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22

Induction of Branch Retinal Vein Occlusion by Photodynamic Therapy with Rose Bengal in a Rabbit Model

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1. Introduction

Branch retinal vein occlusion (BRVO) is a common vascular disorder that is frequently associated with severe vision loss due to its complications. There are two forms of BRVO, the ischaemic and the non-ischemic, both leading to loss of visual acuity. About 20-30% of the BRVOS are ischemic and the majority of them develop secondary glaucoma and rubeosis. [1] Although this disorder was first reported by J. von Michel in 1878, its pathogenesis has still not been fully understood, and no effective therapy has been developed for this disorder [1-3]. Comparative research on this disorder is based on clinical models of BRVO.

The pathological features of BRVO can be more accurately reproduced by a photodynamic method to induce thrombosis in retinal vessels[4–14] than by using other methods such as laser coagulation[15–17], electrocoagulation of the retinal vein via the vitreous body[18], intravenous thrombin instillation[19–21] and endothelin-I injection into the posterior vitreous body[22, 23]. In the photodynamic method, a photosensitizing agent, namely, rose Bengal, is injected intravenously. This dye absorbs light maximally at a specific wavelength of 550 nm. Dye activation in the presence of molecular oxygen leads to the generation of singlet oxygen, which in turn acts locally to injure or destroy the vascular endothelium. The altered endothelium provides a surface for platelet aggregation and subsequent thrombosis. By using this method, thrombi can be produced in vessels. Because relatively low light intensities are required to initiate this process, the thermal effects on the surrounding tissues are minimized.

In this investigation, we used a Xenon arc lamp as the light source, and visible light was transmitted through an intraocular fiber to induce BRVO in pigmented rabbits. The characteristics of BRVO were evaluated quantitatively, and histological studies were performed to support the clinical findings. The results provided a framework for future applications of this technique.

2. Materials and methods

2.1 Animals and husbandry

Thirty-two male Standard Chinchilla rabbits were purchased from the Experimental Animal Center of Tianjin Medical University (Tianjin, China) and allowed to acclimate upon arrival

for 10 d before the initiation of the experiment. At the beginning of the study, the animals were aged 3–4 months and weighed 2.5–3.5 kg. All experimental animals were housed in individual cages under stable environmental conditions: diet, commercial pellet diet (Lushifu, Beijing, China) and tap water ad libitum; relative humidity, 45–55%; temperature, 18°C; and light-dark cycle, 12/12 h (intensity, 300 lux).

The study was approved by the Ethics Committee of Tianjin medical university. All animal experiments were performed in accordance with the conventions of the Association for Research in Vision and Ophthalmology (ARVO) for ethics in animal experimentation.

2.2 Experimental design

The 32 rabbits (32 eyes, 32 vessels) were randomly divided into 15 treatment groups (n = 2) and 1 control group (n = 2). The treatment groups were administered 5 different rose Bengal doses (3, 6, 9, 12 and 15 mg/kg) and exposed to light of 3 different intensities (600, 1000 and 1400 lux) 1 min later. In the control group rabbits, the vessels were exposed to a light intensity of 1400 lux for 20 min without injection of the rose Bengal dye.

2.3 Experimental procedure

In preparation for the treatment and follow-up examinations, the rabbits were fully anesthetized with an intramuscular injection of 60 mg/kg ketamine (Beijing Shuanghe Pharmaceuticals, Beijing, China) and 8 mg/kg xylazine (Beijing Shuanghe Pharmaceuticals, Beijing, China). The pupils were dilated using 0.5% tropicamide eye drops (Beijing Shuanghe Pharmaceuticals, Beijing, China).

Rose Bengal (tetrachloro-tetraiodo-fluorescein sodium; certified purity, 90%; Sigma, St. Louis, MO) was dissolved in normal saline (30 mg/ml), sterilized by passage through a 0.22-μm filter and injected intravenously in doses of 3, 6, 9, 12 or 15 mg/kg before the light treatment. A Xenon arc lamp (Anshijia Instrument Co. Ltd., Beijing, China) was used as a light source, and light was transmitted by using a 0.2-mm intraocular illumination fiber (Alcon (China) Ophthalmic Product Co. Ltd., Shanghai, China). Light intensity was measured using a digital lux meter (LX-9621; Landtek Instrument Co. Ltd., Guangzhou, China). Average values of 600, 1000 and 1400 lux, for the low, medium and high settings, respectively, were obtained with less than 3% variation in repeated measurements throughout the study.

