Diffusion of Macromolecule Through Retina After Experimental Branch Retinal Vein Occlusion and Estimate of Intraretinal Barrier

Yong Tao[1], Xiao-xin Li[1,*], Yan-rong Jiang[1], Xia-bing Bai[2], Bi-dong Wu[3] and Jian-qiang Dong[4]

[1]*Department of Ophthalmology, People's Hospital, Affiliated Peking University;* [2]*School of Material Science and Engineering, Beijing University of Aeronautics and Astronautics;* [3]*Beijing Institute of Pharmacology and Toxicology and* [4]*Pathology Center Laboratory, People's Hospital, Affiliated Peking University*

Abstract: The disposition and diffusion knowledge of intravitreally injected macromolecule drugs through retina in pathological condition is crucial but the related studies are absent. Retinal edema is a common pathological change of fundus diseases and retinal vein occlusion (RVO) pig model were established to emulate it. FITC-dextrans of various molecular weights were dissolved in RPMI-1640 solutions and the rate of transretinal diffusion was determined with a spectrophotometer. Theoretical maximum size of molecule (MSM) was calculated by extrapolating the trend-linear relationship with the diffusion rate. In separate experiments to determine the sites of barrier to diffusion, FITC-dextrans were applied to either the inner or outer retinal surface, processed as frozen sections, and viewed with a fluorescence microscope. Paired-Samples T test was used to compared the diffusion rate of dextrans of the both eyes of one pig. The MSM in RVO tissues and normal tissue was 6.5±0.39nm and 6.18±0.54nm respectively (t=4.143, P=0.0001). FITC-dextrans applying to inner retinal surface, 4.4 kDa dextran were largely arrested at inner nuclear layer (INL). The INL of the 19.6~71.2 kDa dextran diffusion retina section became dark and the nerve fiber layer (NFL) and inner plexiform layer got brighter. As for 150 kDa dextran, the NFL was bright and the other layers were dark. FITC-dextrans applying to outer retinal surface, most dextrans were blocked before outer nuclear layer (ONL). In summary, ONL and INL may act as bottle-neck barriers to diffusion of macromolecules. Compared with normal neuroretina, the MSM of fresh edema retina after RVO increased limitedly.

Key Words: Retinal vein occlusion, drug diffusion, macromolecule, intraretinal diffusion barrier, intravitreous drug delivery.

INTRODUCTION

The limited permeability of the blood-ocular barrier often requires the use of high systemic levels of small-molecule drugs to achieve efficacy, while large molecules such as proteins do not cross this tight barrier. The report that a large molecular weight peptide like insulin can accumulate in the retina and optic nerve after topical application shed light on the treatment of posterior segment diseases [1], but it was not accepted and taken into clinical use widely till now. Recently, intravitreal injection of macromolecule drugs has been tried to treat choroidal diseases and chorioretinopathy [2-6], whereas rare specialized study focused on the size of the molecule which can diffuse through retina and the intraretinal disposition of macromolecular drugs [7]. Although some preclinical pharmacokinetics studies have been performed before clinical use [8, 9,10], the animal model eyes used were normal and what will happen in pathological conditions remains unknown.

Intraretinal barriers to diffusion affect the successful delivery of intravitreous drug to the subretinal space and the removal of intravitreous drug [9, 11-13]. With special anatomic structure, each layer in neuroretina plays a different role in limiting the drug diffusion speed. However, there is limited and sometimes contradictory literature available on the research of the bottle-neck layers in neuroretina: Mordenti *et al.* found that 148 kDa molecules could not penetrate the inner limiting membrane(ILM) of the retina [9], whereas Jackson *et al.* found that inner plexiform layer(IPL) and outer plexiform layer(OPL) were sites of high resistance to the diffusion of 167 kDa macromolecules across the retina [7]. In Bunt-Milam AH's study, the intraretinal barrier was external limiting membrane (ELM) [14]. In addition, experiments in squirrel monkey found that horseradish peroxidase (44 kDa) diffused rapidly across the retina [15], but experiments in rhesus monkey found that it did not [16].

The purpose of the present study was to use the apparatus designed by ourselves to test the maximum size of molecule (MSM) capable of free diffusing across retina in fresh edema condition after retinal vein occlusion (RVO) was established. In order to determine the intraretinal barrier layer, we also performed a series of diffusion tests of variable molecular size in normal pig retina and analyzed the fluorescence distribution of the section, which led to a new finding different from previous reports.

