Multidisciplinary Ophthalmic Imaging

Oxygen-Induced Retinopathy and Choroidopathy: In Vivo Longitudinal Observation of Vascular Changes Using OCTA

Yongjoo Kim,1,2 Hye Kyoung Hong,3 Jang Ryul Park,1,2 WooJhon Choi,2 Se Joon Woo,3 Kyu Hyung Park,3 and Wang-Yuhl Oh1,2

1Department of Mechanical Engineering, Korea Advanced Institute of Science and Technology (KAIST), Yuseong-gu, Daejeon, Republic of Korea
2KI for Health Science and Technology, Korea Advanced Institute of Science and Technology (KAIST), Yuseong-gu, Daejeon, Republic of Korea
3Department of Ophthalmology, Seoul National University College of Medicine, Seoul National University Bundang Hospital (SNUBH), Seongnam, Gyeonggi-do, Republic of Korea

Correspondence: Wang-Yuhl Oh, Department of Mechanical Engineering, Korea Advanced Institute of Science and Technology (KAIST), 291 Dachak-ro, Yuseong-gu, Daejeon 34141, Republic of Korea; wohl@kaist.ac.kr.

METHODS. To induce OIR, Sprague Dawley rat pups were incubated in an 80% oxygen chamber from postnatal day 1 (P1) to P11 and returned to room air. OCTA imaging was performed in six eyes at P15, P18, P21, and P24. All eyes were imaged with ex vivo retinal flat mount immunofluorescence microscopy for comparison with OCTA. The areas of the neovascular tufts, retinal vessel tortuosities and diameters, and vessel densities of different retinal and choroidal layers were quantified.

RESULTS. The neovascular tufts were observed in two OIR eyes. The tuft areas decreased spontaneously from P18 to P24. The increase in arterial tortuosity and venous dilation were observed in the OIR eyes at P15 and P18. The retardation of vascular developments was observed in the deep vascular plexus and the choroidal layer in the OIR group while the superficial vascular plexus did not show developmental delay.

CONCLUSIONS. This study demonstrates an application of OCTA for quantitative and longitudinal studies on in vivo vascular alterations, including neovascular tufts, increase in arterial tortuosity, venous dilation, and developmental delay in the OIR rat model.

Keywords: oxygen-induced retinopathy, optical coherence tomography angiography, rat

The oxygen-induced retinopathy (OIR) model has been widely used in angiogenesis studies and preclinical trials of antiangiogenic drugs. Historically, retinas of various animal species such as cats, dogs, mice, and rats have been used for OIR studies.1–4 Exposing neonatal pups to the hyperoxic condition for multiple days and returning them to the normoxic environment mimics retinopathy of prematurity (ROP) in humans. While OIR is primarily a disease model for ROP, its vascular pathologies also show relevance to other ischemic retinal diseases such as proliferative diabetic retinopathy5 and retinal vein occlusion,6 in which abnormal blood vessel growth in the retina is considered as a hallmark of these diseases.7 The precise mechanisms driving neovascularization depend on diseases; however, the subsequent progressions are similar, resulting in hemorrhage from the leaky pathogenic neovessels and retinal detachment. For this reason, the OIR has been accepted as a valuable animal model in the investigation of the ROP and the ischemic retinopathies, providing the assessment of the pathologic retinal neovascularization.8

In vivo fundus fluorescein angiography (FA) and retinal flat mount immunofluorescence imaging have been widely used for observing retinal vascular abnormalities in the OIR animal models. Several studies investigated the OIR retina in vivo using FA, most of which focused mainly on the retinal vessel diameter, arterial tortuosity, vascular areas, and grades of fluorescein leakage.9–11 The low resolution and the difficulty of focusing the entire retinal surface of small animal eyeballs make FA an inaccurate technique for quantitative measurement of retinal vasculature. Using retinal flat mounts, retinal vascular abnormalities can be visualized, including the neovascularization also known as neovascular tufts. Quantification of the tufts using retinal flat mounts has been considered challenging as area measurement can be subjective and time intensive.12–15 Furthermore, the retinal vasculature is accessible only after euthanizing the animal, which makes it difficult to monitor...
longitudinal changes. The capability to follow up a single animal longitudinally is highly desirable, substantially reducing the number of animals required for statistical analysis and interanimal variability.

