Original Article

Comparison of Dextran Perfusion and GSI-B4 Isolectin Staining in a Mouse Model of Oxygen-induced Retinopathy

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Abstract

Purpose: Oxygen-induced retinopathy (OIR) is a robust and widely used animal model for the study of retinal neovascularization (NV). Dextran perfusion and Griffonia simplicifolia isolectin B4 (GSI-B4) staining are two common methods for examining the occurrence and extent of OIR. This study provides a quantitative comparison of the two for OIR detection.

Methods: At postnatal day 7 (PN7), fifteen C57BL/6J mice were exposed to a 75% hyperoxic condition for 5 days and then returned to room air conditions. At PN17, the mice received intravitreal injection of GSI-B4 Alexa Fluor 568 conjugate. After 10 hours, they were infused with FITC-dextran conjugate via the left ventricle. Retinal flat mounts were photographed by confocal microscopy. Areas with fluorescent signals and the total retinal areas were quantified by Image J software.

Results: Both GSI-B4 and dextran detected the peripheral neovascular area. The mean hyper fluorescence area was 0.33±0.14% of whole retinal area determined by GSI-B4 staining and 0.25±0.28% determined by dextran perfusion. The difference between the two measures was 0.08% (95% CI: -0.59%, 0.43%). The Pearson correlation coefficient between the two methods was 0.386, P =0.035. The mean coincidence rates were 14.3±13.4% and 24.9±18.5% for GSI-B4 and dextran staining, respectively.

Conclusion: Both methods can complement each other in demonstrating and quantitatively evaluating retinal NV. A poor agreement was found between the two methods; GSI-B4 isolectin was more effective than FITC-dextran perfusion in evaluating the extent of retinal NV in a mouse model of OIR. (***Eye Science 2015; 30: 70-74***)

Keywords: oxygen-induced retinopathy; comparison; angiogenesis

Introduction

Retinal neovascularization (NV) is a pathological angiogenesis characterized by a tangle of blood vessels on the retinal surface. These vessels differ from normal vessels that have an organized, bifurcating pattern. Instead, these spontaneous new vessels feature protrusions and outgrowths of capillary buds and sprouts from the pre-existing blood vessels[1]. Most NV vessels are poorly built and readily bleed spontaneously. In the retina, bleeding at the interface between the retina and vitreous will attract fibroglial elements, which form fibrovascular stalks. This induces vitreous contraction and leads to tractional retinal detachment, ultimately resulting in blindness. Retinal NV commonly occurs in different retinopathies, such as retinopathy of prematurity, retinal vein occlusion, and diabetic retinopathy[1].

In 1994, Smith and colleagues first reported a mouse model of oxygen-induced retinopathy (OIR)[2]. The proliferative retinopathy and abnormal retinal vasculature were well described. Since then, the OIR has become a simple and commonly used model in research on retinal NV[3]. Basically, establishment of the model involves two phases. The first phase is the

DOI: 10.3969/j.issn.1000-4432.2015.02.006

Funding: This study was supported by the National Nature Science Foundation of China (30901646 and 81170853), Guangdong Science and Technology Project (2011B031300013), Science and Technology Project of Shantou City, China (2009-70)and Joint Shantou International Eye Center Research and Development Fund (09-012 and 09-013).

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exposure of mouse pups (at postnatal day 7) to 75% oxygen (hyperoxia) for 5 days. Hyperoxia induces retinal vessel loss and cessation of vessel growth. The second phase is the return of the pups to normal room air conditions to induce ischemia. This causes the avascular area induced by hyperoxia to experience severe hypoxia, which subsequently stimulates the pathological angiogenesis[4].

This mouse OIR model has been widely used in anti-angiogenesis drug testing[5], which requires accurate quantitative measurement of retinal NV for the correct interpretation of drug effects. At present, two common techniques are used to quantify the occurrence and extent of retinal NV, namely Griffonia simplicifolia isolectin B4 staining and dextran perfusion[6]. Although both methods are widely used, the consistency between them remains to be established. The purpose of this study was to evaluate the agreement between the staining patterns obtained with fluorescein isothiocyanate (FITC)-dextran perfusion and Alexa Fluor 568-GSI B4 conjugate staining.

