A Model of Retinal Ischemia-Reperfusion Injury in Rats by Subconjunctival Injection of Endothelin-1

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The retinal ischemia-reperfusion model is used in the study of transient ischemia–related diseases, such as central retinal artery occlusion, angle-closure glaucoma, and others. There are two methods for experimentally producing an ischemia-reperfusion model in the rat retina: (i) the intraocular pressure is greatly raised by increasing the height of the infusion bottle connected with the needle in the anterior chamber; or (ii) the blood vessel that accompanies the optic nerve in retina is ligated. However, each method has some drawbacks. For example, in the first method, the needle must be fixed in the anterior chamber for 1 hr, thus, the technique is not stable and mechanical damage to ocular structures sometimes occurs. In the second method, because of the unavoidable involvement of the optic nerve, damage to the nerve induces retinal changes unrelated to ischemia. In this study, we injected endothelin (ET)-1 under the conjunctiva of the eyeball (subconjunctival injection), and evaluated whether a retinal ischemia-reperfusion model could be generated by this method, simply and noninvasively. We injected 4×10^{-5} *M* ET-1 solution into the right eye of the rat and injected a control vehicle (artificial tears) into the left eye. From 5–60 mins after the injection, 50 mg/ml fluorescein isothiocyanate (FITC)-dextran was injected to the left ventricle of heart. Then, the retina was removed and flat mounted. We compared the perfusion conditions of the FITC-dextran to each retina in the right and left eye. There was a complete perfusion of FITC-dextran in the retinal main artery, vein, and the capillary vessels in all of the control eyes. However, perfusion could not be completely observed in the ET-1 injected eye from 5–35 mins after injection; afterwards, the flow was returned. This method of subconjunctival injection of ET-1 is, thus, a feasible technical option for producing a retinal ischemia-reperfusion model in rat.
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Key words: ischemia-reperfusion injury; endothelin-1; subconjunctival injection; retina; rat

Introduction

The retinal ischemia-reperfusion animal model is used in the study of transient ischemia–related diseases, such as central retinal artery occlusion, angle-closure glaucoma, and carotid artery disease. Various methods have been used to create a model of retinal ischemia-reperfusion. The two representative methods for producing a model of retinal ischemia reperfusion already reported are (i) raising the intraocular pressure above the systolic arterial blood pressure through cannulation of the eye (1–11); and (ii) ligation of the central retinal artery together with the optic nerve (1, 12–15). However, both of these methods have drawbacks and limitations (14, 16). For example, the first method is an invasive procedure requiring the penetration of a needle through the cornea, and the needle must be fixed in the anterior chamber for approximately 1 hr; thus, this method is not stable. In addition, the invasiveness of this method often causes inflammation and mechanical damage to ocular structures. The second of these two techniques unavoidably involves the optic nerve, thus, damage to the nerve, as in nerve crush, can induce retinal changes unrelated to ischemia.

Both of these representative methods consequentially create the transient obstruction of the central retinal artery. Therefore, we hypothesized that a similar effect might be

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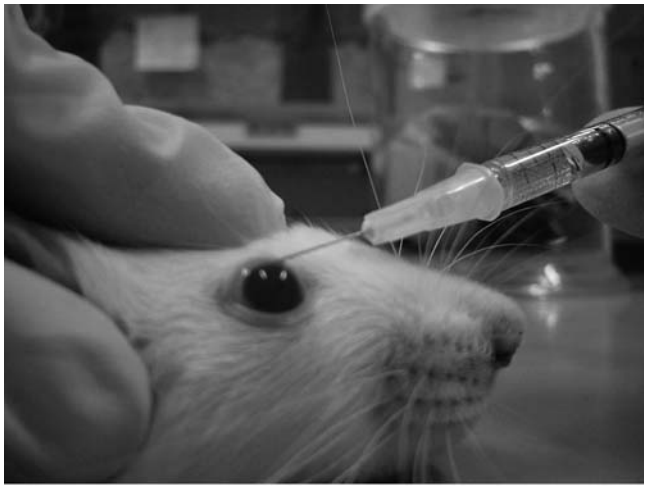
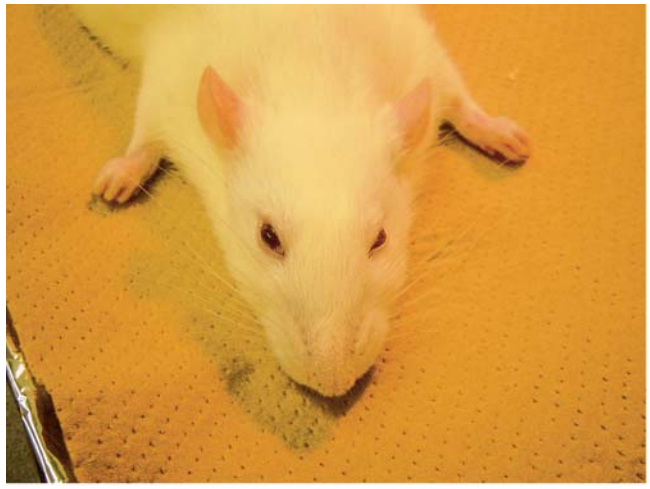


