Retinal Vessel Pathologies in a Rat Model of Periventricular Leukomalacia: A New Model for Retinopathy of Prematurity?

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PURPOSE. To characterize concurrent retinal vessel pathologies reminiscent to retinopathy of prematurity (ROP) in a rat model of periventricular leukomalacia (PVL), in order to identify uniform damage pathways in both organs, the eye and the brain.

METHODS. Ischemia was induced in Long Evans rat pups on postnatal day 6 (P6) with unilateral (left side) carotid ligation (UCL) followed by exposure to different oxygen concentrations. Four different groups were studied: group A, hypoxia/ischemia (UCL) + 6% O2; group B, hyperoxia (80% O2, 24 hours); group C, hypoxia/ischemia + hyperoxia (UCL + 6% O2, 1 hour + 80% O2, 24 hours); and group D, normoxia. In groups A and C, both retinas were examined separately (left retina, group A [A-L], right retina, group A [A-R]; left retina, group C [C-L], right retina, group C [C-R]). Morphologic analysis of vessel development based on flatmounts and cryosections was performed at P11 and P21. Quantitative (q)PCR was performed at P7, P11, and P21 (VEGF-A164, HIF-1α, iNOS, BMP-9, and IGF-1).

RESULTS. On flatmounts, distinct retardation in deeper vascular plexus development was observed, most prominent in A-L and C-L. Retinai of groups A-L and C-L displayed reduced capillary-free zones and an increased number of branching points at P11. Quantitative PCR analysis showed significantly different expression profiles of IGF-1 in A-L and B compared with D over the time course of the experiment.

CONCLUSIONS. This is the first report on concurring damage to the retina that was evaluated in a rat model of white matter injury in the developing brain. The relatively mild damage to the retinal vessel system may represent the basis for a model of moderate forms of ROP and to study vascular remodeling.

Keywords: retinopathy of prematurity, periventricular leukomalacia, unilateral carotid ligation, retinal vasculature, angiogenesis

In developed countries, approximately 10% of all infants are born preterm, and growing technical facilities and theoretical knowledge lead to a decrease in mortality even of those with birth weights lower than 1000 g.1,2 Due to the immaturity of their organ systems, very premature infants are at high risk for serious typical health problems like retinopathy of prematurity (ROP), intraventricular hemorrhage (IVH), periventricular leukomalacia (PVL), bronchopulmonary dysplasia (BPD), and necrotizing enterocolitis (NEC), which often mean a lifelong physical restriction for the children.3,4,5

Increased oxygen availability after birth and use of supplemental oxygen leading to oxidative stress can promote several of these injuries. Although clinicians are aware of the danger and toxicity of oxygen in this vulnerable group of patients,3 the optimal oxygenation of very preterm infants is still unknown,4,5 and as recently published data of large randomized multicenter trials are controversial in this regard.7,8

Infants born with less than 28 weeks of gestation and/or those with birth weights < 1000 g have an increased risk of developing treatment-requiring ROP, which, for example, affects approximately 400 to 600 infants per year in Germany.9 One pathogenic key factor is a dramatic increase of oxygen partial pressure after birth (95–100 mm Hg) compared with intraterine conditions, where the oxygen tissue tension is 25 to 35 mm Hg.10 Several growth factors, such as vascular endothelial growth factor (VEGF) or insulin-like growth factor 1 (IGF-1), which are highly influenced by oxygenation and play important roles in physiological organ and tissue development, are differently expressed and cause a change in the fragile homeostasis around birth.11,12

Retinopathy of prematurity can be separated into two phases, in which most importantly the VEGF expression levels are different.6 The first phase is characterized by a stop of retinal vessel growth because of environmental relative hyperoxia and downregulation of VEGF. The incomplete
maturation of the vasculature will subsequently cause relative hypoxia in unvascularized peripheral retina and upregulation of VEGF expression above normal levels. This can cause exaggerated vessel growth at the peripheral border of the vasculature, the development of a rim, and subsequent outgrowth of vessels into the vitreous, which may lead to serious visual impairment or blindness through retinal detachment in severe cases.\textsuperscript{15} However, the vast majority of ROP cases regress spontaneously and do not require treatment.