Optical coherence tomography angiography (OCTA) has been developed and utilized as an emerging imaging technique for visualization of the retinal and the choroidal vasculatures in clinical studies.20–22 OCTA detects the motion-induced decorrelation mainly caused by the movement of the erythrocytes in blood vessels by comparing repeatedly acquired OCT B-scans at the same location. This provides excellent contrast for visualizing retinal and choroidal vasculature. OCTA has several advantages over conventional retinal-imaging modalities. First, it is an in vivo imaging technique, which allows longitudinal observation of an individual subject. Second, it visualizes three-dimensional vasculature, providing exact three-dimensional locations of vascular changes and separately visualizing vasculatures in different depths or layers. Previous studies have also demonstrated the utility of OCTA in preclinical models, including laser-induced choroidal neovascularization, elevated intraocular pressure, and targeted retinal vessel occlusion.19–22

In this study, we present longitudinal observation of vascular changes in the OIR rat model using a prototype swept-source OCTA system. In the OIR eyes, the neovascular tufts were readily distinguished from the retinal vasculature, and the tuft areas were measured longitudinally. The longitudinal changes in diameters and tortuosities of the retinal arteries and veins were also observed and quantitatively compared between the control and the OIR eyes. In addition, two distinct intraretinal vascular plexuses and the choroidal vasculature were separately visualized to reveal vascular development delay in the OIR eyes by measuring vessel densities in each layer.

**METHODS**

**Animal Preparation**

Nonpigmented Sprague Dawley (SD) rat pups were used in this study. To induce OIR, pups with their nurturing mother were incubated in an 80% oxygen chamber cycled with 20% oxygen for 3 hours per day from postnatal day 1 (P1) to P11.23 At P12, the animals were returned to normal housing with 12-hour light and dark cycles. Another group of SD rat pups and a mother were prepared in normal housing as a control group. The corresponding three en face OCTA images, acquired at the center of optic nerve head (ONH), nasal retina, and temporal retina, were manually stitched together to visualize wide field retinal vasculature.

**OCTA Imaging**

A prototype swept-source OCTA system was used in this study. A detailed schematic and specification of the system were described in the previous publication.20 An area of 2 × 2 mm² was scanned for OCTA imaging. For angiographic imaging, nine B-scans were repeatedly acquired at each of 768 locations in the slow axis direction, resulting in a total of 6912 B-scans with 820 A-lines/B-scan. The inter-B-scan time interval and total imaging time were 4.45 millisecond and 30.77 seconds, respectively. At each cross-sectional location, the subpixel motion compensation was applied to repeated pairs of B-scans, and the intensity projection image was used to mask the shadows of hyaloid vessels by calculating decorrelation signal between each pair of consecutive B-scans, generating OCTA B-scan angiograms.20 A total of eight OCTA B-scan angiograms were produced at each cross-sectional location and averaged to enhance OCTA image quality. En face OCTA images were obtained by projecting maximum decorrelation signals within each A-line. En face mean intensity projection images, which are intrinsically coregistered to en face OCTA images, were additionally created. Residual hyaloid vessels in the vitreous attenuated the OCT beam significantly, leaving local regions of the signal void. To distinguish the hyaloid vessel shadow from the avascular retina in the en face OCTA image, the intensity projection image was used to mask the shadows of hyaloid vessels where the intensity signal was below a threshold value, which was determined as the three quartiles of the most frequent bin of the image histogram. The hyaloid vessel shadows were overlaid as the blue color in the en face OCTA image to distinguish true avascular regions from the shadows. Since the vascular response in the rat OIR model, in general, is initiated from the periphery of the retina, OCTA imaging was performed at three different locations on the retina by rotating the pup’s body with respect to the imaging optics. The corresponding three en face OCTA images, acquired at the center of optic nerve head (ONH), nasal retina, and temporal retina, were manually stitched together to visualize wide field retinal vasculature.