According to the treatment protocol, the rabbits were placed on a stage under a surgical microscope (OPTON Universal S3; Germany). 2% methylcellulose solution (Zhengda Co. Ltd., Shandong, China) is dropped on the cornea to couple the input light into the 30-degree prism lens (Anshijia Instrument Co. Ltd., Beijing, China). The operations were observed by using a contact lens and a surgical microscope. A 25-gauge trocar (Alcon (China) Ophthalmic Product Co. Ltd., Shanghai, China) was used to make an incisions in the conjunctiva and sclera at the projection holes located supratemporally 3 mm from the corneal limbus, then the needle was removed and the intraocular fiber was inserted through the incision and its direction was toward optic disc, the depth was 10 mm. Rose Bengal was injected intravenously through the marginal ear vein. After 1 min, the trunk vessels adjacent to the optic disc of 1~ 3 mm were exposed to a beam of white light. The duration of continuous exposure to light did not exceed 20 min, and the exposure time was recorded if complete vascular occlusion was observed. The fiber finally was then taken out and the sclera was finally sutured.

Fundus fluorescein angiography (FFA) was performed using a fundus camera (Zeiss FF450IR; Carl Zeiss Far East Co. Ltd., Beijing, China) before the treatment and everyday after the treatment until the occluded vein reopened. The 5 mg aqueous fluorescein sodium (100 mg/ml, Baiyunshan Pharmaceuticals Co. Ltd., Guangzhou, China) was injected into ear veins immediately prior to FFA under anesthesia and pupil dilation those were as same as the prior.

2.4 Experimental observation

The rate and duration of vessel occlusion are confirmed with an ophthalmoscope and by FFA. The blood flow was confirmed by a scleral depressor transiently applied pressure on the equator of the eyeball, thereby the higher intraocular pressure (IOP) reduced the flow rate until freely moving red blood cells were seen. Once the scleral depressor was removed, a rapid uninterrupted column of blood flow passed through without the presence of stagnant red blood cells. [9] These dynamic phenomenons were monitored by an ophthalmoscope and it was the character of reperfused vessels.

When reperfusion was noted on post-treatment day 1, the number of days of occlusion was defined as 0. When the vessel was observed to be occluded on day 1 but not on day 2, the number of days of occlusion was defined as 1 and so on.

Two rabbits in the control group and 1 rabbit in the treatment groups in whom BRVO was confirmed by FFA were euthanized using intravenous overdose of pentobarbital sodium (Beijing Shuanghe Pharmaceuticals, Beijing, China). Their eyes were enucleated and prepared for light microscopic examination by using standard techniques. Sections (5-μm-thick) were stained with haematoxylin/eosin. The remaining rabbits were euthanized by administering an overdose of pentobarbital sodium at the end of the experiment.

2.5 Statistical analysis

A partial correlation coefficient was used to analyze the relationship between the time of thrombosis, the drug dose, and light intensity. A P value of 0.05 or less was considered statistically significant. All data were analysed with the SPSS 11.5 software.

3. Results

3.1 Evaluation of BRVO animal model

BRVO models were developed in 22 of the 30 rabbits in the treatment group. The fundus could be observed in detail by using a surgical microscope (please see supplemental video 1). In the site exposed to light, photodynamic injury resulted in white debris (plaque-like thrombi) and the stagnation of blood flow. FFA showed the engorged distal ends of the venules proximal to the occlusion site and peripheral retinal oedema in the BRVO area (Figure 1).

Histological sections were studied to confirm the presence and extent of the photodynamic lesions. Retinal vein damage was confined to the area of direct light exposure in the treatment groups. Histological examination revealed thrombi consisting of platelet aggregates, stagnant red blood cells and a few white blood cells. Occluded retinal vein histological section indicated that compact thrombus filled the entire vein cavity and occlusion is evident. (Figure 2).