A wealth of knowledge about mechanisms of angiogenesis and in particular retinal vessel development has been gained from the classic murine oxygen-induced retinopathy (OIR) model that was developed by Smith and colleagues.\textsuperscript{14–19} This model also serves as the standard model for ROP, even though clinical signs in these animals only partly mimic the situation in preterm infants. Mice are kept from P5 to P12 in 75% oxygen atmosphere, followed by normoxic conditions. During and after the hyperoxic situation, vessel growth stops due to relative hyperoxia, and the avascular area in the peripheral retina reaches its maximum at P14. Few days after return to room air, the animals develop peripheral neurovascular tufts additionally to the central avascular regions. The group of Penn and colleagues adopted a different approach by establishing a rat model of OIR by altering the oxygen concentration in the atmosphere in 24-hour cycles between 10% and 50% from birth until P14 followed by room air inhalation from P14 to P18.\textsuperscript{20} Peripheral avascular regions appear most prominent at P14 and peripheral hypervascula-

The brain disorder PVL is caused by oxygen imbalances and ischemia and evokes motoric, visual,\textsuperscript{24} and mental\textsuperscript{25} disabilities in later life.\textsuperscript{26,27} The disease is characterized by an increased sensitivity of immature oligodendrocytes to hypo- and hyper-

A model for PVL has existed in rats for more than a decade, and is induced by unilateral ligation of the common carotid artery (UCL) at P6 and exposure to hypoxia thereafter for 1 hour.\textsuperscript{51–53} The white matter damage is typically characterized by scoring the presence of myelin basic protein 1 (MBP-1), that is, the presence of oligodendrocytes in the periventricular region.\textsuperscript{28–30} Compared with the classic OIR models in mice and rats, the PVL model is induced by ischemia (UCL) and a short period of altered oxygen levels, but these insults are sufficient to induce oligodendrocyte cell death.

Even though both pathologic entities, ROP and PVL, develop regularly in preterm infants and are both related to altered oxygen conditions and hypoxia within the first week of life, any possible correlations between both diseases have been only sparsely examined. It has been shown long ago that very preterm infants with severe ROP are at high risk of also developing brain damage.\textsuperscript{54} This observation was confirmed recently in a study including 173 children with severe ROP, who were at higher risk than their peers without ROP to have Bayley scales of mental and psychomotor development of 2 to 3 SD below the expected mean.\textsuperscript{55} The authors ascribed this increased risk either to shared risk factors between ROP and brain lesions or, less likely, to anesthetic neurotoxicity associated with ocular examinations. Ng and colleagues\textsuperscript{56} reported in 1989 a small series of six patients that suffered from ROP and PVL, in which they postulated that since cerebral and ocular blood supply in premature infants arises both from the internal carotid artery and episodes of hypoperfusion are frequent in premature neonates, cerebral ischemia and retinal ischemia can happen concomitantly; thus causing PVL and ROP simultaneously. Huang and colleagues\textsuperscript{57} reported recently that 33 out of 195 infants with ROP of any stage did suffer from PVL (17%), but could not correlate the presence of PVL with the severity of ROP or the necessity of treatment. Yang et al.\textsuperscript{58} determined that even 26% of ROP patients in their study also suffered from PVL, which led to restricted visual outcome of these children after 7 years of observation.

In order to shed more light on the correlation of cerebral and retinal vessel abnormalities under ischemic and altering oxygen conditions, we aimed at studying retinal vessel abnormalities in the standard rat model of PVL. Even though the period of altered oxygenation in this model is rather short, we hypothesized that ischemia/hypoxia alone or in combination with transient hyperoxia may provoke vessel changes in the retina. We found significant alterations of the normal development of the retinal vasculature reminiscent of certain features of ROP. The long-term goal is to establish potential biomarkers that are significant for the onset of both retinal vessel abnormalities and PVL symptoms, first in the rat PVL model and subsequently in human preterm infants.

**Materials and Methods**

### Animals

Pregnant wildtype Long Evans rats were purchased from Janvier (Saint-Berthevin Cedex, France). Animal experiments were accomplished according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the German animal protection act. The local institutional animal care and use committee (IACUC) approved the study (MR-31/2011 and MR-93/2012).

