이중 윤부주위둘레 봉합술을 이용한 쥐 망막허혈/재관류 모델

Retinal Ischemia/Reperfusion Rat Model with Double Circumlimbal Suture

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Purpose: To introduce a simple, novel method to develop retinal ischemia/reperfusion (I/R) rat model using double circumlimbal suture technique.

Methods: Ten Sprague Dawley rats were used for the experiment, and animals were divided into the non-I/R control (n = 5) and I/R group (n = 5). To produce retinal I/R rat model, double circumlimbal suture was performed in unilateral eyes of the rats, and intraocular pressure (IOP) was measured before and immediately after the procedure, 1 and 2 hour(s) after the suturing, and daily until 1 week after the suture release. Animals were sacrificed at 1 week of the surgery, and immunohistochemistry for BRN-3a using retinal whole mounts was conducted to examine whether proper retinal I/R condition was established using double circumlimbal suture. terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was performed to observe RGC apoptosis according to time after I/R insult.

Results: After the double circumlimbal suture surgery, IOP was immediately elevated up to nineties, and immediately came down to normal range after suture release, while IOP level non-I/R control eyes were measured at between low to mid ten. Significantly decrease in number of RGCs was detected in the retina underwent the procedure compared to the retina from control. From TUNEL assay, increased RGC apoptosis was visible at early time after the surgery, at 24 and 48 hours after suture release.

Conclusions: Our novel double circumlimbal suture technique effectively produced retinal I/R rat model, and this model may be an alternative to produce retinal I/R model other than conventional anterior chamber puncturing method.

Key words: Experimental animal model; Glaucoma; Intraocular pressure; Ischemia-reperfusion injury; Suture technique

Introduction

Retinal ganglion cells (RGCs) are responsible primary neurons for conducting visual stimuli to the brain, and damage to this neuron results in its axonal degeneration and irreversible blindness. Several pathological ocular conditions may lead to RGC death, which include diabetic retinopathy,^{1,2} retinopathy of prematurity,^{3,4} and glaucoma.⁵ While several different animal models have been introduced to induce

RGC death, retinal ischemia/reperfusion (I/R) animal models mimic retinal vascular occlusion conditions involving hypoxic/ischemic events eventually leading to RGC loss. Also, since retinal hypoperfusion and vascular insufficiency has been shown to play a role in the development of glaucoma,^{6,7} retinal ischemia/reperfusion animal model is also widely used for animal studies for investigating glaucoma.

Conventional retinal ischemia/reperfusion model uses invasive procedure, puncturing the cornea with an infusion needle and connecting to sterile saline bottle to induce acute elevation of intraocular pressure (IOP) over 100 mmHg for an hour.⁸ While the procedure is quite simple and straight forward, there is a risk of damaging intraocular structure, such as iris and lens while puncturing the cornea, and also disconnection of infusion due to instability of infusion nee-

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dle position. Therefore, there is a need for developing more safe, non-invasive and meanwhile also reproducible method to develop retinal ischemia/reperfusion model.

Recently, novel and simple technique to develop a chronic IOP elevation animal model was introduced using a circumlimbal suture, which involves placing a simple circumferential suture around the globe approximately 1.0 mm behind the limbus.^{9,10} In this model, acute IOP spike was observed immediately after the suturing, which gradually came down to mid to high twenties and maintained until 15 weeks after the surgery. In this study, we modified this technique to produce retinal I/R model using double circumlimbal sutures, and observed the effectiveness of this model on RGC apoptosis in rats.

Materials and Methods

Animals

Ten male Sprague Dawley rats, aged 7-8 weeks (Orient Bio, Sungnam, Korea), were used in this study. Animals were housed under standard conditions and a 12/12-hours light/dark cycle with food and water ad libitum. Animal care and experimental procedures were carried out in compliance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research, and this study was approved by the Animal Care Committee of Soonchunhyang University Bucheon Hospital (Permit Number: SCHBCA201708). Deep sedation in rats was performed by intraperitoneal injection of a mixture containing 40 mg/kg zolazepam/tiletamine (Zoletil; Virbac, Carros, France) and 5 mg/kg xylazine (Rompun; Bayer Healthcare, Leverkusen, Germany) before proceeding to any surgical procedures.