Figure 1. Subconjunctival injection of ET-1. After general and local anesthesia, we injected 4×10^{-5} *M* ET-1 solution in the subconjunctival space of the rat eye (top). After the subconjunctival injection, the presence of ET-1 or vehicle solution in the ocular circumference (indicated by black arrow) was seen for approximately 1 hr, similar to conjunctival chemosis (bottom).



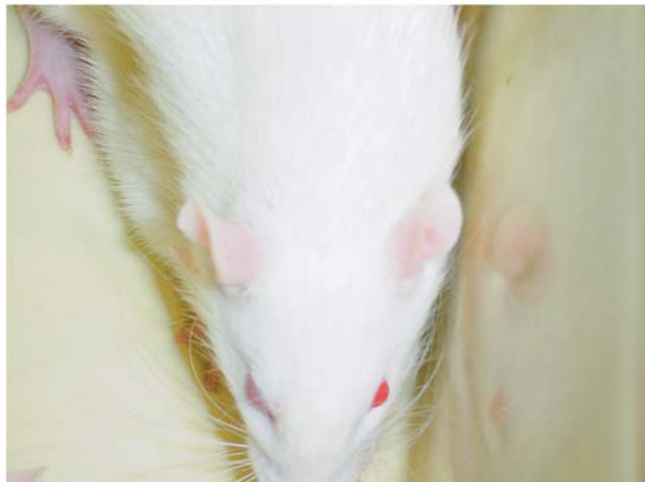


Figure 2. Five minutes after the subconjunctival injection, the ET-1–injected right eye was relatively dark compared with the vehicle-injected left eye (top). This difference was highlighted using a flashlight (bottom).

obtained if a vasoconstrictive drug was injected in the circumference of the central retinal artery. In this study, we propose a new method to produce a rat model of ischemia reperfusion that is less invasive, is simple, and does not require special equipment for the induction of retinal ischemia in the rat. We injected endothelin (ET)-1, a potent vasoconstrictive peptide, under the conjunctiva of the eyeball (subconjunctival injection) to occlude the central retinal artery and evaluated whether the retinal ischemia could be obtained using fluorescein isothiocyanate (FITC)-dextran angiography. In the preliminary study, in the retina, 30 mins after the subconjunctival ET-1 injection, retinal perfusion could not be completely observed. Therefore, we think that if the degree of the ischemia and duration of the ischemia are both appropriate, an ischemia-reperfusion model can be generated. Thus, in the present study, we propose a new, simple, and noninvasive method for producing a retinal ischemia-reperfusion injury animal model.

Materials and Methods

Animals. Male Sprague-Dawley rats weighing 250–300 g (Charles River Japan, Inc., Yokohama, Japan) were used. The experimental protocols were approved by the Committee on Animal Research at the University of Tsukuba, Tsukuba, Japan, and conformed to the Association for Research in Vision and Ophthalmology resolution on the use of animals in research.

Subconjunctival Injection of ET-1. Rats were anesthetized with an intraperitoneal injection of 50 mg/kg pentobarbital sodium for general anesthesia and with 0.4% oxybuprocaine hydrochloride eyedrops (Santen Seiyaku, Osaka, Japan) for local anesthesia. Then, we injected 0.3 ml of 4×10^{-5} *M* ET-1 solution into the subconjunctival space of the right eye using a 30-gauge needle. We injected ET-1 in the posterior part of the eyeball (at least behind the