### Unilateral Carotid Ligation (UCL)

Rat pups underwent surgery at P6. Animals were anesthetized with fentanyl (0.05 μg/10g BW, Fentanyl 0.1 mg; Ratiopharm, Ulm, Germany); medetomidine (1.5 μg/10g BW, Domitor 1 mg/mL; Janssen, Neuss, Germany); and midazolam (20 μg/10g BW, Dormicium V 5 mg/5 mL; Roche, Grenzach-Wyhlen, Germany) mixture. An incision (1 cm) was made on the left side of the animals’ necks. The left common carotid artery was separated from the vagus nerve and the jugular vein and ligated using electrocauterization. Antagonization of the drugs was accomplished with naloxone 1.2 μg/10g BW, (Naloxon Intresa 0.4 μg; Intresa, Freiburg, Germany); atipamezole 7.5 μg/10 g (Anti-sedan 5 mg/ml; Janssen); and flumazenil 2 μg/10 g BW (Flumazenil Intresa, 0.5 mg/5 mL; Intresa), leading to full recovery after 5 minutes. For pain inhibition, animals received metamizole natrium 1 mg/10 g BW (Novaminsulfon 1 g/2 mL, Ratiopharm).

### Oxygen Treatment

Following UCL, rat pups were left to recover for approximately 1 hour and subsequently exposed to different oxygen conditions. Four different experimental groups were created (Fig. 1). Animals in group A (ischemia/hypoxia) received UCL and were then exposed to 6% oxygen for 1 hour. Distinction between left and right eyes of group A (A-L and A-R) was obtained because of unilateral ligation. Animals in group B (hyperoxia) did not receive UCL (no side differentiation), but 80% oxygen for 24 hours. Animals in group C (ischemia/hypoxia + hyperoxia) received UCL and were treated with 6% oxygen for 1 hour, followed immediately with 80% oxygen for 24 hours (differentiation between left
and right eyes of group C, C-L and C-R). Animals in group D served as control with sham operation but without UCL and oxygen treatment.

Figure 1 shows experimental setup and processing of animal experiments and treatment of the different groups. In each experimental round, one animal per group was killed at P7 for gene expression analysis (GEA). At P11 and P21 each, one animal per group was killed for GEA and one of each group for IHC. Four experimental rounds were performed totaling 80 animals with four animals per group and experimental time point for GEA as well as for IHC.

**Immunohistochemical Staining and Quantification**

At P11 and P21, rat pups were killed by perfusion with 4% paraformaldehyde (PFA). Eyes were enucleated and received additional fixation in 4% PFA for 2 hours at room temperature. Neuroretina was isolated and stained with isoelectin B4 (Life Technologies, Darmstadt, Germany) for 2 hours.

Fluorescence microscopy was performed with a digital microscope, viewer, and software (Keyence Bioproto fluorescence microscope, Keyence Bioproto II Viewer, and Biozero II Analyzer Software; Keyence, Neu-Isenburg, Germany). In total, 32 eyes were examined at P11 (×4 A-L, ×4 A-R, ×8 B, ×4 C-L, ×4 C-R, ×8 D) and 28 eyes on P21 (×4 A-L, ×4 A-R, ×8 B, ×3 C-L, ×3 C-R, ×6 D).

Expansion of the deep vascular plexus was measured at P11 and P21 and indicated in relation to total retinal area. Diameters of big retinal arteries were measured at P11 and P21 in central retina and midperiphery (halfway between center and ora serrata). Only arteries with diameters larger than 20 μm were included. Capillary-free zones (CFZ) were determined at P11 along 300 μm length of big retinal arteries in the midperipheral retina and set in relation to the corresponding vessel diameter. We used a modified version of a published method for counting branching points (BPs)/intercapillary junctions. The number of BPs was determined per millimeter of vascular front of P11 superficial vascular plexus in at least four fields per retina. One junction was counted where three capillaries met, two junctions were counted where four capillaries met, and so on.

**Gene Expression Analysis**

Animals were killed by decapitation, eyes were enucleated, and neuroretina was isolated immediately. Retinas were homogenized with a tissue homogenizer (Precellys 24; Peqlab Biotechnologie GmbH, Erlangen, Germany); RNA was extracted with a commercial kit (RNeasy Mini Kit; Qiagen GmbH, Hilden, Germany); and DNase digestion was done by RNase-free DNase (Qiagen GmbH). According to the minimum information for publication of quantitative real time PCR experiments (MIQE) guidelines, three cDNAs were prepared out of one RNA sample. We used 800-ng RNA per reaction, and RNA quality was checked with a commercial RNA cartridge (QIAxcel RNA Quality Control Cartridge; Qiagen GmbH).