**Retinal Vessel Tortuosity and Diameter Measurement**

The tortuosities and diameters of retinal arteries and veins were measured in OCTA images. To distinguish between arteries and veins, flat mount images, which provide arterial-specific contrast by additionally staining abundant smooth muscle cells in the arterial wall, were compared with the corresponding OCTA images. The tortuosity measurements were performed over the vessel segments between two points located at 100 μm and 800 μm from the ONH. The tortuosity of each vessel segment was calculated as a ratio of the actual vessel segment length to a straight line distance between the two points. Diameters were measured at a predetermined distance 800 ± 50 μm from the optic nerve where the first bifurcation point resulted in the smallest variation between measurements at P24.11

**Layer-Specific Visualization of OCTA**

To identify the neovascular tufts that sprout above the surface of the retina, the inner limiting membrane (ILM) and retinal pigment epithelium (RPE) were segmented semi-automatically.
Immunofluorescence Retinal Flat Mount Imaging

After OCTA imaging at P24, the eyes were enucleated and fixed in 2% paraformaldehyde/PBS (pH 7.4) for 5 minutes. The retina and choroid were then isolated from eyecups and permeabilized with 0.5% Triton X-100 (Sigma-Aldrich Corp., St. Louis, MO, USA), 5% fetal bovine serum, and 20% dimethyl sulfoxide (DMSO) for 3 hours at room temperature. After washing, the retinas were incubated with anti-mouse CD31/PECAM (EDM Millipore Corp., Temecula, CA, USA) for the first antibody and conjugated goat anti-hamster IgG (H+L) (Alexa Fluor 488; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) for second antibody at 4°C for 2 days each. After staining, four cuts were made from the edges to the center of the tissue, which was flattened and mounted with the vitreous side up on a microscope slide and visualized with a confocal microscope (LSM710; Carl Zeiss, Oberkochen, Germany). The flat mount images were obtained by opening a 38-μm laser pinhole of the microscope and at 100× magnification using image analysis software (Zen 2011; Carl Zeiss). The superficial and deep vascular plexuses were separately visualized in the retinal flat mount imaging for comparison with OCTA. The superficial vascular plexus imaging was performed by axially positioning the scanning plane at the retinal tissue surface. The deep vascular plexus images were acquired by positioning the scanning plane 30 μm deeper than the retinal tissue surface.

Statistical Analysis

For statistical analysis of vascular parameters, means and standard errors of the mean were calculated for the control and the OIR groups at each time point. A 2-way ANOVA with Tukey post hoc test was used, and a P value of less than 0.05 was considered statistically significant. All analyses were performed using statistical software (GraphPad Prism 7; GraphPad Software, San Diego, CA, USA).

RESULTS

Wide Field OCTA Imaging

Figure 1 shows the OCTA retina observed at P24. Imaging fields that match with each of three single-shot en face OCTA images are indicated as red dashed boxes on the retinal flat mount image (Fig. 1A). A wide field en face OCTA image was obtained by stitching three single-shot images as shown in Figure 1B. The vascular patterns in two images, the retinal flat mount and the en face OCTA, show good correlation. The small bright areas delineated by the red-dotted contours in each en face OCTA image are artifacts caused by spurious reflection from the imaging optics of the OCTA system.

Observation and Longitudinal Monitoring of the Neovascular Tufts in the OIR Eyes

Among the six eyes of the OIR rats, the neovascular tufts were identified in two eyes when observed with OCTA. Figure 2 shows the neovascular tufts observed in a single eye at P24. The tufts were identified on only a single side of the entire wide OCTA imaging field. Figure 2B shows the middle right side area of the flat mount image (Fig. 2A) that corresponds to the field of the OCTA image containing the neovascular tufts (Fig. 2D). Figure 2C shows the magnified view of the tufts inside the dashed magenta rectangle in Figure 2B, and the yellow asterisks indicate each tuft. The same vascular tufts were observed in the OCTA image, and the segmented tufts were overlaid with magenta color as shown in Figures 2D and 2E. A horizontal line in Figure 2D is due to the residual sample motion. The cross-sectional OCT intensity B-scan corresponding to the location where the vascular tufts were observed (indicated by the red dashed line in Fig. 2C) is shown in Figure 2F. The decorrelation signals are overlaid with colors representing flows in different layers, retinal flow (green), choroidal flow (red), and flow in the tufts (magenta).