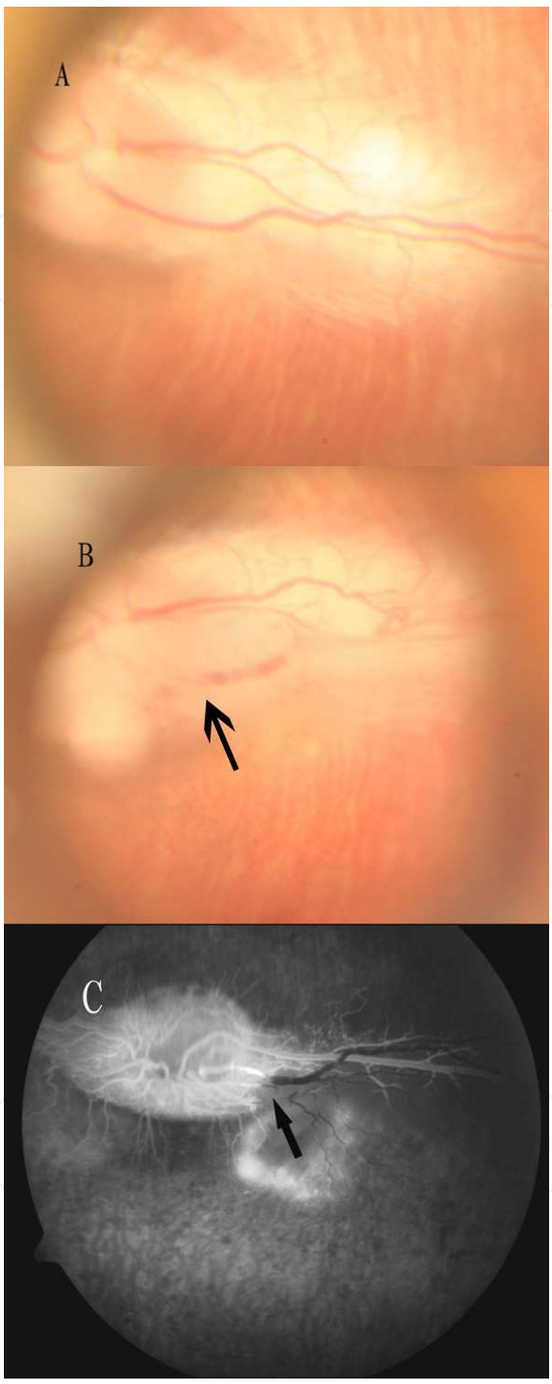

Fig. 1. **Photodynamic thrombosis of a branch retinal vein in a pigmented rabbit eye.** Initial formation of a thrombosis in the target vein (A) and complete obliteration of the vessel lumen (B). FFA showed a delay in the time required for angiosclerosis in the distal end of the vein and discontinuous intravenous blood flow in the vicinity of the optic disc (C). Arrows indicate the occlusion site and peripheral retinal oedema.