In total, gene expression of 96 retinae (32 at P7, 32 at P11, and 32 at P21) was examined. Quantitative PCR was performed with Realplex 4 Cycler (Eppendorf AG, Hamburg, Germany) according to the MIQE guidelines. Two PCR runs were done per gene and sample. Primers for hypoxia inducible factor (HIF)-1α, VEGF-A164, and endothelial nitric oxide synthase (NOS)-2 were taken from Chidlow et al., primers for bone morphogenetic protein (BMP)-9 were modified from Ricard et al., and primers for insulin-like growth factor IGF-1 were taken from El-Bahr. Hypoxanthine phosphoribosyltransferase 1 (HPRT-1) was used as housekeeping gene (Table).

**Statistical Analysis**

Statistical analysis was performed using the R program for statistical computing (version 3.1.1). Immunohistologic data at P11 and P21 were analyzed using a linear-mixed model that included main effects for—as well as possible interaction between—the hypoxia/ischemia and hyperoxia treatments. A nested factor, indicating which side (left/right) the measurement was taken on, was included for UCL groups due to the stronger effect of UCL on the animal’s ligated left side. The mixed model incorporated the intrasubject correlation between measurements taken on the same animal (left/right side) into the analysis as well. As an exception, beta regression for measurements made on closed intervals was used to analyze the data for deep plexus expansion at P21, since these values were close to or reached the maximum of 100% in several groups.
For analyses regarding the changes in artery diameter from P11 to P21 and gene expression from P7 to P11 and P21, a mixed model was used to compare each of the five treatment groups (A-L, A-R, B, C-L, and C-R) to the control group D, while accounting for intrasubject correlation.

Statistical significance was assumed at $P < 0.05$.

### RESULTS

#### Mild Changes to the Plexus Outgrowth in A-L and C-L

Representative images of ipsi- and contralateral retinal flatmounts stained with isoelectin B4 of each group at P11 and P21 are presented in Figures 2 and 3, respectively. These time points represent the vasoproliferation phase in the model. We did not observe dramatic vascular changes reminiscent of those observed in the classic OIR mouse or rat model. Vessel abnormalities, such as tortuositas, extravasations, vasoconstriction, arteriovenous crossing, or neovascular tufts, were observed mostly in flatmounts of A-L and C-L at P21 (Supplementary Fig. S1). However, no significant increase was detected for any type of vascular abnormality on its own.

#### Retardation of the Deep Vascular Plexus Growth in Groups A-L and C-L

When looking more closely at the plexus outgrowth, we found that the expansion of the deep vascular plexus was retarded in all intervention groups at P11, most prominent in A-L and C-L (Figs. 4b, 4c). Those groups showed a reduction of the deep plexus expansion of 26% and 17%, respectively, whereas the remaining groups featured reductions of 2% to 12%. Ischemia/hypoxia had a significant effect on the values of the left eyes of groups A and C compared with the effects on the right eye (Fig. 4e, $P < 0.001$). At P21, full vascularization of the retina was observed in D (control group), A-R, B, and C-R. In contrast, the deep plexus had not yet reached the retinal periphery (ora serrata) in A-L and C-L, covering only 88% of the retina. The combination of ischemia and hypoxia had significant effects on the left eyes compared with all other eyes and compared with the right eyes only (Fig. 4e).

In order to confirm our findings, cryosections were prepared out of the flatmounts (P11) of one experimental round (Fig. 4d). Distances between ora serrata and first sprouts of the deep vascular plexus were measured and were almost equal on flatmounts and cryosections. Furthermore, largest distances were found in A-L (2321 $\mu$m on flatmounts/2282 $\mu$m on cryosection) and C-L (1574 $\mu$m on flatmounts/1615 $\mu$m on cryosections).

#### Diameters of Retinal Arteries Are Enlarged

Alterations of retinal vessel diameter are a typical sign of ongoing ROP in humans. In the rat PVL model, we did not observe any significant changes to venous diameter at P11 or P21 (data not shown). Likewise, we found no differences in the diameters of big retinal arteries in the intervention groups compared with control retinæ at P11 (Fig. 5c). At P21, however, clear enlargements of artery diameters in the central