Figure 3 shows the longitudinal progression of the neovascular tufts in a single eye. Note that the orientation of the OCTA image at P18 was adjusted to correlate with the OCTA images at P21 and P24. From P18 to P24, three clusters of the tufts were identified and labeled as L1, L2, and L3. No tuft was identified in the OCTA image acquired at P15, and we attribute this to the shadows of abundant hyaloid vessels at the early stage. The neovascular tufts in all three clusters exhibited regression from P18 to P24 as shown in the en face OCTA images and the intensity B-scans intersecting each of three tuft clusters. While the tufts in L3 regressed the least, the cluster in L2 completely disappeared. For quantitative analysis of neovascular tuft size, the tuft areas within each cluster were measured after binarization of the en face OCTA images. The effect of eye size growth during the study period was examined by measuring a distance from ONH to the periphery of the retina in the flat mount image (Supplementary Fig. S2), and it
was concluded that there is negligible change in eye size and the physical dimension of en face OCTA images taken from P15 to P24. The graph in Figure 4 represents a relative change of neovascular tuft areas normalized to the areas measured at P18. It is noteworthy that the neovascular tuft areas decreased gradually, showing that the tufts were regressed spontaneously after P18.

Retinal Vessel Tortuosity and Diameter Measurement

Figure 5 represents measurements of retinal vessel tortuosities and diameters. Figure 5A shows a flat mount image of a control eye at P24. Figures 5B and 5C are flat mount and OCTA images of the same eye corresponding to the region indicated by the red-dotted box in Figure 5A, respectively. In the flat mount...
images, arteries appeared orange due to the smooth muscle cells, whereas veins appeared as green. The arteries and veins indicated in the flat mount were correspondingly observed in the OCTA image. Figure 5D and 5E show two representative cases of the OIR retina exhibiting arterial tortuosity. Tortuous arteries were well observed at P15 (orange arrows), whereas veins were not noticeably tortuous. The same arteries were observed at P18, which appeared less tortuous than at P15. Quantitative tortuosity measurements confirmed tortuous arteries at P15 and a significant reduction of arterial tortuosity from P18, while the veins were not tortuous at any time point in the OIR eyes, as shown in Figures 5D and 5E. The arterial diameter measurements (Fig. 5H) show no significant differences between the control and the OIR groups at all time points. The venous diameter at P15 was measured to be larger in the OIR retinas than in the controls, but no significant difference in the vein diameter was observed from P18 as shown in Figure 5I.

Vascular Development in Retinal Vascular Plexuses

The retinal vascular structures in superficial and deep vascular plexuses were visualized using the OCTA and the retinal flat mount imaging. Figure 6 shows images of superficial and deep vascular plexuses of the same eye acquired by the two imaging modalities at P24. The retinal flat mount images (Figs. 6A1, 6A2) were reprocessed to match the imaging field of OCTA (Figs. 6B1, 6B2). In the superficial vascular plexus images (Figs. 6A1, 6B1), the vascular patterns show a good agreement. Cases of the OIR retina exhibiting arterial tortuosity. Tortuous arteries were well observed at P15 (orange arrows), whereas veins were not noticeably tortuous. The same arteries were observed at P18, which appeared less tortuous than at P15. Quantitative tortuosity measurements confirmed tortuous arteries at P15 and a significant reduction of arterial tortuosity from P18, while the veins were not tortuous at any time point in the OIR eyes, as shown in Figures 5D and 5E. The arterial diameter measurements (Fig. 5H) show no significant differences between the control and the OIR groups at all time points. The venous diameter at P15 was measured to be larger in the OIR retinas than in the controls, but no significant difference in the vein diameter was observed from P18 as shown in Figure 5I.

Vascular Development in Retinal Vascular Plexuses

The retinal vascular structures in superficial and deep vascular plexuses were visualized using the OCTA and the retinal flat mount imaging. Figure 6 shows images of superficial and deep vascular plexuses of the same eye acquired by the two imaging modalities at P24. The retinal flat mount images (Figs. 6A1, 6A2) were reprocessed to match the imaging field of OCTA (Figs. 6B1, 6B2). In the superficial vascular plexus images (Figs. 6A1, 6B1), the vascular patterns show a good agreement. Cases of the OIR retina exhibiting arterial tortuosity. Tortuous arteries were well observed at P15 (orange arrows), whereas veins were not noticeably tortuous. The same arteries were observed at P18, which appeared less tortuous than at P15. Quantitative tortuosity measurements confirmed tortuous arteries at P15 and a significant reduction of arterial tortuosity from P18, while the veins were not tortuous at any time point in the OIR eyes, as shown in Figures 5D and 5E. The arterial diameter measurements (Fig. 5H) show no significant differences between the control and the OIR groups at all time points. The venous diameter at P15 was measured to be larger in the OIR retinas than in the controls, but no significant difference in the vein diameter was observed from P18 as shown in Figure 5I.