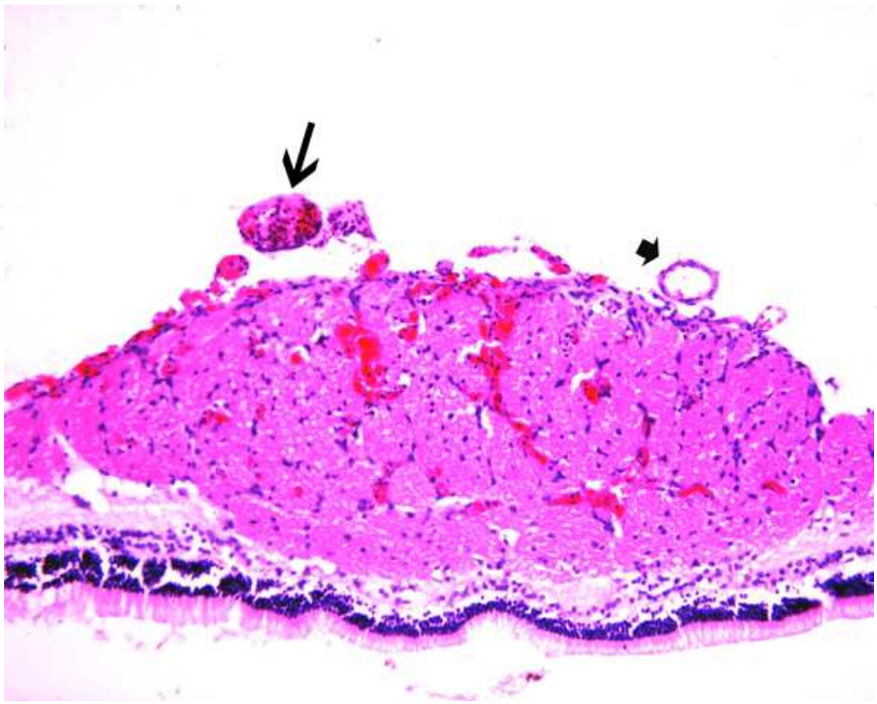

Fig. 2. **Histological findings.** Two hours after thrombosis, the thrombus filled the entire vein cavity (long arrow), and the artery was normal (short arrow). The thrombus consisted of platelet aggregates, stagnant red blood cells and a few white blood cells. Magnification, 200×.

The control vessels and the blood flow through them were as same as those in healthy animals. The histological sections from the rabbits that were not injected with rose Bengal showed no evidence of photochemical or thermal retinal damage in the site that was exposed to a light intensity of 1400 lux (maximum light intensity) for 20 min.

3.2 Dose- and light-response studies

Vein occlusion in the pigmented rabbits was considered as the end point for determining dose- and light-response relationships. In 22 successful BRVO models, the time required to produce an occlusion decreased with an increase in the dose of rose Bengal administered and the light intensity. Rose Bengal at doses of more than 9 mg/kg was effective in producing BRVO; a dose of 6 mg/kg was ineffective in producing BRVO at a light intensity of 600 lux. At a dose of 3 mg/kg, small plaques also developed in the target vessel; however, they did not progress to vascular occlusions in 20 min even if the light intensity was as high as 1400 lux. The total light energy required to produce an occlusion increased from an average of 600 lux with 9 mg/kg of rose Bengal to 1000 lux with 6 mg/kg of rose Bengal. The time of occurrence of thrombosis showed a negative correlation with the drug dose and light intensity. A decrease in the time required for the development of BRVO corresponded to a higher drug dose (partial $r = -0.7895$; $P < 0.001$) and a higher light intensity (partial $r = -0.9060$; $P < 0.001$) (Figure 3).

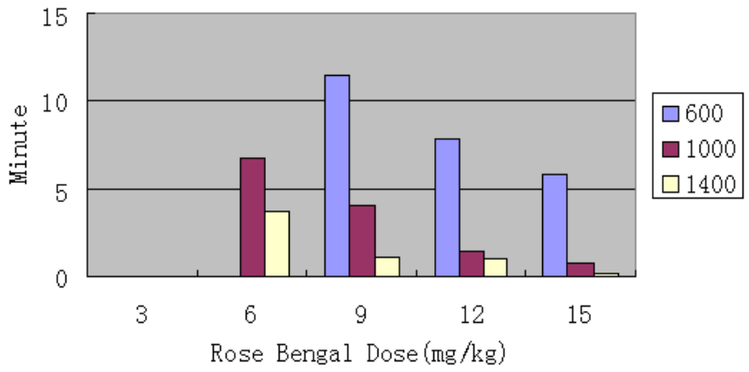

Fig. 3. **The relationship between the time required for thrombosis and the dose of rose Bengal and the light intensity.** The mean value of the time required for thrombosis was determined (n = 2). Complete BRVO formation did not occur when a dose of 3 mg/kg rose Bengal was used, and a dose of 6 mg/kg rose Bengal was ineffective in producing BRVO at a light intensity of 600 lux.

3.3 Duration of occlusion

In the early period of BRVO in the animal models, FFA and fundus photography documented the blood along the circuitous expanded vein, and retinal oedema that was similar to the lesion in humans. The area of the white thrombus gradually decreased with time, and the angiostenosis in the distal end of the retinal vein also gradually decreased. In 21 cases, the minimum duration record for the reopening of an occlusion was 3 d and the maximum, 10 d (Figure 4).