MATERIALS AND METHODS

Animals

Miniature pigs weighing 10 to 12 kg were obtained from the Department of Animal Science, Beijing Agricultural University, Beijing, China, and were housed by the Department of Animal Studies, People's Hospital, Peking University, Beijing. They were treated following the ARVO State-

*Address correspondence to this author at the 11 XiZhiMen south street, Department of Ophthalmology, People's Hospital, Affiliated Peking University, Beijing 100044, China; Tel: (8610) 65224572; Fax: (8610) 82211370; E-mail: drlixiaoxin@vip.sina.com

1389-2002/07 $50.00+.00

ment for the Use of Animals in Ophthalmic and Vision Research. Animals were systemically anesthetized with intramuscular injections of ketamine hydrochloride (15 mg/kg) and xylazine hydrochloride (15 mg/kg) followed by a retrobulbar injection of 3mL of 2% lidocaine hydrochloride. Pupils were dilated using 0.5% phenylephrine hydrochloride and 0.5% tropicamide eyedrops.

Experimental Branch Retinal Vein Occlusion

The technique for producing experimental branch retinal vein occlusion (BRVO) in miniature pigs has been described previously [17]. In brief, after a superionasal sclera puncture was performed, 20mg/kg rose bengal (Sigma, USA) solution was injected *via* an ear vein in each miniature pig just before light exposure. Then, an endo-illuminator was inserted into the eye and a spot of light was focused on the superior retinal vein 0.5–1.0 disc diameter away from optic disc for 10 minutes. After surgery, the vein became tortuous and hemorrhages appeared in the retina distal to the site of the block (Fig. (**1**)). Surgery was performed in a randomly chosen eye of 23 pigs in total.

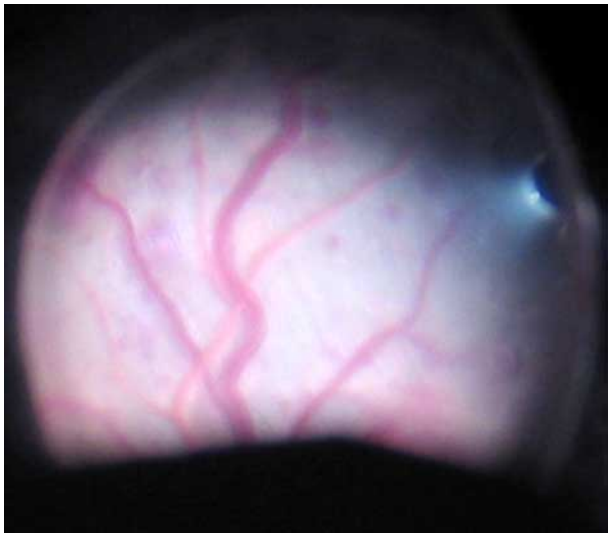

Fig. (1). Fundus photograph of miniature pig several minutes after superior branch retina vein occlusion was performed. Retinal vein became tortuous and dilated, and retinal hemorrhage could be observed.

Tissue Preparation

Before enucleation was performed, excessive pentobarbital sodium was injected. The eyes of miniature pigs were enucleated 2 days postoperatively and the same parts of both eyes were chosen for study. As what we needed was the retinal edema status which was somewhat macroscopical, the fundus fluorescence angiography was not performed to avoid the interfering effect of fluorescein sodium left in retinal tissue. In addition, the fresh porcine eyes obtained from a local abattoir were used to test the diffusion apparatus and to access the intraretinal barriers to diffusion. The eyes were opened circumferentially approximately 6 mm behind the limbus and the anterior tissues and the vitreous was separated gently from the neuroretina. The neural retina was soaked in RPMI 1640 solution (including 5958 mg/L HEPES, 100U/ml Penicillin and 100μg/ml Streptomycin, PH 7.4, from Tianrunshanda, China), separated gently with a glass rod and supported on ashless filter paper with a 20- to 25- μm pore size (no.541; Whatman, Maidstone, UK). The availability of filter paper use has been tested previously [7].

In Vitro Diffusion Apparatus

An apparatus with a 4-mm interchamber aperture processed with nonmagnetic stainless steel was designed and used to test the transretinal diffusion of fluorophore labeled dextran (Fig. (**2**)). Firstly, a 8-mm round retina supported on filter paper was put on bedplate (Fig. (**2A**)) with ILM of the retina facing upward or downward, then, aligning the groove on the bedplate, the upper chamber part (Fig. (**2B**)) was fixed by gravity and a small amount of cyanoacrylate adhesive was applied to seal the rim. 1ml RPMI 1640 solution containing 0.1mM FITC-dextran was added into the central chamber and 40ml RPMI 1640 solution was added into the outer chamber. The fluid plane of both chambers was held at the same horizontal level. A small glass-encased magnetic stirrer was inserted into the bottom chamber (Fig. (**2C**)). Experiments were conducted at 25°C and were protected from ambient illumination. A series of pilot studies using waterproof plastic membrane and pig retina from abattoir were performed to test that the apparatus was tight (data not shown).