### TABLE. List of Primer Pairs Used in This Study

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<th>Gene Fragment Size</th>
<th>Primer Sequence</th>
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<tr>
<td>BMP9 (bone-morphogenetic protein/GDF2) 293 bp</td>
<td>Sense GAAAGGGAGCACTGCTGCGAGCC Antisense GGTTGCGACGCCATGCTATCC</td>
</tr>
<tr>
<td>EpoR (erythropoietin-receptor) 115 bp</td>
<td>Sense CGGCTCATCGTTTCTGGT Antisense ATGGCCTGTGAGGAGAGATC</td>
</tr>
<tr>
<td>HIF-1 (hypoxia inducible factor 1, alpha subunit) 180 bp</td>
<td>Sense GCACCCATCGCCATGGAGG Antisense TCACTTTGTGGAAGTCC</td>
</tr>
<tr>
<td>HPRT (hypoxanthine phosphoribosyltransferase 1) 105 bp</td>
<td>Sense TTGTTGATATGCCCTTGACT Antisense CCGCGTCTTTTATGGGTT</td>
</tr>
<tr>
<td>IGF-1 (insulin like growth factor 1) 170 bp</td>
<td>Sense CTGGGTGTGCCAAATGTA CT Antisense GATACTTATGAGGAGAT</td>
</tr>
<tr>
<td>NOS-2 (inducible nitric oxide synthase 2) 225 bp</td>
<td>Sense CTGGAGGTGCTGGAAGAGTT Antisense CTGCGGCTTCCAGTTATG</td>
</tr>
<tr>
<td>TNFz (tumor-necrosis-factor alpha/TNF superfamily, member 2) 111 bp</td>
<td>Sense AAATTGGCTCCCTCTCATCAGTT Antisense CTGCTTTGGTTGTGGATAGACC</td>
</tr>
<tr>
<td>VEGF-A (vascular endothelial growth factor A/164) 180 bp</td>
<td>Sense ATGAACTTTCTGCTCTGGTGC Antisense ATGCGGACAGCAATAGCTGCCT</td>
</tr>
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retina were observed in all intervention groups compared with the control group (Fig. 5d). Most prominent changes were found in A-L (+61%) and C-L (+74%). Groups that received ischemia/hypoxia treatment always had significantly increased artery diameters (Fig. 5i).

By comparing artery diameters at P11 and P21 in the central retina, physiological reduction of the vessel diameter (control group: from 41.83 μm at P11 to 32.19 μm at P21) was disturbed in all intervention groups and had turned into enlargement. Group C-L showed the most striking increase from 36.84 μm at P11 to 56.13 μm at P21, but similar results were found in groups A-L, A-R, B, and C-R (Fig. 5c). These changes in vessel growth from P11 to P21 compared with the control group were significant to a varying degree for all groups except B (Fig. 5i). In the midperipheral retina, modifications of vessel diameters compared with the control group were less prominent (Fig. 5f) and not significant (Fig. 5i).

Comparing central and midperipheral values at P11 (Fig. 5g), a distinct reduction of artery diameter was noticed in group D representing the physiological situation, and similar results were observed in all other groups.

Central and midperipheral artery diameters were unaltered at P21 in group D (32.17 μm and 31.17 μm; Fig. 5h). In contrast, enlarged central artery diameters led to nonphysiological reductions of vessel diameters between central and midperipheral measuring points in all intervention groups. This reduction was most prominent in C-L and A-L (highly significant for C-L; Fig. 5i).

FIGURE 2. Representative images of retinal flatmounts stained with isolectin B4 at P11 for each group. (a, b) Group A. (c, d) Group B. (e, f) Group C. (g, h) Group D. (a, c, e, g) Flatmounts of left eyes. (b, d, f, h) Flatmounts of right eyes.

FIGURE 3. Representative images of retinal flatmounts stained with isolectin B4 at P21 for each group. (a, b) Group A. (c, d) Group B. (e, f) Group C. (g, h) Group D. (a, c, e, g) Flatmounts of left eyes. (b, d, f, h) Flatmounts of right eyes.
Values for CFZ and BP Are Altered in All Groups With Modified Oxygen Supply

To analyze the effects of oxygen supply and the maturation status of experimental retinae at P11, we measured capillary-free zones in relation to vessel diameter (VD; Fig. 6a). We found that the ratio of CFZ and VD (CFZ/VD) was reduced to 3.15 in A-L, to 4.12 in A-R, and to 4.30 in C-L compared with control group D (4.59), and was increased to 6.29 in B and to 5.60 C-R (Fig. 6b). Significant effects could be attributed to hyperoxia, as well as to ischemia/hypoxia in the left eyes (Fig. 6c).