Materials and methods

The mouse model of oxygen-induced retinopathy

All animal experiments conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Animal Care Committee of Joint Shantou International Eye Center. OIR mice were induced according to the protocol described previously[2]. Fifteen C57BL/6J pups at postnatal day (PN) 7, with their nursing mothers, were exposed to a hyperoxic condition (O2:N2 75%:25%, O_2 level controlled by ProOx110, BioSpherix, NY, US) in an airtight chamber for 5 days. They were then returned to room air conditions.

Intravitreal injection of the GSI-B4 Alexa Fluor 568 conjugate

At PN17, each pup received an intravitreal injection under an operating microscope (Topcon OMS-800). The animal was anesthetized and its pupil was dilated. A glass micropipette (Blaubrand, Germany) was inserted at a site just beneath the limbus through the sclera into the vitreous cavity. A micro-injector was depressed to deliver the drug into the vitreous cavity. GSI-B4 Alexa Fluor 568 conjugate (1 μg/μl, Invitrogen) was injected with 1 μl as the injection volume[9].

Cardiac infusion of the FITC-dextran conjugate

Ten hours after intravitreal injection of the GSI-B4 conjugate, the mice were anesthetized again and infused with 50 mg/mL FITC-dextran conjugate (molecular weight 2000K, Sigma, MO) (1 ml infusion volume) through the left ventricle.

Retinal whole mounts

The mice were sacrificed at 3 minutes after dextran infusion, when a marked yellow color appeared in the limbs and tongue. The eyes were enucleated and fixed in 4% neutral buffered paraformaldehyde in phosphate buffered saline (PBS) for 2 hours. The anterior segment was removed and the retina was flattened by making four radial cuts and flat mounted on a glass slide.

Measurements of retinopathy and statistical analysis

Retinal whole mounts were photographed under laser scanning confocal microscopy (TCS SP5, Leica, Germany). All digital images were captured under the same parameters. The fluorescence areas (stained by FITC and by Alexa Fluor 568) were quantified using Image J software (National Institute of Health, MD) by two independent investigators blinded to the experimental details. The percentage of NV area compared to the whole retinal area was calculated and compared for the two dyes using a paired T-test, Pearson correlation analysis, and a Bland-Altman plot, using SPSS software (Version 17.0, SPSS Inc., IL).

Results

Staining by FITC-dextran infusion (Figure 1A) and GSI-B4 Alexa Fluor 568 conjugate injection (Figure 1B) revealed the central avascular and peripheral vascular areas. The high fluorescence area was determined as 0.33±0.14% of the whole retinal area by isolectin staining and 0.25±0.28% by FITC-dextran perfusion. No statistically significant difference was noted between the two measurements ($P>0.05$, paired *t*-test).

However, the correlation between the high fluorescence areas determined by isolectin staining and FITC–dextran perfusion was moderate, with a Pear-