Vascular Development in Retinal Vascular Plexuses

The retinal vascular structures in superficial and deep vascular plexuses were visualized using the OCTA and the retinal flat mount imaging. Figure 6 shows images of superficial and deep vascular plexuses of the same eye acquired by the two imaging modalities at P24. The retinal flat mount images (Figs. 6A1, 6A2) were reprocessed to match the imaging field of OCTA (Figs. 6B1, 6B2). In the superficial vascular plexus images (Figs. 6A1, 6B1), the vascular patterns show a good agreement. Cases of the OIR retina exhibiting arterial tortuosity. Tortuous arteries were well observed at P15 (orange arrows), whereas veins were not noticeably tortuous. The same arteries were observed at P18, which appeared less tortuous than at P15. Quantitative tortuosity measurements confirmed tortuous arteries at P15 and a significant reduction of arterial tortuosity from P18, while the veins were not tortuous at any time point in the OIR eyes, as shown in Figures 5D and 5E. The arterial diameter measurements (Fig. 5H) show no significant differences between the control and the OIR groups at all time points. The venous diameter at P15 was measured to be larger in the OIR retinas than in the controls, but no significant difference in the vein diameter was observed from P18 as shown in Figure 5I.

Vascular Development in Retinal Vascular Plexuses

The retinal vascular structures in superficial and deep vascular plexuses were visualized using the OCTA and the retinal flat mount imaging. Figure 6 shows images of superficial and deep vascular plexuses of the same eye acquired by the two imaging modalities at P24. The retinal flat mount images (Figs. 6A1, 6A2) were reprocessed to match the imaging field of OCTA (Figs. 6B1, 6B2). In the superficial vascular plexus images (Figs. 6A1, 6B1), the vascular patterns show a good agreement. Cases of the OIR retina exhibiting arterial tortuosity. Tortuous arteries were well observed at P15 (orange arrows), whereas veins were not noticeably tortuous. The same arteries were observed at P18, which appeared less tortuous than at P15. Quantitative tortuosity measurements confirmed tortuous arteries at P15 and a significant reduction of arterial tortuosity from P18, while the veins were not tortuous at any time point in the OIR eyes, as shown in Figures 5D and 5E. The arterial diameter measurements (Fig. 5H) show no significant differences between the control and the OIR groups at all time points. The venous diameter at P15 was measured to be larger in the OIR retinas than in the controls, but no significant difference in the vein diameter was observed from P18 as shown in Figure 5I.

Vascular Development in Retinal Vascular Plexuses

The retinal vascular structures in superficial and deep vascular plexuses were visualized using the OCTA and the retinal flat mount imaging. Figure 6 shows images of superficial and deep vascular plexuses of the same eye acquired by the two imaging modalities at P24. The retinal flat mount images (Figs. 6A1, 6A2) were reprocessed to match the imaging field of OCTA (Figs. 6B1, 6B2). In the superficial vascular plexus images (Figs. 6A1, 6B1), the vascular patterns show a good agreement. Cases of the OIR retina exhibiting arterial tortuosity. Tortuous arteries were well observed at P15 (orange arrows), whereas veins were not noticeably tortuous. The same arteries were observed at P18, which appeared less tortuous than at P15. Quantitative tortuosity measurements confirmed tortuous arteries at P15 and a significant reduction of arterial tortuosity from P18, while the veins were not tortuous at any time point in the OIR eyes, as shown in Figures 5D and 5E. The arterial diameter measurements (Fig. 5H) show no significant differences between the control and the OIR groups at all time points. The venous diameter at P15 was measured to be larger in the OIR retinas than in the controls, but no significant difference in the vein diameter was observed from P18 as shown in Figure 5I.