In counting BPs of the superficial vascular plexus at P11 (Fig. 6c), we verified the maturation status of the retinal vessel system. An increase in the number of BP is a distinct feature of an immature vessel system at this time point. Figure 6d shows an increase in the number of BP in all groups compared with group D (75.23 BP/mm²). Most noticeable and significant changes were found in A-L with 177.77 BP/mm² and C-L with 117.80 BP/mm², showing a significant effect of ischemia/hypoxia on the left eyes in general and in particular compared with the right eyes (Fig. 6e).

Gene Expression Analysis

In order to analyze changes to classic parameters of angiogenesis, inflammation, and vessel maturation, we determined the expression profiles of HIF-1α, VEGF-A isoform 164, EpoR, TNFα (inducible) NOS-2, BMP-9, and IGF-1 at P7, P11,
FIGURE 5. Diameter of large retinal arteries. (a, b) P11 flatmount (group D) stained with isolectin B4 (green). (a) Measurement of diameter of big arteries (red marks) and veins (orange marks) in the central retina and in (b) the midperipheral retina. (c, d) Variation of artery diameter at P11 (c) and P21 (d) in the central and the midperipheral retina. Graphs show moderate changes in vessel diameter at P11 but significant increase of artery diameter in central retina at P21, most remarkable in A-L and C-L. (e, f) Changes in artery diameter between at P11 and P21 in the central retina (e) and in the midperipheral retina (f). In the central retina, physiological reduction of vessel diameter between P11 and P21 is disturbed in all intervention groups. In the midperiphery, physiological assimilation of vessel diameter between P11 and P21 is not visible in intervention groups.
and P21 (Fig. 7). In total, changes in gene expression over the
time course of the experiment compared with the control
group were only mild and not significant, with the only
exception of the profile for IGF-1 (Fig. 7g). Expression of IGF-1
in groups A and B was increased at P7, and a significant
reduction of gene expression over time was observed for A-L
compared with control group D at P11 and P21 and for group
B over time at P21.

The remaining groups and expression profiles did indeed
not show significant alterations, but some changes indicated

**FIGURE 6.** Measurement of CFZ and BP. (a) Description of the measurement technique of capillary-free zones around large arteries on a P11
flatmount (group D) stained with isoelectric B4. Yellow bracket: 300-μm artery piece. Red lines: Maximum distance of first capillary sprouts within
300 μm. Orange lines: Minimum distance of first capillary sprouts within 300 μm. Blue marks: Artery diameter at the start, midterm, and end of a
300-μm artery piece. (b) Graphical illustration of CFZ/VD for all groups: A reduction of the quotient is most obvious and significant in A-L, followed
by A-R. An increase of quotient/widening of CFZ can be observed in B and C-R. (c) Description of the quantification method of branching points in
500 × 500 μm retina. (d) An increase in the number of BP is present in all intervention groups, most prominent in A-L and C-L. (e) Statistical analysis.

**Table:**

<table>
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<tr>
<th>Experiment</th>
<th>Linear hypothesis</th>
<th>p value</th>
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<tr>
<td>CFZ/VD ratio at P11</td>
<td>H ypoxia</td>
<td>0.015*</td>
</tr>
<tr>
<td></td>
<td>Hypoxia/Hypoxia L</td>
<td>0.004**</td>
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<tr>
<td></td>
<td>Hypoxia/Hypoxia R</td>
<td>0.327</td>
</tr>
<tr>
<td></td>
<td>Hypoxia/Hypoxia L-R</td>
<td>0.014*</td>
</tr>
<tr>
<td>Branching points at</td>
<td>H ypoxia</td>
<td>0.022*</td>
</tr>
<tr>
<td>P11</td>
<td>Hypoxia/Hypoxia L</td>
<td>&lt;0.001***</td>
</tr>
<tr>
<td></td>
<td>Hypoxia/Hypoxia R</td>
<td>0.121</td>
</tr>
<tr>
<td></td>
<td>Hypoxia/Hypoxia L-R</td>
<td>&lt;0.001***</td>
</tr>
</tbody>
</table>

The linear hypotheses for each main effect are the following. Hypoxia: Did the hyperoxia groups have lower/higher values of the parameter in question averaged across the ischemia groups? Ischemia/hypoxia L: Were the values of the ischemia/hypoxia groups (the left eye) lower/higher than those for the groups without ischemia/hypoxia? Ischemia/hypoxia R: Were the values of the ischemia/hypoxia groups (the right eye) lower/higher than those for the groups without ischemia/hypoxia? Ischemia/hypoxia L-R: Were the values of the left eye lower/higher than those for the right eye in the ischemia/hypoxia groups?
DISCUSSION