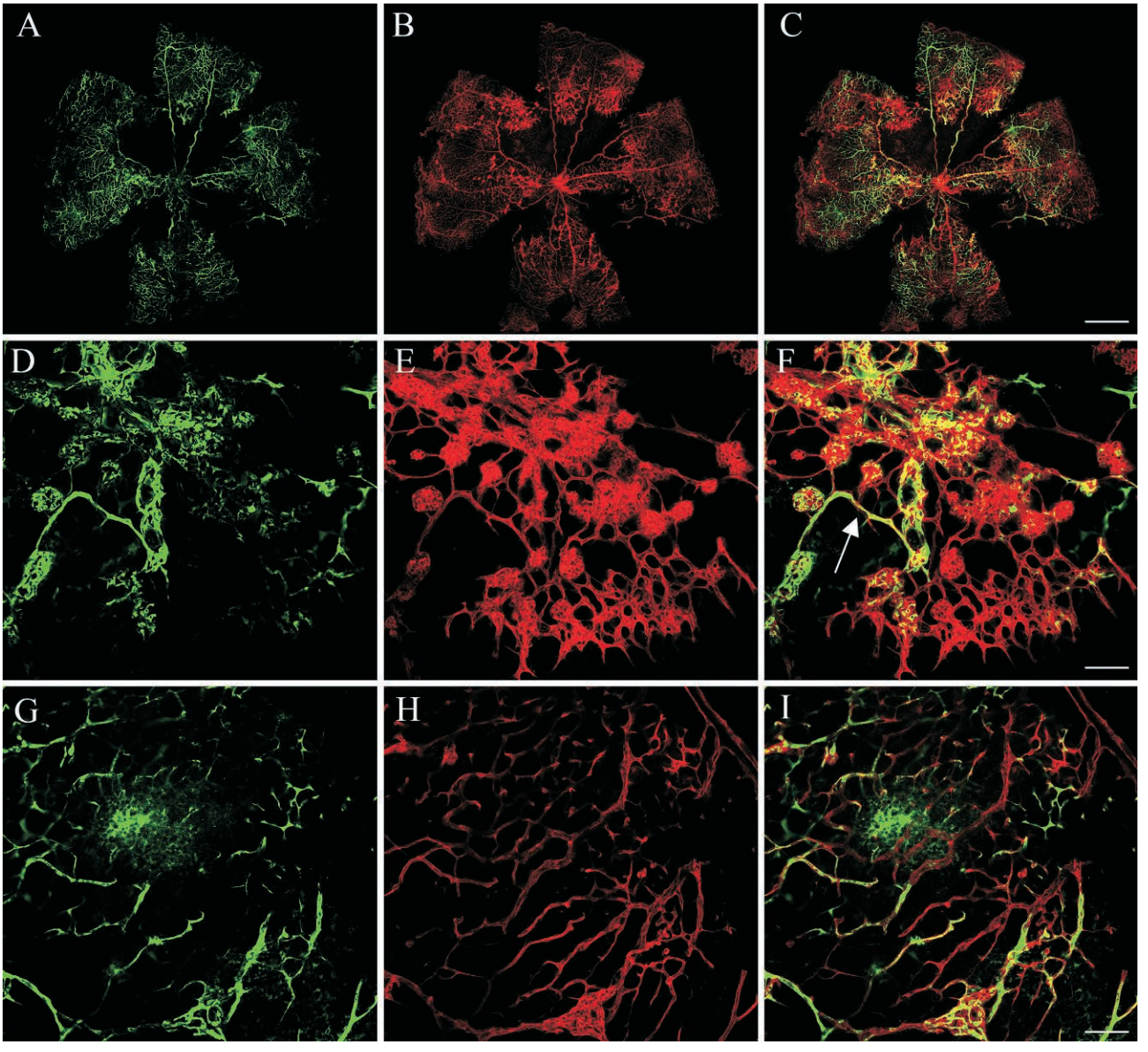

Figure 1 Retinal flat-mount of oxygen-induced retinopathy mouse model. A.D.G: FITC-dextran perfusion; B.E.H: isolectin staining; C.F.I: coincidence of the two measures; A-C: overview of the retinal flat-mount; D-F: some areas stained with isolectin but not perfused with FITC dextran. G-I: some areas perfused with FITC dextran but not stained with isolectin.

son correlation coefficient = 0.386, P=0.035. The Bland-Altman plot (Figure 2) showed a difference between the two measures of 0.08%, with a 95% confidence interval (-0.59%, 0.43%). The two types of fluorescent signal did not coincide well with each other in some cases. Some FITC-dextran perfused areas were unstained by isolectin (Figure 1 D, E, F) and some isolectin-stained areas were not perfused by FITC-dextran (Figure 1 G, H, I). The mean coincidence rate was 14.3 ± 13.4% and 24.9 ± 18.5 % for isolectin staining and FITC-dextran perfusion, respectively (Figure 1C).