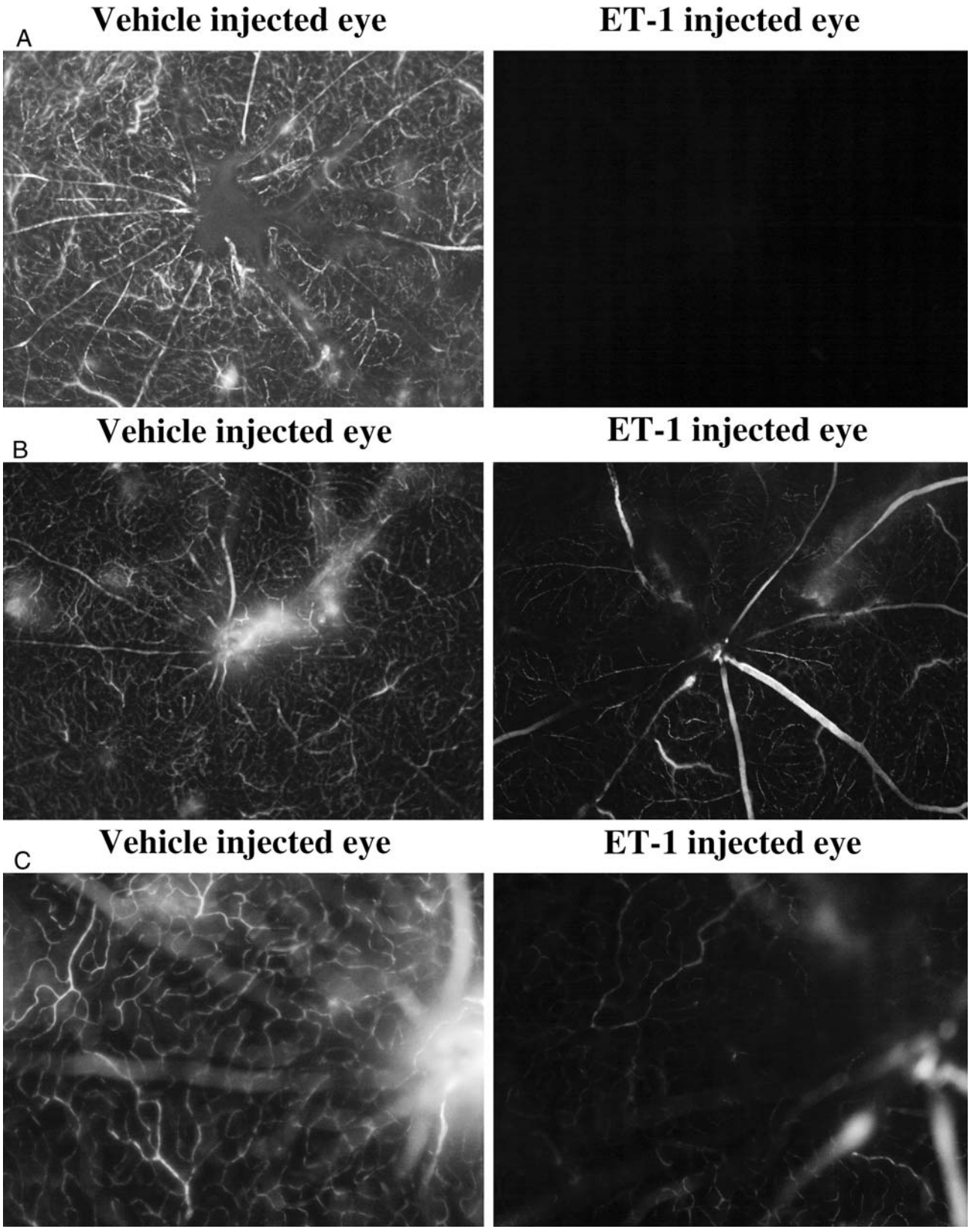


Figure 3. FITC-dextran angiography of the retina. (A) Thirty minutes after injection. There was a complete perfusion of FITC-dextran in the retinal main artery, vein, and the capillary vessels in the control eyes (left). However, perfusion could not be completely observed in the ET-1–injected eye (right). (B) Sixty minutes after injection. Again, there was a complete perfusion of FITC-dextran in the control eye (left). Similar to the control eye, the blood flow has been resumed in the ET-1–injected eye (right). (C) However, the fluorescein intensity was relatively low and the perfusion was not yet normalized in the ET-1–injected eye (right) at the capillary level after 60 mins of injection.

equator) at our best possible level so that the solution could reach the central retinal artery circumference (Fig. 1). The left eye was injected similarly with artificial tears and was considered as a control. The preliminary judgment of the retinal ischemia was confirmed by a pale color of the eyeball (Fig. 2).