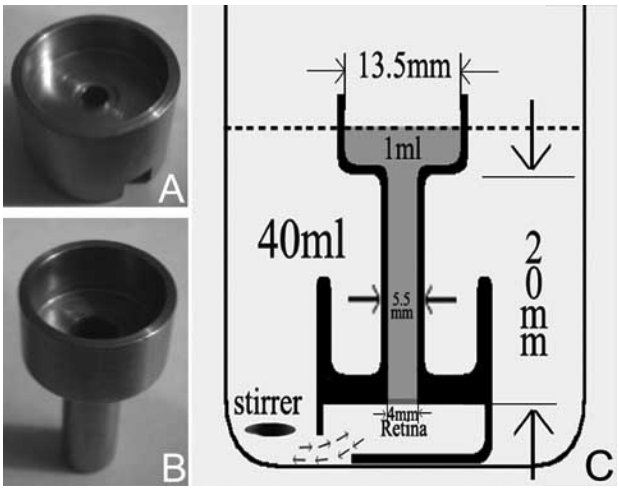

Fig. (2). The apparatus used in this study to test the diffusion of macromolecules across neuroretina.

Fluorescent Compounds and Sample Collection

Separate experiments were conducted using Carboxyfluorescein (376 Da) and FITC-conjugated dextran 4.4, 9.3, 19.6, 38.9, 71.2 and 150 kDa (Sigma-Aldrich, St.Louis, MO). To confirm that the parent compound was not cleaved from FITC, selective samples were analyzed using gel-filtration chromatography to detect the presence of any unconjugated fluorescein. 1ml sample was taken from the outer chamber at 0, 30, 60, 90, 120, 180 and 240 minutes and was supplemented with pure RPMI 1640 solution. Samples were stored at -80 ℃ until being analyzed. Fluorescence was measured at room temperature (25℃) with a fluorescence spectrophotometer (F-4010; HITACHI, Japan). The excitation and emission wavelengths were 490 and 520nm respectively. Standard curves of fluorescence versus concentration

were obtained by serial dilution of fluorescent compounds in diffusion medium. Concentrations in samples were determined by linear regression analysis within the linear portion of the standard curve. If there was a rapid and high increase of fluorescence, a minimal retinal hole was doubted and the data was discarded. Tissue was removed after 1 or 4 hours' exposure, placed into embedding compound and frozen. 6 μm sections were cut on a cryotome (CM1900; Leica, Wetzlar, Germany), viewed with a fluorescence microscope (DFC300 FX; LEICA, Cambridge, UK) and photographed (IM50; LEICA, Cambridge, UK). Then, the sections remained immersed in 4% formaldehyde for 10 minutes and were stained with hematoxylin-eosin.

Control Experiments and Data Calculation

After enucleation, the same parts of superior retina of BRVO eye and contralateral normal eye were used for fluorescence diffusion tests. Dextrans of 9.3, 19.6, 38.9, 71.2 kDa were used to calculate the MSM. Calculated from standard concentration-fluorescence curve for each dextran, the batch of samples collected at different time points were processed to determine the transretinal diffusion rate of FITC-dextrans. Because pilot studies indicated that the rate of diffusion showed an exponential decline with increasing molecular weight and the natural log (Ln) was the most appropriate log scale [18], the rate of diffusion were plotted against the natural log of molecular weight (x-axis) and a straight trendline graph was created. The straight line was extrapolated and the antilog of the point where it crossed the x-axis was taken as the theoretical maximum molecular weight for retinal diffusion. The MSM were calculated using the Stokes-Einstein radius for each dextran. The rates of diffusion displayed an inverse linear relationship with the molecular radius, and an MSM could be calculated without log transformation. The above method has been reported before [7]. Paired-Samples T test was used to compare the diffusion rates of dextrans of both eyes of one pig.

RESULTS

No free fluorescein was detected in the FITC-dextran stock solutions or after exposure to experimental conditions. Figure 3 showed the fluorescein distribution in neuroretina observed under fluorescence microscope after 1 hour diffusion test with ILM facing upward or downward: the inner nuclear layer(INL) and outer nuclear layer(ONL) were the brightest in carboxyfluorescein group, no matter whether ILM faced upward or downward (Fig. (**3A&B**)). In 4.4 kDa dextrans group, when ILM faced upward, INL was still bright but the ONL became dark to some extent (Fig. (**3.C**)); and when ILM faced downward, lots of fluorescence dextrans were blocked before ONL, but the INL and GCL were still bright (Fig. (**3D**)). In 19.6 kDa dextrans group, when ILM faced upward, the INL darkened and the nerve fiber layer (NFL) and IPL became bright (Fig. (**3E**), the photos using 9.3, 38.9 and 71.2 kDa were similar but not shown); and when ILM faced downward, the part near the ONL was bright and other parts were relatively dark (Fig. (**3F**)). In 150 kDa dextrans group, when ILM faced upward, the NFL became the brightest part in the section and the other layers behind it were dark (Fig. (**3G**)); and when ILM faced downward, only the part out of the ONL was bright (Fig. (**3H**)).