Discussion

This study showed no statistically significant difference between the results of isolectin staining and FITC-dextran perfusion measures in the OIR mouse model. However, the findings of a correlation coefficient of only 0.386, the large variation of the difference, and the low coincidence, suggest that some differences exist between the two methods.

Angiography with high molecular weight dextran conjugated with FITC[2] has been used to observe retinal blood vessels in OIR mice because of its advantage

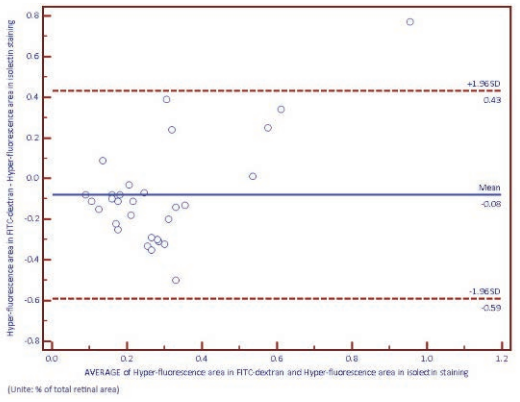

Figure 2 Bland-Altman plot of the retinal neovascularization area in the oxygen induced retinopathy mouse model measured by FITC-dextran perfusion and isolectin staining.

of faster speed compared to cross-section immunohistochemistry. The intravascularly-perfused high-molecular-weight FITC-dextran would remain in the vascular structure without spreading, thereby aiding the observation of mouse blood vessels[7]. High fluorescence, in turn, would indicate leakage of FITC-dextran from abnormal vasculature such as NV[8]. However, the results obtained with FITC-dextran perfusion are affected by several factors: (1) depth of anesthesia, which may influence the retinal capillary perfusion; (2) perfusion pressure and blood flow speed variation when pushing the syringe[9]; and (3) constriction or expansion of the vessels caused by the pH or temperature of the perfusion solution. Therefore, caution is needed when interpreting the results of dextran perfusion. Endothelial-specific markers, such as isolectin[10], collagen IV[11] and anti-CD31 antibody[12], have been used for immunohistochemistry as they bind to the surface of endothelial cells and indicate vascular structure, including NV. Traditionally, immunohistochemistry was performed on cross sections, which is time and manpower consuming compared to FITC-dextran perfusion[2]. Subsequently, whole mounts immunohistochemistry has been introduced into OIR models[13]. This saves time and manpower by eliminating the need for preparing, staining, and counting over 100 cross-sections.

The finding of poor agreement between high molecular dextran perfusion and isolectin staining in the quantification of retinal NV was not unexpected. On the one hand, the high fluorescence region arising from leaked dextran following perfusion may not be caused by retinal NV but by the breakdown of the blood-retinal barrier. On the other hand, retinal NV may not leak fluorescent dextran if the neovascular tissue becomes scarred or the neovascular tufts are not well perfused[14]. Therefore, FITC dextran may not accurately reflect retinal NV. We therefore recommend using whole-mount isolectin immunohistochemistry for quantification of retinal NV in OIR mice[15]. Compared with the dextran perfusion method, the isolectin immunostaining is more objective and efficient. The procedure is simpler and easier, and it provides a clear display of NV distribution and avascular areas in the retina when a blind area is present in dextran perfusion. Immunostaining can also label retinal NV easier, in order to distinguish NV from the normal vessels.

Unbiased and reliable comparisons were ensured by performing the two measurements on the same eye, which eliminated interocular variation and made possible the measurement of the coincidence rate. The investigators were blinded when quantifying neovascular areas to avoid subjective measurement bias. However, slight errors could still have occurred during the manual measurements.

In conclusion, the agreement was poor between FITC-dextran perfusion and isolectin staining in quantification of OIR mice. Isolectin staining is recommended for quantifying retinal NV in OIR mice.

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