforceps was inserted under the lip of the incision and gently blunt-dissected through the stroma;C:Completed corneal micropocket;D:Pellet was positioned at the base of the pocket

teflon. After drying at room temperature for 1 to 2 hours in a sterile environment the pellets were stored at 4℃.

Pellet implantation Pellets were implanted into rat corneas as described previously with some modifications [5-7]. Following anesthesia with an combined intraperitoneal injection of katamine (1mL/kg) and chlorpromazine (1mL/kg), a rat was placed under a surgical microscope, and a drop of Tetracaine was applied on the cornea of one eye. A 1.5mm superficial incision was made at the cornea center with a discission needle, and a micropocket was created by bluntly separating the lamella of the stroma toward the limbus with a microforceps. The distance between the bottom of the micropocket and the limbus was 1.0/1.5mm. A prepared sterile Hydron pellet was rehydrated with a drop of sterile saline and placed into the corneal micropocket (Figure 1). The cornea was covered with tobramycin ophthalmic ointment after surgery.

Quantification of CNV Area Rats were examined under a slit-lamp microscope on 1, 3, 5, and 7th day after implantation of pellet. Both the length and clocks of CNV were measured. The formula below was used to determine the area of neovascularization as described before[8].

$$A=\sum_{i=1}^{n}=13.1416\times C\,i\times(R^2-(R-L)^2)/12$$

C: the clocks of CNV, L : the length of CNV, R: measured radius of the rat cornea. Ten rats were randomly selected to determine the R value as 7mm.

Statistical Analysis All statistical analysis were done using SPSS 10.0, $P<0.05$ was considered to be statistically significant. The area of CNV was expressed by mean±s and variables of different days were tested for statistical significance using One-Way ANOVA test.

RESULTS

Morphology of CNV The response was a vasodilation of pericorneal vessels on the 1st day after surgery. Then the

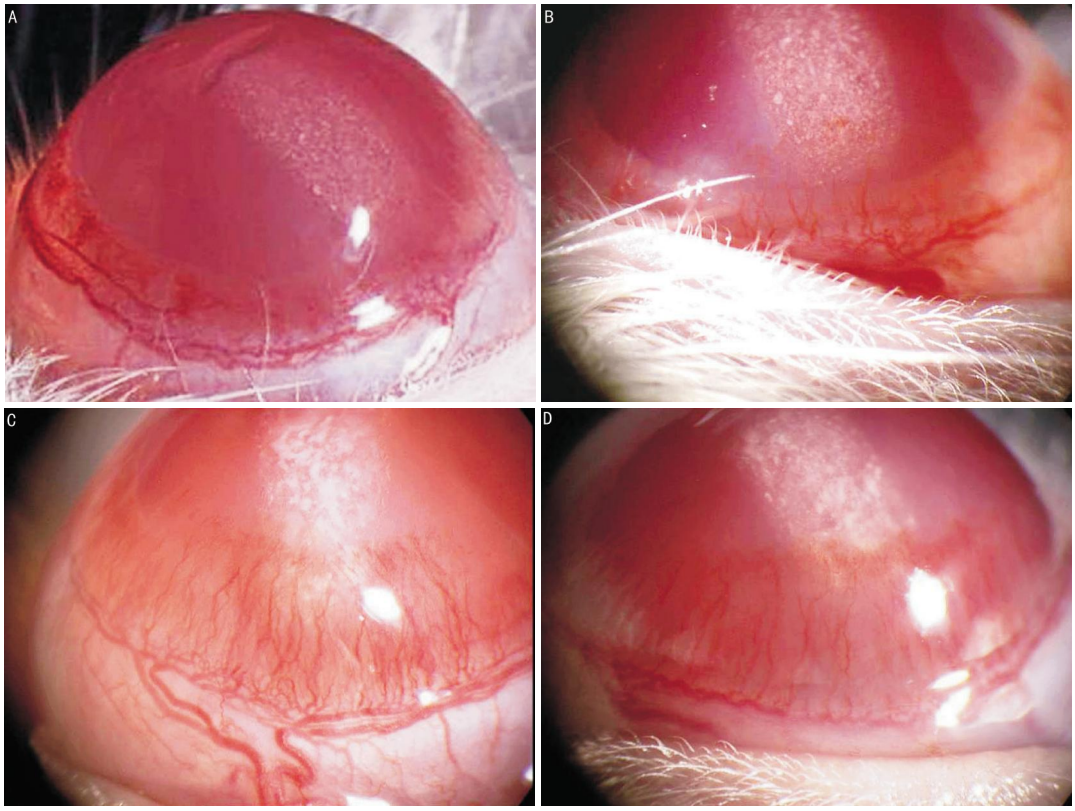

Figure 2 Slit lamp photographs of rat corneal neovascularization A: lst day after implantation with VEGF pellet; B: 3rd day after implantation with VEGF pellet; C: 5th day after implantation with VEGF pellet; D: 7th day after implantation with VEGF pellet