In 18 surgery-performed pigs, an obvious thicker and hemorrhagic retina were observed after enucleated. Histopathological examinations showed the edema and hemorrhage in the inner part and intercellular junction of INL was destroyed, however the ONL remained relatively normal (Fig. (**4**)). Fluorescence test showed that the ONL was relatively bright in the section and the fluorescence in the inner part of retina was dark (Fig. (**4**)). In the other 5 eyes, fundus appearance was almost normal except the slightly enlarged superior retinal vein or some dot-blot intraretinal hemorrhage in 3 eyes. In these 5 eyes, recanalization was doubted and these data were discarded. In the 18 eyes in which BRVO was successfully established, the rapidly increasing fluorescence data of 2 eyes among them were also discarded due to a potential retinal break.

The theoretical data extrapolated from the straight-line portion of the graph plotting rate of diffusion against the x-axis (Ln of molecular weight and Stokes-Einstein radius of dextrans) were as follows: RVO eyes 83.4$\pm$2.2kDa (6.5$\pm$ 0.39nm) and contralateral normal eyes 70.1$\pm$2.4 kDa (6.18$\pm$0.54nm) (Fig. (**5**)). The result of Paired-Samples T test was t=4.143, P=0.0001. With the molecular weight or radius increasing, the disparity of diffusion rate between RVO eyes and contralateral normal eyes reduced (Fig. (**5**)).

DISCUSSION

In the present results, edema occurred in the inner part of retina and the intercellular junctions in INL were destroyed after BRVO was successfully established (Fig. (**4**)). Previous work using electron microscope showed the separation of neural and glial elements in the nerve fiber-ganglion cell layer and cystic cavities in the INL [21]. Under fluorescence examination, the inner part of neuroretina was dark (Fig. (**4**)), which may mean the loss of barrier function in inner part, therefore the MSM calculated from the curve (Fig. (**5**)) may mainly represent the diffusion limitation of the outer part of neuroretina. Compared with the normal retina, there was a limited increase of the MSM of RVO retina (6.5$\pm$ 0.39nm vs 6.18$\pm$0.54nm, Fig. (**5B**)). In this sense, we may infer that the barrier effect of the outer part of neuroretina is strong. An evidence was that FITC-dextran were predominantly arrested at photoreceptor layer if the tissue was exposed to dextran on the outer retinal surface (Fig. (**3D,F,H**)).

The MSM of normal pig neuroretina detected by using the apparatus designed by ourselves is 70.1$\pm$2.4 kDa (6.18$\pm$ 0.54nm) which is similar to 60$\pm$11.5 kDa (5.68$\pm$0.45nm) detected by Jackson *et al.* [7]. The present findings are consistent with the result of subretinal albumin diffusion study undertaken by Takeuchi A *et al.* which indicated that albumin (69 kDa, 3.6 nm) could diffuse into the rabbit subretinal space from vitreous [22]. In addition, the dextran diffusion studies in rabbits undertaken by Marmor *et al.* showed that the subretinal FITC-dextran 10-S (smaller than albumin) could diffuse readily into the vitreous, but the diffusion of FITC-dextran 70-S and 150-S (both larger than albumin) was markedly slower [23].

Jackson *et al.* once performed a 167 kDa FITC-dextran diffusion test and found that fluorescence was arrested predominantly at the IPL if tissue was exposed to dextrans on the inner retinal surface and it was arrested predominantly at