The present study describes for the first time retinal vessel abnormalities in a standard rat model of PVL, which can very likely be used as biomarkers in further studies on the correlation of retinal vascular changes and cerebral damage. The observed abnormalities include a retarded growth of the deep vascular plexus, increased numbers of local vascular pathologies, increased artery diameters, as well as alterations in the size of capillary-free zones and elevated numbers of branching points. It remains to be seen whether one of these markers can be further developed into a clinically relevant tool in premature infants. Regardless of any potential clinical outcome, the standard PVL model in rats represents a valuable model for analyzing the importance of ischemic insults to the vessel maturation in the retina.

The intervention groups can be sorted into two groups depending on the degree of vessel alteration. Moderate vessel modifications were observed in groups A-L and C-L (UCL ipsilateral), and mild vessel modifications in the contralateral retinae A-R and C-R, as well as in group B. The stronger phenotype in the retina ipsilateral to the UCL is likely due to the transitory ischemia, even though complete absence of perfusion is prevented by collateral vessels. The generally milder phenotype in eyes contralateral to UCL was expected. A similar observation was made in brain hemispheres following UCL and correlated with the death of nerve cells in the ipsilateral hemisphere, while cells in the contralateral hemisphere were almost not affected. Retinace from group B showed prominent retardation in vessel maturation that can be directly correlated with the transitory hyperoxia (for significance levels, see Fig. 6e). Similar experiments to examine the microvasculature of the neonatal rat brain demonstrated that hyperoxia alone has a degenerative effect on brain endothelial cells due to upregulation of NOS-3.47

We did not observe significant differences between A-L and C-L, which indicates that the major trigger for the manifestation of vessel pathologies/retardation of vessel maturation is indeed the ULC-induced ischemia in combination with subsequent hypoxia. Hyperoxia by itself does not seem to have a large effect following UCL. This would favor the hypothesis that ischemia is a common pathologic feature in both PVL and ROP, as it was postulated by Ng and colleagues.36 It is, however, not enough to induce dramatic vascular changes observed in classic OIR models.

As stated above, compared with the classic OIR models,14,23 vessel abnormalities in our ligation model are rather mild. Considering the data from the classic retinal ischemia model, this result is not surprising.48,49 In the former model, permanent ligation of the pterygopalatine artery (PPA) and external carotid artery (ECA) was performed, which—with respect to retinal ischemia—outlines a more effective method than the ligation of the common carotid artery (CCA). This is due to vessel anastomoses between ECA and internal carotid artery (ICA), and concomitant retrograde blood flow from ECA to ICA in case of CCA ligation. This leads to complete stop of blood flow within the first hours after ligation, and recovery of vascularization occurs partially until day 5 after surgery. The mild to moderate effects on the retinal vascularization of a permanent ligation of the CCA in the PVL model are probably related to the transitory character of the ischemia due to the above-mentioned anastomoses.

In terms of retinal vascularization, the PVL model is a mixture of a retina ischemia model and models with altering oxygen treatment regimens like in the ROP/OIR models. It was originally developed to induce maximum damage to immature oligodendrocytes in the brain. We show for the first time that changes in the retinal vessel system can also be observed, albeit at a slight to moderate level. As stated above, hyperoxia is only a minor contributor to the observed changes, and indeed, oxygen exposition time in the PVL model is much shorter (24 hours) than in the OIR mouse model (Smith,14 5 days) or the
ROP rat model (Penn, 14 days). Exposure time and fluctuation of oxygen levels are crucial for the manifestation of vessel pathologies, while constant high and low oxygen concentrations are not. Lower “hyperoxia” concentrations lead to peripheral avascular regions. In addition, retinal vessels in mice and rats do react differently to altering oxygen exposition. In particular, rats that were exposed to the OIR mouse oxygen regime showed a much milder phenotype.

Concerning groups A and C, the deep vascular plexus size at P11 is reduced in the left eye but not the right eye, showing the negative effect of ischemia in combination with hypoxia on the plexus outgrowth (significance at  0.001; see Fig. 4e). This demonstrates that ischemia itself could play a role in the pathology but may not be sufficient to trigger dramatic vessel alterations.