Vascular Development in Retinal Vascular Plexuses

The retinal vascular structures in superficial and deep vascular plexuses were visualized using the OCTA and the retinal flat mount imaging. Figure 6 shows images of superficial and deep vascular plexuses of the same eye acquired by the two imaging modalities at P24. The retinal flat mount images (Figs. 6A1, 6A2) were reprocessed to match the imaging field of OCTA (Figs. 6B1, 6B2). In the superficial vascular plexus images (Figs. 6A1, 6B1), the vascular patterns show a good agreement. Cases of the OIR retina exhibiting arterial tortuosity. Tortuous arteries were well observed at P15 (orange arrows), whereas veins were not noticeably tortuous. The same arteries were observed at P18, which appeared less tortuous than at P15. Quantitative tortuosity measurements confirmed tortuous arteries at P15 and a significant reduction of arterial tortuosity from P18, while the veins were not tortuous at any time point in the OIR eyes, as shown in Figures 5D and 5E. The arterial diameter measurements (Fig. 5H) show no significant differences between the control and the OIR groups at all time points. The venous diameter at P15 was measured to be larger in the OIR retinas than in the controls, but no significant difference in the vein diameter was observed from P18 as shown in Figure 5I.
FIGURE 5. Retinal vessel tortuosities and diameters measurement. (A) A flat mount image of a control eye at P24. (B) A flat mount image of the same eye reprocessed from the whole mount image to visualize the red-dotted rectangle in (A). (C) Wide field OCTA image of the same eye visualizing the region indicated by the red-dotted rectangle in (A). A and V denote retinal arteries and veins, respectively. (D, E) Retinal vessel tortuosity progression of OIR eyes from P15 to P18. The red circles indicate points located 100 μm and 800 μm from the optic nerve head. Scale bars: 200 μm. (F, G) Retinal arterial and venous tortuosity measurement. (H, I) Retinal arterial and venous diameter measurement. *P < 0.05, **P < 0.01.
the lowest at P15 and gradually increased over time, although it was still lower than that in the control eyes at P24.

Hyaloid Vasculature

When performing retinal OCTA imaging, the existence of the hyaloid vasculature and its morphology were confirmed indirectly as shadows (regions with low OCT signal) in an intensity projection image, as shown in Figure 9A. Figure 9B shows the en face OCTA image of the hyaloid vasculature acquired by adjusting the focus of the OCT imaging beam to the center of the vitreous space. The vascular patterns in Figures 9A and 9B are similar, which confirms the low-intensity regions observed on the retina were a consequence of the attenuation of OCT beam when passing the hyaloid vessels. Figure 9C shows the OCTA cross-sectional image intersecting blue dashed line in Figure 9B. While the hyaloid vessels in the vitreous space appeared as strong OCTA signals, the OCTA signals in the retinal layer were masked under the hyaloid vessels.

**DISCUSSION**

We performed longitudinal OCTA imaging to investigate vascular changes in the OIR rat model in vivo and correlated them with ex vivo histopathology obtained from retinal flat mount imaging.

OCTA enabled localization of the neovascular tufts and quantitative and longitudinal analysis of the tuft area change in each cluster. Although each tuft cluster showed a different rate, all clusters regressed gradually over time, which is consistent with the pathophysiology of the neovascular tufts in the OIR model.30,31 Quantitative and longitudinal analysis on retinal neovascularization using OCTA provides a significant advantage over FA since the assessment of vascular leakage, which is usually used for evaluation of disease severity in FA, involves subjectivity. Although ex vivo retinal flat mount imaging can also present the quantitative analysis of retinal neovascularization, the serial in vivo imaging is a significant advantage of OCTA. In addition to the neovascular tufts, changes in vascular parameters, including retinal vessel tortuosities and diameters, were investigated using OCTA. It was difficult to discern the retinal arteries and veins by OCTA alone, but the flat mount imaging complementary to OCTA allowed identifying each vessel. The venous dilation and the arterial tortuosity were observed in the OIR eyes at P15, which support the previous studies.11,32

The vascular development in superficial and deep plexuses of the OIR retina was also investigated. The vessel densities measured by OCTA revealed that there was no significant change in the development of superficial vascular plexus between the control and the OIR groups. In contrast, the development of deep vascular plexus was significantly retarded at P15 in the OIR retina. It is known that hyperoxia disturbs the vascular formation in developing retina, possibly due to the toxic effect of high oxygen level on vascular endothelial cells

The vascular development in the choroidal layer was further investigated. While the majority of OIR studies so far have focused on retinal vascular abnormalities, a previous study revealed choroidal involution in the OIR rat model.38 According to this study, the vascular area of choriocapillaris and choroidal layer thickness in the central region of the OIR...
eye were reduced at P14 and P60 with less severity. Considering that the imaging field of view of the en face OCTA images mainly covers the central region, our result is consistent with the previous study showing that the vessel density in the OIR choroid was the lowest at P15 and increased thereafter but still lower than the density in the control eye at P24. Although we also observed a clue of choriocapillaris flow reduction at P15 in the OIR eye, quantitative validation of the choriocapillaris alteration using OCTA was challenging due to the limited imaging resolution and robustness of choriocapillaris segmentation in cross-sectional OCTA images.