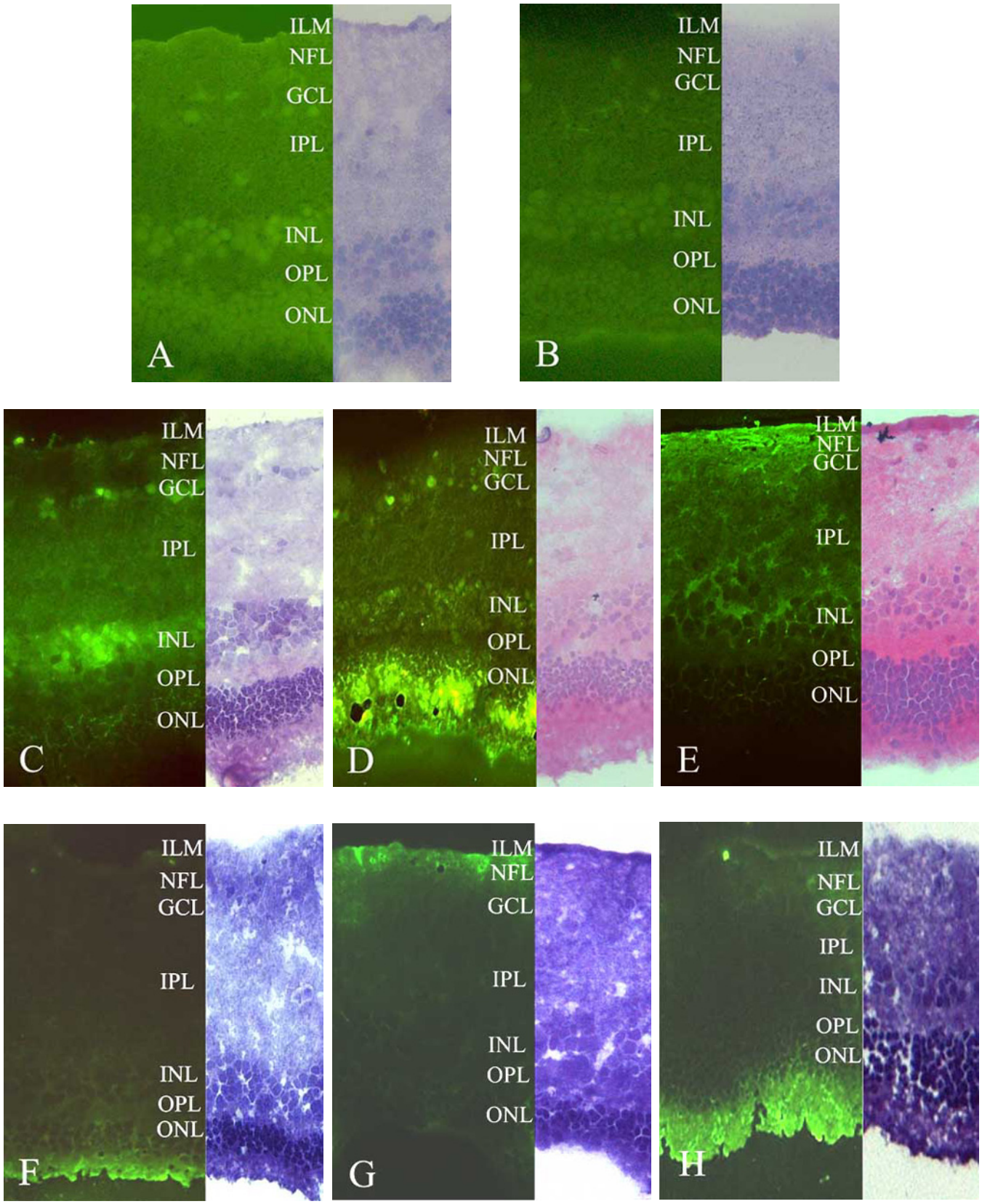

Fig. (3). Fluorescence (left) and hematoxylin-eosin staining (right) micrograph of frozen sections of normal pig neuroretina after 1 hour diffusion test for carboxyfluorescein and variable molecular weight FITC-dextrans with ILM facing upward (A,C,E&G) or downward (B,D,F&H). The fluorescence-labelled molecules were carboxyfluorescein (A&B), 4.4 kDa FITC-dextrans (C&D), 19.6 kDa FITC-dextrans (E&F) and 150kDa FITC-dextrans (G&H). (original magnification×200).

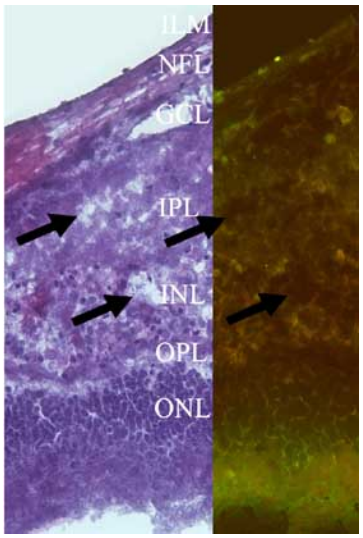

Fig. (4). Fluorescence (right) and hematoxylin-eosin staining (left) micrograph of frozen sections of RVO model in miniature pig neuroretina after 4-hour diffusion test for 38.9 kDa FITC-dextrans with ILM facing upward. The cystic cavities (arrows) in IPL and INL were dark (original magnification×200).

the OPL if tissue was exposed to dextrans on the outer retinal surface for 24 hours [7]. Therefore, in their report, a conclusion that IPL and OPL were the sites of high resistance to the diffusion of macromolecules across retina was drawn [7]. Adding the similar tests of smaller molecular weight FITC-dextran (Fig. (**3A,B,D,E**)), we found that the nuclear layers of neuroretina may be the real barrier for macromolecules diffusion rather than the plexiform layers.