Double circumlimbal suture for I/R model development

Animals were divided into I/R ($n = 5$) and non-I/R control ($n = 5$) groups. For animals in the I/R group, we performed double circumlimbal suture, by modifying previously introduced single circumlimbal suture technique to produce chronic ocular hypertension rat model.^{9,10} Briefly, single circumferential suturing (7/0 nylon) was performed around

the globe at an approximate distance of 0.5-1.0 mm behind the limbus, and another suturing was performed at 0.5-1.0 mm distance behind the first suture. The contralateral eye was left untreated. After 2 hours of double circumlimbal suturing, both sutures were released and removed from the eyeball to induce reperfusion condition. Anterior segment photography was taken before and immediately after single suturing, and also after double circumlimbal suturing.

IOP measurement and fundus photography

Baseline IOP readings were measured in animals with a rebound tonometer (TonoLab; iCare, Helsinki, Finland), and IOP was measured between 11 AM and 12 PM to exclude the effects of diurnal IOP variation. After baseline IOP measurement, IOP was measured at immediately after double circumlimbal suturing, and 1 and 2 hours after the suturing. To confirm that IOP returned to normal range after suture release, IOP was measured immediately after suture release, and at 12 hours, 1, 2, 3, 4, 5, 6, and 7 days after the suture release. Animals were under sedation when IOP was measured at immediate, 1, and 2 hours after the suturing, and at the time of suture release. In other cases, IOP was measured in awake condition. Fundus photography (Eyemera; IIScience, Seoul, Korea) was taken to ensure that retinal ischemia and reperfusion status was established after the suturing and release of the suture, respectively.

Tissue preparation

Ocular tissues were processed for further analyses, as described previously.^{11,12} Briefly, rats were deeply anesthetized and intracardially perfused with 0.1 M phosphate buffered saline (PBS) containing 150 U/mL heparin, followed by perfusion with 4% paraformaldehyde (PFA) in 0.1 M PBS. Subsequently, the eyes were enucleated, and a 360-degrees sclerotomy around the limbus was done to obtain the posterior segment of eyeball. Tissues were fixed in 4% PFA, followed by overnight incubation in 30% sucrose (in PBS) and embedding in Optimal Cutting Temperature compound. Sections were cut serially with 10 μ m thickness and mounted on adhesive microscope slides (Histobond; Paul Marrenfeld GmbH & Co. KG, Lauda-Königshofen, Germany).

For preparing retinal whole mounts, posterior eyecups were fixed in 4% PFA and flattened by making four equidistant cuts.

Immunohistochemistry (IHC) and TUNEL staining

Sections of retinas were permeabilized in 0.1% Triton X-100 containing 5% goat serum for 1 hour, followed by

Figure 1. Anterior photographs of control, single, and double circumlimbal sutured rat eyes. While control (A) and single circumlimbal sutured eyes (B) showed no definite abnormality in anterior photography, double circumlimbal suture (C) induced pale-looking appearance of the anterior segment, suggesting ischemia status of the eye. White arrowheads indicate circumlimbal suture performed on the eyeball.

Figure 2. Fundus photographs and intraocular pressure (IOP) measurement after double circumlimbal suture. In control eyes, normally perfused retina and choroid was observed in fundus photography (A). Upon double circumlimbal suture, impaired retinal and choroid circulation was detected (B), indicating retinal ischemia condition. After release of sutures, reactive hyperemia possibly in the retina and choroid was visible (C). IOP was significantly elevated from the time immediately after double circumlimbal suturing (91 ± 7 mmHg) and after suture release, IOP significantly decreased to 10 ± 2 mmHg and remained in normal range until 1 week of the surgery (D). I/R = ischemia/reperfusion.

overnight incubation at 4°C with the NeuN (MAB377; Chemicon, Burlington, MA, USA). For the TUNEL assay, the tissue was stained in accordance with the protocol provided by the manufacturer (12156792910, *In Situ* Cell Death Detection Kit, Roche Diagnostics). Sections were then washed and incubated with for 1 hour at room temperature with secondary antibodies: Alexa Fluor 488-conjugated donkey anti-rabbit IgG (Invitrogen Corp., Carlsbad, CA, USA) and Alexa Fluor 568-conjugated donkey anti-mouse IgG (Invitrogen Corp.). Nuclei were counterstained with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI, 0.1 mg/mL; Sigma-Aldrich, St. Louis, MO, USA) for

3 minutes. Retinal whole mounts were also stained following the same procedure with BRN-3a (MAB1585, Chemicon) as primary antibody. Confocal microscopy (LSM510 Meta; Carl Zeiss Meditec AG, Jena, Germany) was used to examine and photograph the samples. For counting RGCs, images from the peripheral (eight images), middle (five images), and central (three images) regions of the whole mounts were used.