FITC-Dextran Angiography. The chest cavities of the rats were opened between 5 and 60 mins after ET-1 injection and perfused *via* an intracardiac route with 50 mg/ml FITC-dextran (Sigma Chemical Co., St. Louis, MO), and eyes were immediately enucleated. The enucleated eyes were fixed briefly in 10% buffer formalin for 15 mins. Retinas were dissected free from the choroid and sclera, and flat mounted on a glass slide in a mounting medium for fluorescence. Fluorescein-perfused sections were visualized with fluorescence microscopy (PROVIS AX70; Olympus, Tokyo, Japan). We compared the perfusion conditions of the FITC-dextran between each retina in the right and left eye.

Results

There was a complete perfusion of FITC-dextran in the retinal main artery, vein, and the capillary vessels in all of the control eyes. However, perfusion could not be completely observed in the ET-1–injected eye between 5 and 35 mins after ET-1 injection (data not shown). Figure 3 shows representative photographs of the retina 30 mins (Fig. 3A) and 60 mins (Fig. 3B) after ET-1 or vehicle injection. Thirty minutes after the injection of ET-1, perfusion in the retina was not complete (Fig. 3A). However, perfusion in the ET-1 injected eye resumed 60 mins after the ET-1 injection (Fig. 3B). However, at 60 mins after the ET-1 injection, the perfusion style was slightly different at the capillary level (relatively low fluorescein intensity was detected); the perfusion status at capillary level was not yet completely resumed (Fig. 3C).

Discussion

In the present study, we achieved an obstruction of 30 mins of the central retinal artery by the subconjunctival injection of ET-1. The former representative methods for producing retinal ischemia-reperfusion injury models are to ligate the central retinal artery with the optic nerve or to raise the intraocular pressure to create a transient obstruction of the retinal artery. In the present study, we demonstrated a similar type of occlusion of the retinal blood flow as evident in the results of the retinal angiography.

The important point is the pattern of the vascular obstruction. In the clinical field, central retinal arterial obstruction is observed, for example because of thrombus formation in the vessels and, in such a case, only the central retinal artery is obstructed. The method introduced in the present study also exhibited the obstruction of the central retinal artery without any damage to other tissues, such as the optic nerve. In the anatomic concept, all of the retinal nerve fibers finally join the optic nerve. Thus, retinal constitutions are closely linked to the optic nerve. Therefore, maintaining the anatomical structure of the optic nerve is crucial for retinal experiments. The method of ligating the central retinal artery unavoidably involves the optic nerve; this drawback adds extra merit to the method we proposed in the present study.

The less invasive or noninvasive quality and the technical simplicity of the method introduced in the present study are beneficial for the ocular structure. The method of raising the intraocular pressure requires the penetration of the cornea and even if it is only a small penetration of the cornea, the perforation between the anterior chamber and the outside of the eye causes infection and inflammation that may affect the retina. In addition, continuous monitoring is required to fix the needle for 1 hr in the anterior chamber. Thus, to prepare a single rat model of retinal ischemia reperfusion by this method is expensive. Moreover, a microscope is required to fix the needle in the anterior chamber. Our present method does not need either special equipment or extraordinary facilities, the subconjunctival injection can easily be performed without special equipment, such as a microscope. After the injection is performed, no special care or attention is needed. Moreover, because the conjunctiva and the subconjunctival space are not intraocular structures, serious inflammation or infection rarely occurs.

Although the method used in the present study to produce the model of ischemia-reperfusion injury of the retina has many advantages, it may not be completely free from some drawbacks. The dose of ET-1 used in the present study is quite high, and this high dose of ET-1 may pass into the systemic circulation, where it may exert some systemic effects. It may cause a number of biologic and physiologic effects in other organs and tissues. Although these effects may not contribute much to the retina itself, it would be preferable to monitor systemic indices, such as blood pressure, pulse rate, and organ perfusion.

In many of the representative models to date, the duration of the retinal ischemia is approximately 1 hr. From the results of the retinal angiography, our model does not produce occlusion for 1 hr. Therefore, we must confirm whether a complete 30-min obstruction might be good enough for the retinal ischemia-reperfusion injury model, as demonstrated in the present study. To further validate this model, the retina should be subjected to intensive histopathologic and morphologic examinations, in a time-dependent manner.

In the present study, we achieved transient and complete obstruction of the central retinal artery after the subconjunctival injection of ET-1. This method seems to be a feasible technical option for producing a retinal ischemia-reperfusion injury model in rat.

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