The explanation is as follows: among the area that FITC-dextrans are small enough to diffuse across freely, the density of fluorophore labeled molecules is the highest in the diffusion barrier layers which will become the brightest under fluorescence microscope. If the FITC-dextran are too large to diffuse across retina freely, those macromolecules will be prevented before the first barrier which will become dark relatively. In the test using carboxyfluorescein that is only 376 Da, the INL and ONL were the brightest (Fig. (**3A**), which may suggest that ***the INL and ONL be the bottle-neck barrier of molecules diffusing across retina***. In the test using 4.4 kDa FITC-dextran diffusing from the ILM to the outer retina, INL was the brightest (Fig. (**3A**)) and the ONL behind it was dark. So, we may infer that ***the ONL was a site of resistance to 4.4 kDa dextran but INL was not***. If 9.3~71.2 kDa dextrans instead of 4.4 kDa dextran were used for diffusion tests, the INL was not bright any more and the inner retina before INL showed stronger fluorescence (Fig. (**3B**)), which implied that ***the INL was a site of resistance to 9.3~71.2 kDa dextran but IPL, NFL, GCL and ILM were not***. The image of 150 kDa FITC-dextran diffusion test showed that the NFL became the brightest part in the section and the other layers behind it were dark (Fig. (**3C**)), which implied that ***the IPL or GCL was a site of resistance to 150 kDa dextran but NFL and ILM were not***. Therefore, we can rank the different layers in neuroretina according to their barrier limitation of molecular weight or theoretical radius (Fig. (**6**)). Something interesting was that the cell nucleus of

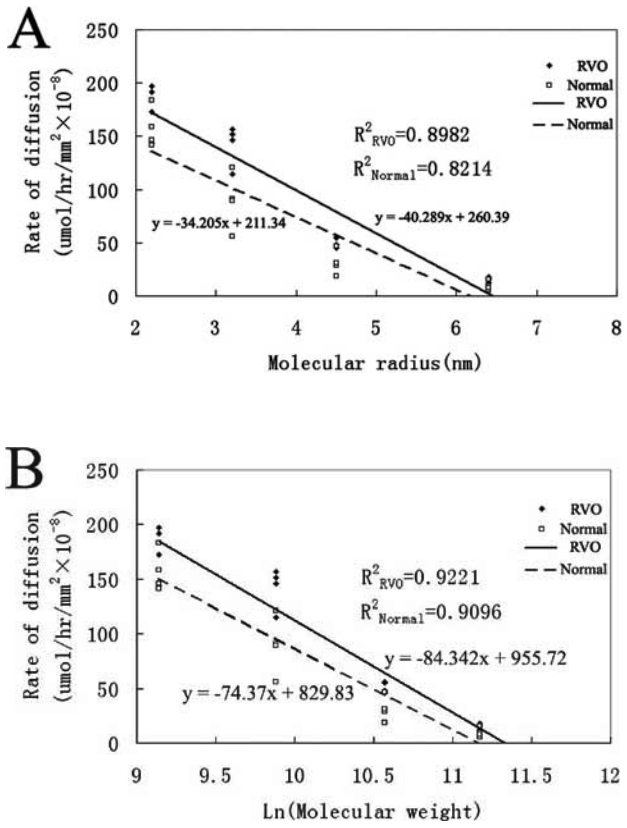

Fig. (5). Data in the two graphs represent the straight-line portion of the graph plotting the rate of diffusion versus the natural log (Ln) of molecular weight (9.3, 19.6, 38.9 and 71.2 kDa dextrans) (A) or molecular radius for each dextrans (B). The molecular (Stokes-Einstein) radii were culled from the literature [19,20].

GCL was also bright in 4.4 kDa test no matter whether ILM or photoreceptor was exposed to FITC-dextran (Fig. (**3A, D**)). Nevertheless, the neural ganglion cell nucleus are a single layer and array loosely, therefore the macromolecules can penetrate across GCL through the intervals. Despite that the surrounding region of ganglion cell nucleus caused barrier effect to dextrans and the nucleus in GCL could become bright under fluorescence examination, the GCL as a whole was not a barrier.