Statistical analyses

Quantitative comparison of the number of RGCs between control and I/R eyes was performed using the ImageJ soft-

Figure 3. Retinal whole-mount images of control and OHT eyes immunostained for BRN-3a (red) at 1 week after double circumlimbal suture. (A, B) TUNEL staining (×20). Compared to the control retina (A), immunostaining of BRN-3a at 1 week of retinal ischemia/reperfusion (I/R) revealed a markedly decreased number of retinal ganglion cells in OHT eyes (B), which was statistically significant (C). Data are means ± SEM (scale bar, 40 μm). * $p < 0.01$.

ware, and the Mann-Whitney *U* test was used to determine statistically significant differences among the groups. Three eyes from each group were used for whole-mount image analysis, and two eyes from each group were used for retinal cross section imaging. Statistical analyses were conducted using SPSS for Windows software (ver. 20.0; IBM Corp., Armonk, NY, USA). Differences at $p < 0.05$ were considered statistically significant.

Results

Rat model of retinal I/R was established using double circumlimbal suture

Upon double circumlimbal suturing, anterior segment photography showed grossly pale-looking eyeball (Fig. 1C) compared to eyes with single circumlimbal suturing (Fig. 1B) and non-IR control eyes (Fig. 1A). Fundus photographs of the retina before suture placement showed normally perfused retinal and choroidal vessels (Fig. 2A), while double circumlimbal suture placement effectively diminished retinal and choroidal circulation, producing retinal and choroidal ischemia condition (Fig. 2B). Releasing sutures led

Figure 4. Time-dependent results from terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay using retinal cross sections from control and ischemia/reperfusion (I/R) eyes. In control eyes (A, E, I), TUNEL positive cells were not visible in nuclei of retinal ganglion cells (RGCs) while eyes after 24 hours of I/R (B, F, J), markedly increased number of TUNEL positive cells in RGC layer were detected, which continued at least until 48 hours after I/R insult (C, G, K). After 1 week of IR insult (D, H, L), TUNEL positive cells were mainly observed in outer nuclear layer with remarkable decrease in total retinal thickness (scale bar, 40 μ m). INL = inner nuclear layer; ONL = outer nuclear layer.

to reperfusion status of the retina and choroid, which was observed as flushing back of blood into the retinal and choroidal vessels as seen in Fig. 2C. IOP increased significantly (91 ± 7 mmHg) immediately after double circumlimbal suturing and then slightly decreased to 79 ± 7 mmHg and 76 ± 6 mmHg after 1 hour and 2 hours after the procedure. After release of sutures, IOP in I/R eyes significantly decreased to 10 ± 2 mmHg and remained in normal range until 1 week of the surgery (Fig. 2D). IOP of control eyes was 14 ± 2 mmHg at baseline and 11 ± 1 mmHg at the end of the experiment.

RGC death upon retinal I/R insult

To observe whether retinal I/R induced damage RGC, we counted numbers of RGCs in retinal whole mounts after 1 week of I/R insult and compared them with those from control retina. Markedly decreased number of RGCs was observed in the retinal whole mount from 1 week of ischemia/reperfusion eyes (Fig. 3B) compared to the control (Fig. 3A), which showed statistical significance (Fig. 3C). To further study time-dependent pattern of RGC apoptosis upon I/R insult, we performed TUNEL assay using retinal cross-sections of eyes from the control, as well as rats of 24 hours, 48 hours, and 1 week after I/R insult. After 24 hours of I/R, TUNEL/NeuN positive cells were markedly increased compared to control, and similar pattern of TUNEL staining was observed in the retinas from 48 hours of I/R insult. After 1 week of I/R, TUNEL positive cells were mainly found in outer nuclear layer and decreased total retinal thickness was also detected (Fig. 4).

Discussion

In this study, we developed a novel retinal I/R model using double circumlimbal suture technique, which was a modification from previously introduced single circumlimbal suture to produce chronic ocular hypertensive model.^{9,10} Immediately after double circumlimbal suturing, acute IOP elevation up to 90 seconds was observed which came down to normal range after release of sutures at 2 hours of suturing. Results from IHC and TUNEL assay showed that retinal I/R model was effectively established using this suture

technique.