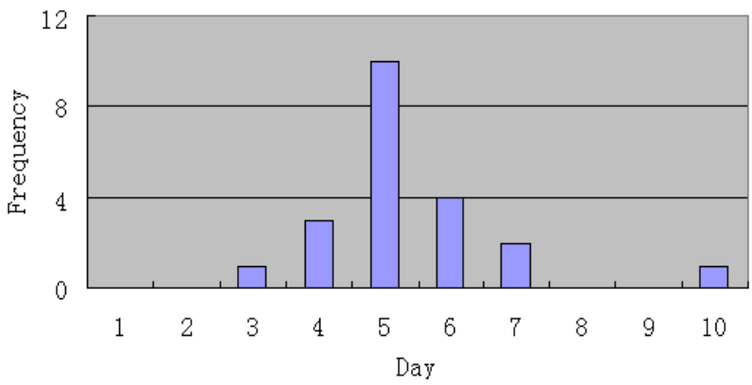

Fig. 4. **The frequency of the duration of vessel occlusion.** The duration of BRVO varied from 3 to 10 d. The data correspond to a Gaussian distribution ($P < 0.01$); the 95% confidence bound was 4.73–6.03 d, and the mean duration of vessel occlusion was 5.38 ± 1.43 d (n = 21).

4. Discussion

While developing an animal model, the following factors should be considered: the animal species used, the ease of development and reproducibility of the model and the degree of similarity of the model with the human disease. In this study, we focus on optimizing the method used to induce experimental thrombosis.

The animal species that are usually selected for the development of BRVO animal models are mouse, rat, rabbit, cat, pig, dog and monkey. Mice[14] and rats[5–7, 9] are suitable for the development of large numbers of RVO models for research because of their low price, easy availability and breeding management. Further, these models enable easy identification of the retinal artery and vein. However, the eye of mice or rats is too small to permit investigative surgical procedures. The structure of the retina in pigs[12, 15], cats[8] or dogs[4] is similar to that in humans except that it does not have a macular region. The retinal blood supply in rhesus monkeys[16, 17] is similar to that in human; however, the sources of these animals are limited, and they are difficult to breed and manage. Compared with the eyeballs of other animal species, the rabbit eyeball is sufficiently large to operate[19–23]. Further, rabbits are inexpensive, can be easily bred and the FFA of the rabbit eye is stable. Therefore, we selected pigmented rabbits for our study.

The combination of intraocular fibre illumination with the administration of rose Bengal should be classified as a photodynamic method of inducing BRVO because the wavelength of visible light include 550 nm. These findings correspond to Virchow's hypothesis of thrombosis in humans. Compared with the photodynamic method, laser coagulation[15–17] requires higher energy and a longer exposure time, which would inevitably result in heat injury and disruption of the vessel wall, and subsequently, frequent bleeding. Electrocoagulation of the retinal vein via the vitreous body[18] utilizes electrothermal energy to destroy the vascular endothelium and block the vessels, which is different from the process of thrombosis. In intravitreous thrombin instillation method[19–21], the location of the retinal thrombus could not be determined; thus, the thrombus may occlude other regions but not the target vessels. The injection of endothelin-I into the posterior vitreous body[22, 23] caused complete occlusion of the temporal retinal vessels, but this vasospastic mechanism is also unsuitable for application in the experimental induction of thrombosis because actual clinical BRVO was occluded at one or some spots in vein.