One weakness is that the present method was not fine enough to evaluate the MSM of external limiting membrane (ELM) which is a line of junctions between photoreceptor cells and Müller cells [24]. A study in rabbits found that ELM is a barrier to the transretinal diffusion of albumin [14].

The present findings may be helpful in explaining some clinical observations. The hard exudates observed in hypertensive and diabetic retinopathy are characterized by intraretinal deposits of serum and glial breakdown products and distributed at the level of OPL [25]. Given the large size of these macromolecules, if the ONL and INL act as barriers to diffusion *in vivo*, then it is not surprising that these deposits between the two layers may persist for many months. Based on their study result mentioned above that OPL was one of the diffusion barriers, Jackson *et al*. explained it otherwise [7].

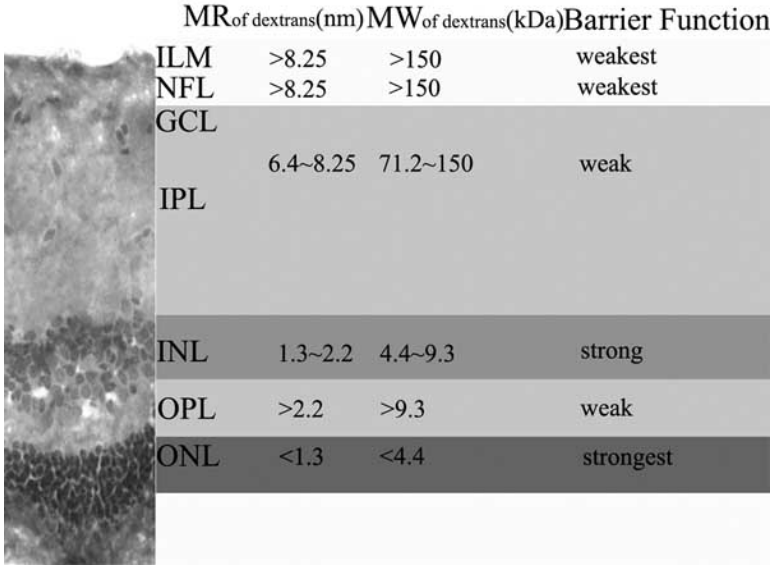

Fig. (6). The maximum molecular weight (MW) and theoretical molecular radii (MR) of dextrans which can freely diffuse through different layers in neuroretina. The shade of color is consistent with the barrier ability in that layer. The molecular (Stokes-Einstein) radii were culled from the literature [19,20].

The results of some basic research seemed to be contrary to the effect of drugs observed by clinical use. The improvement in visual acuity, decreased retinal thickness and reduction in angiographic leakage were observed in most neovascular age-related macular degeneration (AMD) patients after intravitreal injection of bevacizumab (Avastin) [3-5], whereas experiment in rhesus monkey showed that this full-length antibody (148 kD) could not penetrate the retina [9]. One possible reason may be that the MSM of neuroretina of AMD patients is much larger than that under normal condition. In some degenerative retinal diseases, such as retinitis pigmentosa and age-related macular degeneration, the ONL are damaged [26-29]. If ONL acts as a major barrier as we found, it will not be surprising that the diffusion limitation and rate of macromolecules may largely increase.

In summary, this study indicated that ONL and INL may act as the major barriers to diffusion. Compared with normal neuroretina, the MSM of fresh edema retina after RVO increased slightly, which may be helpful in estimating the diffusion of macromolecular drugs injected intravitreally in fresh retinal edema. Such data may also be useful in estimating the intraretinal distribution of drugs, but further studies are needed to understand the change of transretinal diffusion of macromolecules under other different pathological conditions.

ACKNOWLEDGEMENTS

This research was supported by the National Basic Research Program of China under the project: 2005CB724307.