Rodent glaucoma model using circumlimbal suture technique was recently introduced in previous studies.^{9,10,13-15} It only requires one 8-0 nylon suture to effectively induce IOP elevation, enabling investigators to more simply and effectively develop chronic OHT model compared to conventional OHT models such as episcleral vein cauterization¹⁶⁻¹⁸ and limbal laser photocoagulation model.¹⁹⁻²¹ For I/R models, conventional method to produce retinal I/R condition requires invasive procedure, including puncturing the cornea to introduce cannula to anterior chamber of the eye. It is obvious that damage to intraocular structures such as iris, lens and corneal endothelium may occur quite often when performing this procedure, which may affect and alter the results of the whole experiments. In our double circumlimbal suture induced retinal I/R model, simple two circumferential sutures effectively induced retinal ischemia condition without damaging any intraocular structures, and release of the suture after 2 hours of ischemia led to RGC apoptosis and retinal thinning at 7 days of the procedure.

The results from IHC and TUNEL assay shows markedly increased cell apoptosis in RGC and inner nuclear layer after 24 hours of I/R insult, which continued, at least, up to 48 hours of the injury. This result coincide with previous reports using conventional retinal I/R model that retinal I/R injury induced damage in the inner retina first.^{22,23} Also, after 1 week of I/R insult, increased TUNEL positive cells were mainly visible in the outer nuclear layer, showing subsequent outer retinal damage after inner retinal injury. Substantial thinning of the total retinal thickness was also observed after 1 week of I/R insult, which was also consistent findings from previous studies using conventional I/R models.²²

There are several limitations to consider when interpreting the results of our study. First, we did not perform any examinations to measure functional activity, such as electroretinogram, of the retina in our novel I/R model. However, there was significant damage visible in RGCs after 1 week of the insult in both cross-sectional and whole mount IHC images, showing sufficient evidences that this I/R model was effective in producing RGC and axonal dam-

age. Second, we conducted our investigations only until 1 week after the surgery. While there may be more prominent changes in the retina at 2 weeks and 4 weeks of the suturing, we observed noticeable damage occurring in the inner retina as well as in the outer retina at 1 week of the surgery. Indeed, future researches should look for any further damage or changes taking place in the retina after a few weeks or a month of the I/R insult using this I/R model.

Overall, this study introduced a simple method to effectively produce retinal I/R condition using double circumlimbal suture technique. This novel animal model may aid investigators to more easily develop retinal I/R condition and may be another optional animal model for conducting experiments regarding RGC and axonal damage.

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국문초록

이중 윤부주위둘레 봉합술을 이용한 쥐 망막허혈/재관류 모델

목적: 이중 윤부주위둘레 봉합술을 이용하여 생성한 새로운 망막허혈/재관류 쥐 모델에 대해 소개하고자 한다.

대상과 방법: 10마리의 Sprague Dawley 쥐를 사용하였고 대조군(n=5)과 망막허혈/재관류(n=5)로 나누어 실험을 진행하였다. 망막허혈/재관류 쥐 모델 생성을 위해 이중 윤부주위둘레 봉합술을 단안에 시행하여 생성하였으며, 안압은 수술 직후 측정된 후 술 후 1시간, 2시간 후 측정하였으며, 1주째까지 매일 측정하였다. 술 후 1주째 동물을 희생하였으며, 면역조직염색을 시행하여 망막 허혈/재관류 모델 생성 여부를 확인하였다. TUNEL assay를 통해 허혈/재관류 손상 후 시간에 따른 망막신경절세포 사멸 정도를 확인하였다.

결과: 이중 윤부주위둘레 봉합술 직후 안압은 90 mmHg 초반 및 중반까지 상승하였고 봉합을 푼 후부터 안압은 정상 범위로 측정되었으며, 대조군에서는 안압은 10 mmHg 초반 및 중반으로 측정되었다. 봉합술을 시행한 눈에서 망막신경절세포 수가 유의하게 감소한 것을 확인할 수 있었고, TUNEL assay 결과상 봉합 제거 후 24시간, 48시간 후 시점에 망막신경절세포 사멸이 증가되어 있음을 확인할 수 있었다.

결론: 이중 윤부주위둘레 봉합술을 이용하여 망막허혈/재관류 모델을 성공적으로 생성할 수 있었으며, 본 모델은 전방천자를 시행해야하는 기존의 망막허혈/재관류 모델의 새로운 대안 모델이 될 수 있을 것으로 사료된다.