**FIGURE 7.** Longitudinal OCTA imaging of the superficial and deep vascular plexuses in (A) the control retina and (B) the OIR retina. The blue areas on OCTA images are shadows of residual hyaloid vessels. In deep vascular plexus images, projection artifacts caused by major retinal vessels in the superficial vascular plexus are shaded in pink. Artifacts enclosed by the red-dotted contours are caused by spurious reflections from the imaging optics of the OCTA system. Scale bars: 200 µm. Longitudinal measurements of vessel densities in (C) the superficial vascular plexus and (D) the deep vascular plexus. The error bar represents SEM. **P < 0.01.
Retinal avascularity is one of the pathologic features observed in earlier stages of the OIR model. A wide avascular area in the central retina was observed in a single eye (Supplementary Fig. S3), which was excluded from the longitudinal study because of an unintended death of the pup at P21. The OIR retina exhibits different avascular location on the retina (central or peripheral) depending on oxygen exposure protocols. In our study, the avascular area in the central retina was not observed in the rest of the eyes. Nonetheless, a single case with the central avascular area validates the capability of the en face OCTA to visualize vascular recovery in the avascular area. Direct visualization of the hyaloid vasculature was performed revealing the precise morphology of hyaloid vessels. It was confirmed that the local dark regions on en face intensity
projection images were certainly originated from the hyaloid vessels, which attenuated the OCT beam substantially. The visualization of the hyaloid vessel in this study shows that OCTA can be a potential imaging tool for studying anatomy and physiology of the hyaloid vascular system.

There are limitations in this investigation of the OIR rat model using OCTA. The sample size (n = 6) was not sufficient to generalize the results. In particular, the neovascular tufts were observed in only two eyes among six eyes enrolled in the study. This is because the neovascular tufts usually develop in the peripheral retina, and they do not always locate within the imaging field of OCTA. It would be beneficial to increase the imaging field by acquiring OCTA images on multiple locations on the retina. For instance, additionally imaging superior and inferior parts of the retina can increase the chance of observing the tuft by approximately twofold. However, this inevitably increases total imaging time required for an eye as well and increases the likelihood of acute cataract development induced by xylazine that disturbs OCTA imaging. Moreover, corneal calcification, the presence of hyaloid vessel shadows, and the permanent cataract induced by oxidative stress are inherent constraints of in vivo imaging, which should be taken into consideration when performing in vivo OCTA imaging. Although the imaging field of OCTA cannot cover the entire retina and might have missed part of retinal neovascularization, OCTA showed promising results providing detailed vascular images of different retinal layers, which is difficult from other imaging methods. There are various OIR protocols in rat retina with different onset, duration, and concentration of hyperoxia. Temporal and spatial vascular response can be different between models. Furthermore, there is strain dependency of vascular response in the same model. Therefore, when studying other models, the imaging results can be different from those shown in this study, and it is required to confirm results in each model. However, this is beyond the scope of this study, and further investigations are needed.

In conclusion, the current investigation has verified and shown OCTA as a prospective in vivo imaging technique for the OIR study in the rat retina and choroid. Quantification of the neovascular tufts was readily performed with a consistent layer segmentation algorithm. Depth-resolved OCTA visualized detailed vasculatures in different retinal and choroidal layers reliably, which was not easily achieved with other imaging methods. The serial quantitative measurement of vascular density, vascular tortuosity, and vessel diameter could be good measurement outcomes in addition to retinal neovascularization. These vascular features of the OIR model imaged using OCTA could be used in the preclinical studies evaluating the severity of retinal diseases and the therapeutic efficacy of antiangiogenic drugs.

Acknowledgments

Supported by the Ministry of Health & Welfare of Korea (H115C0001), the National Research Foundation of Korea (2016M3C7A1913843), and Seoul National University Bundang Hospital (SNUBH) fund (16-2015-005).

Disclosure: Y. Kim, None; H.K. Hong, None; J.R. Park, None; W. Choi, None; S.J. Woo, None; K.H. Park, None; W.-Y. Oh, None

References