Diameters of big retinal arteries are enlarged in the central retina at P21 in all groups exposed to ischemia/hypoxia (highly significant  0.001 for both left and right eyes; see Fig. 5i), leading to an opposing growth of vessel diameters between P11 and P21 in these groups (increase in diameter) compared with control group D (decrease in vessel diameter). The physiological reduction of the vessel diameter in group D results from the different demand of oxygen in the remodeling retina compared to the initial high demand during plexus outgrowth. In contrast, ischemia/hypoxia seems to prolong the need for high oxygen levels, which may indicate retarded retinal remodeling. Furthermore, the physiological reduction of the vessel diameter between central and midperipheral retina at P11 is attenuated in A-L and C-L and takes place only until P21, again indicating a retarded vessel remodeling.

Based on data concerning the vessel diameters, it seems that ischemia and hypoxia in A-L indeed lead to reduced oxygen diffusion through big retinal arteries in the early stages and, therefore, to reduced CFZ areas (Fig. 6b). In contrast, the CFZ area and the CFZ/VD quotient are increased in B (Figs. 6b, 6e), reflecting the high oxygen tissue pressure. This effect is even likely the cause of physiological values for CFZ/VD quotient in group C (ischemia, hypoxia, and hyperoxia), where the hypoxia neutralizes the hypoxia effect.

The increased number of BP in A-L and C-L at P11 again indicates retardation in vascular remodeling in these retinae because of ischemia/hypoxia (highly significant  0.001; see Fig. 6e). Increased numbers of BP at a given time point are a typical sign of delayed vascular remodeling mechanisms, as the maturation status of a vessel system is expressed by the number of BP. Alteration processes in the vasculature lead to apoptosis of endothelial cells and therefore reduction of the number of intercapillary junctions (branching points).

Local vessel pathologies, like neovascular tufts, which were repeatedly observed exclusively in A-L and C-L, are very likely related to the induced manipulations. However, the insult of ischemia and the subsequent hypoxia might not have been strong enough to produce these signs more often, as it is the case in classic OIR models.

Gene expression analyses were performed at P7, P11, and P21 in order to cover the entire experimental period. All of the seven mRNA targets were chosen because of their known involvement in the regulation of angiogenesis, in the development of ROP, or in inflammatory mechanisms potentially leading to retinal vascular changes.

Even though HIF-1z has been shown to be the master regulator of hypoxia-related gene expression, and was typically found to be upregulated during the proliferative phase in the OIR mouse model, overexpression of this factor was not observed at any of the investigated time points. Interestingly, VEGF mRNA levels dropped immediately in those tissues that were exposed to 24-hour hypoxia. This, on one hand, demonstrates the influence of hypoxia in this model but is surprising given the unchanged HIF-1z levels at P7. The reason may be that HIF-1z expression is differently regulated at time points, which we do not cover, or that VEGF expression here is independent of HIF-1z activity, a mechanism proposed to be effective in some forms of cancer.

This demonstrates that ischemia itself could play a role in the development of ROP in the OIR mouse model, where VEGF levels dropped early and increased over time in groups B and C, while they were normal in group A. This dissociation of the expression profiles for the two genes in our model could not be explained so far.

Inflammatory targets TNF-z and iNOS have been shown to be dramatically upregulated in the proliferative phase of the murine OIR model, but only iNOS was upregulated at P7 in the PVL rat model. The inducible NOS has even been shown to be connected to increase HIF-1z activity in the OIR model. This correlation was not observed here. While upregulation in the classic OIR model was effective throughout the proliferative period until P21, this was not observed in our study for both inflammatory markers.

Expression of BMP-9, a marker shown to be important during vessel maturation in the retina, was not altered throughout the experiment.

In conclusion, the ischemia, which was induced through a UCL together with a subsequent hypoxia phase or in combination with hyperoxia, is suitable to induce moderate changes/pathologies of the developing retinal system in rats. Since most ROP patients do not develop severe forms of the disease, this model may have a high reference to the clinical situation and can as such very well serve as a disease model for mild to moderate forms of ROP. Furthermore, moderate alterations can be quantified and therefore be used as biomarkers in studies for the development of clinical diagnostic markers as well as experimental treatment studies.

In addition, since most of the pathologic features go along with typical signs of vascular remodeling, the model may also serve for the research of this physiological mechanism.

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