Presently, an argon (Ar) laser is often used as the excitation light source in the photodynamic method because its wavelength of 514 nm is similar to the light absorption peak of rose Bengal. However, heat injury from laser-coagulation to the retinal tissue and its vessels is observed. Moreover, due to the pulsed excitation of the laser, continuous exposure of the vessel to the laser beam is impossible. Consequently, it is difficult to adjust the level of energy used in this procedure: if the energy level is too low, no thrombus formation occurs and if it is too high, vessel hemorrhage occurs[8]. Some researchers utilize common light sources such as slit lamps and Xenon lamps to develop BRVO animal models. However, the use of these light sources resulted in an extensive arteriovenous obstruction caused due to the exposure of a large area to the light source, therefore, these light sources are not suitable for studying a typical BRVO[9, 10, 13]. In a previous study, we use a microsurgical illumination system, which comprised a 0.9-mm intraocular fibre that served as an endoilluminator to produce RVO in pigs[11]; the intraocular fibre illuminated the nearby retinal vascular target

and by localizing energy, it is able to produce a thrombus as effectively as laser irradiation. However, the diameter of the retinal trunk vein in rabbits was only 150–200 μm, and the microsurgical system was too wide to the rabbit. Therefore, in this study, we used a Xenon arc lamp as a light source and designed a 0.2 mm fiber for illumination to ensure that the light beam irradiate the trunk vein and not the accompanying artery. We find that this is a simple and reliable system for developing BRVO models in rabbits.

The process of formation of BRVO in our rabbit model can be visualized and photographed using a surgical microscope. At the site exposed to light, the following course of thrombogenesis is recognized: photodynamic injury resulted in a decrease in the rate of venous blood flow; this is followed by the appearance of stagnant red blood cells in the vessel, the gradual accumulation of white debris (plaque-like thrombi) and the development of a vascular occlusion. The distal ends of the venules are engorged proximal to the occlusion site. The morphological and histopathological changes are similar to Kohner's results[24]. No changes were observe in the light microscopic images of the control vessels; this confirmes that illumination do not produce heat injury in the retinal vessels and tissue.

The dose- and light-response studies show that the time of occurrence of thrombosis correlate negatively with the drug dose and light intensity; further, the basic set of parameters require to produce an occlusion is an average light intensity of 600 lux, a rose Bengal dose of 9 mg/kg and an exposure time of 11.5 min or an average light intensity of 1000 lux, a rose Bengal dose of 6 mg/kg and an exposure time of 6.75 min. Thus, we suggest that the optimum parameters should be 1000 lux, 9 mg/kg of rose Bengal and an exposure time of 3–5 min. This suggestion is based on the following considerations: the experimental dose of rose Bengal in this model is maintained as low as possible to prevent the leakage of rose Bengal into the vitreous humour and the anterior chamber[12]; the energy level of the light used to induce thrombosis is higher than that in the breeding conditions (300 lux); this ensures that thrombosis occur only in the area exposed to the light and not in other vessels. On the other hand, the exposure time of 3–5 min was suitable for operation. The optimization of this method may aid investigators in controlling thrombus formation more precisely.

Clinical BRVO is a disease of long duration and various types [1-3]; however, the experimental occlusion is maintained for 3 to 10 d (average, 5.38 d) in our model. This may be attributed to the use of young and healthy experimental animals, the sudden generation of an obstruction in the normal vascular bed and the activation of an autochthonous repair mechanism; all these factors result in rapid thrombolysis. Therefore, this BRVO model is very suitable for observation of immediate therapy experiment, for example, surgery; and not suitable for long-term drug treatment studies.

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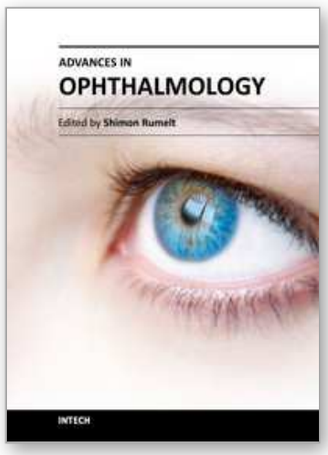

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This book focuses on the different aspects of ophthalmology - the medical science of diagnosis and treatment of eye disorders. Ophthalmology is divided into various clinical subspecialties, such as cornea, cataract, glaucoma, uveitis, retina, neuro-ophthalmology, pediatric ophthalmology, oncology, pathology, and oculoplastics. This book incorporates new developments as well as future perspectives in ophthalmology and is a balanced product between covering a wide range of diseases and expedited publication. It is intended to be the appetizer for other books to follow. Ophthalmologists, researchers, specialists, trainees, and general practitioners with an interest in ophthalmology will find this book interesting and useful.

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