REFERENCES

[1] Koevary, S.B. (2003) *Curr. Drug Metab*., **4**(3), 213-222.
[2] Gragoudas, E.S.; Adamis, A.P.; Cunningham, ET. Jr.; Feinsod, M. and Guyer, D.R. (2004) *N. Engl. J. Med*., **351**(27), 2805-2816.
[3] Avery, R.L.; Pieramici, D.J.; Rabena, M.D.; Castellarin, A.A.; Nasir, M.A. and Giust, M.J. (2006) *Ophthalmology*, **113**(3), 363-372.
[4] Rosenfeld, P.J.; Moshfeghi, A.A. and Puliafito, C.A. (2005) *Ophthalmic. Surg. Lasers Imaging*, **36**(4), 331-335.
[5] Maturi, R.K.; Bleau, L.A. and Wilson, D.L. (2006) *Retina*, **26**(3), 270-274.
[6] Ghazi, N.G.; Noureddine, B.; Haddad, R.S.; Jurdi, F.A. and Bashshur, Z.F. (2003) *Retina*, **23**(6), 780-784.
[7] Jackson, T.L.; Antcliff, R.J.; Hillenkamp, J. and Marshall, J. (2003) *Invest. Ophthalmol. Vis. Sci*., **44**(5), 2141-2146.
[8] Gaudreault, J.; Fei, D.; Rusit, J.; Suboc, P. and Shiu, V. (2005) *Invest. Ophthalmol. Vis. Sci*., **46**(2), 726-733.
[9] Mordenti, J.; Cuthbertson, R.A.; Ferrara, N.; Thomsen, K.; Berleau, L.; Licko, V.; Allen, P.C.; Valverde, C.R.; Meng, Y.G.; Fei, D.T.; Fourre, K.M. and Ryan, A.M. (1999) *Toxicol. Pathol*., **27**(5), 536-544.
[10] Kamei, M.; Misono, K. and Lewis, H. (1999) *Am. J. Ophthalmol*., **128**(6), 739-746.
[11] Kimura, A.E.; Reddy, C.V.; Folk, J.C. and Farmer, S.G. (1994) *Retina*, **14**(1), 83-84.
[12] Boone, D.E.; Boldt, H.C.; Ross, R.D.; Folk, J.C. and Kimura, A.E. (1996) *Retina*, **16**(6), 518-524.
[13] Coll, G.E.; Sparrow, J.R.; Marinovic, A. and Chang, S. (1995) *Retina*, **15**(4), 319-326.
[14] Bunt-Milam, A.H.; Saari, J.C.; Klock, I.B. and Garwin, G.G. (1985) *Invest. Ophthalmol. Vis. Sci*., **26**(10), 1377-1380.
[15] Peyman, G.A. and Bok, D. (1972) *Invest. Ophthalmol*., **11**(1), 35-45.
[16] Tso, M.O.M. and Shih, C.Y. (1977) *Invest. Ophthalmol. Vis. Sci*., **16**(5), 381-392.
[17] Zhang, X.; Ma, Z.; Hu, Y. and Fan, J. (2002) *Curr. Eye Res*., **24**(4), 263-267.
[18] Curry, F.E. (1984) in *Handbook of Physiology*, (Renkin, E.M. and Michel, C.C. Eds.), Am Physiological Society Press, Bethesda, pp. 251-309.
[19] Ambati, J.; Canakis, C.S.; Miller, J.W.; Gragoudas, E.S.; Edwards, A.; Weissgold, D.J.; Kim, I.; Delori, F.C. and Adamis, A.P. (2000) *Invest. Ophthalmol. Vis. Sci*., **41**(5), 1181-1185.
[20] Pitkanen, L.; Ranta, V.P.; Moilanen, H. and Urtti, A. (2005) *Invest. Ophthalmol. Vis. Sci*., **46**(2), 641-646.
[21] Kohner, E.M.; Dollery, C.T.; Shakib, M.; Henkind, P.; Paterson, J.W.; De Oliveira, L.N. and Bulpitt, C.J. (1970) *Am. J. Ophthalmol*., **69**(5), 778-825.
[22] Takeuchi, A.; Kricorian, G.; Yao, X.Y.; Kenny, J.W. and Marmor, MF. (1994) *Invest. Ophthalmol. Vis. Sci*., **35**(11), 3792-3798.
[23] Marmor, M.F.; Negi, A. and Maurice, D.M. (1985). *Exp. Eye Res*., **40**(5), 687-696.
[24] Bernstein, M.H. (1985) *Scan Electron Microsc*., (Pt 2), 859-868.
[25] Yannof, M. and Fine, B.S. (1996) in *Ocular Pathology*, (Yannof, M. and Fine, B.S. Eds.), Mosby-Wolfe Press, London, pp.551–575.
[26] Humayun, M.S.; Prince, M.; de Juan E Jr; Barron, Y.; Moskowitz, M.; Klock, I.B. and Milam, A.H. (1999) *Invest. Ophthalmol. Vis. Sci*., **40**(1), 143-148.
[27] Milam, A.H.; Li, Z.Y. and Fariss, R.N. (1998) *Prog. Retin. Eye Res*., **17**(2), 175-205.
[28] Santos, A.; Humayun, M.S.; de Juan, E. Jr.; Greenburg, R.J.; Marsh, M.J.; Klock, I.B. and Milam, A.H. (1997) *Arch. Ophthalmol*., **115**(4), 511-515.
[29] Papermaster, D.S. and Windle, J. (1995) *Invest. Ophthalmol. Vis. Sci*., **36**(6), 977-983.

Received: May 4, 2006 **Accepted: November 2, 2006**