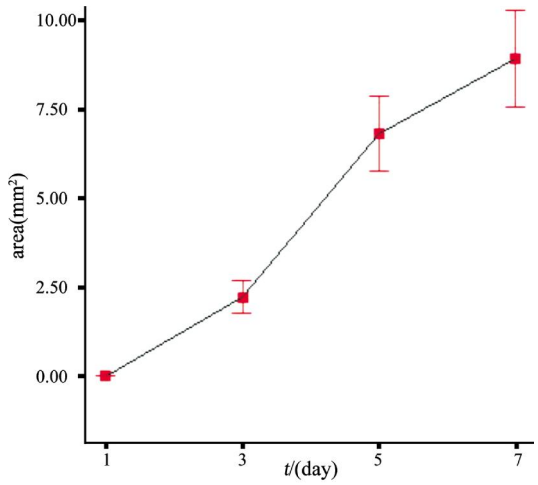

Figure 3 Time course of CNV area after implantation with VEGF pellet

new vascular buds emerged from the pericorneal venules and capillaries on 3rd day after micropellet implantation as brush. On 5th day, these sprouts lengthened and multiplied to produce a rich anastomosing plexus, directional nature of them toward central cornea and micropellet. Channels growed continually and reached the underside of pellet by 7 days, and part of these vessels inosculated as looping channels (Figure 2).

Area of CNV On day 1 after operation, the limbal vessels were dilated, with no angiogenesis appeared. On day 3, angiogenesis began to invade pericornea with a brush shape, the area of CNV was $(2.23 \pm 0.59)mm^2$. On day 5, new vessels reached the lower margin of pellet densely, and the area of CNV was $(6.81 \pm 1.35)mm^2$. On day 7, new vessels continued to elongate, parts of them extended as loops toward the pellet, and the area of CNV was $(8.92 \pm 1.79)mm^2$. However, there was no significant difference in vessel areas of day 5 and day 7 (P=0.075>0.05,Figure 3).

Complications No corneal ulcer, hyphema and other complications occurred.

DISCUSSION

CNV model is the most important means to study the CNV mechanism and evaluate the treatment. CNV can be induced by physical, chemical, and biologic means artificially. Alkali burn model is adopted widely in angiogenesis research in our country. The mechanism by which NV occurs in this model is similar to that of inflammatory corneal disease [4]. However, this model has been criticized for the inability to exclude inflammation as the predominant neovascular stimulus. The micropocket assay is a recently developed CNV model which is dependent on direct stimulation of blood vessels

rather than on indirect stimulation by the induction of inflammation. In the study, we prepared and implanted pellets containing VEGF into rat corneas, and found the angiogenic response is reproducible and reliable. In addition, in contrast with other reports [5,6], we improved the preparation of micropocket. A 1.5mm superficial incision was made at the cornea center with a discission needle, and a micropocket was created by bluntly separating the lamella of the stroma toward the limbus with a microforceps instead of a dissector. This technique made fixation at limbus with forceps unnecessarily, reduced the separation resistance, avoided intrabulbar haemorrhage and other injury during the operation. Thus, the improved micropocket assay is more convenient and reliable.

In our study, the area of neovascularization was measured after VEGF pellet implantation for statistically analyzed from postoperative day 1 to 7. Vessels were first noted on postoperative day 3. As progressed, the areas of the vessels gradually increased. This is in agreement with the observation of Kenyon [9] that neovascularization induced by bFGF began on day 3 and was sustained through to day 8. These results showed that VEGF has the potential to be used in angiogenic studies, as an angiogenic inducer. VEGF is a 34 to 42kDa, dimeric, disulfide-bound glycoprotein, and it is one of the most important proangiogenic factors for controlling angiogenesis [10]. The effects of VEGF are mediated by binding to VEGFR-1 and VEGFR-2, receptor tyrosine kinases expressed on the blood vascular endothelium [11]. VEGF may modulate angiogenesis by different mechanisms: it facilitates proliferation, migration and sprouting of endothelial cells [12-14]; it has the ability to induce vascular leakage, which would result in the formation of an extravascular fibrin gel, a substrate for endothelial cell growth [15,16]; it may promote chemotaxis of monocyte and myoctye during the construction of new vessels [17]; it acts as para-secretion medium, which in turn promote other growth factors[18]; and etc.

We think this straightforward and reproducible model of angiogenesis offers distinct advantages and affords new applications. Because the neovascular stimulus is induced directly by an angiogenic factor, the efficacy of specific angiogenesis inhibitors can be monitored easily. Serial observations and measurements of the localized sector of neovascular response, or inhibition thereof, can be readily and noninvasively documented with slit lamp biomicroscopy. Furthermore, the rat model is especially advantageous given the low cost of animal maintenance. In conclusion, micropocket assay is a perfect CNV model and will be applied widely in future.

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