Medical Sciences

Impact of Chronic Neonatal Intermittent Hypoxia on Severity of Retinal Damage in a Rat Model of Oxygen-Induced Retinopathy

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Neonatal intermittent hypoxia (IH) followed by re-oxygenation in normoxia or supplemental oxygen (IHR) increases the risk for severe retinopathy of prematurity (ROP). The exact timing for the onset of retinal damage which may guide strategic interventions during retinal development, is unknown. We tested the hypothesis that chronic exposure of the immature retina to neonatal IH induces early manifestations of retinal damage that can be utilized as key time points for strategic pharmacologic intervention. Newborn rats were exposed to IH within 2 hours of birth (P0) until P14, or allowed to recover in room air (RA) from P14 to P21 (IHR). Retinal integrity and angiogenesis biomarkers were progressively assessed before (P0), during IH, and post IH (recovery in RA), or IHR, and compared to normoxic age-matched controls. Retinal damage occurred as early as day 3 of neonatal IH, consistent with vascular abnormalities and disturbances in the astrocytic template. These abnormalities worsened during IHR. Pharmacologic and non-pharmacologic interventions to identify, prevent, or minimize neonatal IH should be implemented shortly after birth in high risk preterm newborns. This strategy may lead to a reduction in the outcome of severe ROP requiring later invasive treatments.

Angiogenesis | Astrocytes | Neonatal Intermittent Hypoxia | Oxygen-Induced Retinopathy

Introduction
Retinopathy of prematurity (ROP) is a leading cause of childhood blindness worldwide [1]. It is a developmental vascular disorder characterized by abnormal growth of retinal blood vessels in the incompletely vascularized retina of extremely low gestational age neonates (ELGANs) who are <28 weeks gestation [2-4], and is especially severe in the sickest, most immature infant requiring long-term supplemental oxygen therapy[5,6]. The etiology of ROP is multifactorial and highly complex. It is now well established that intermittent hypoxia (IH), defined as brief, repetitive arterial oxygen desaturations followed by re-oxygenation in normoxia or hyperoxia with supplemental oxygen (IHR), plays a major role in the pathogenesis, progression, and severity of the ROP [7-15] and oxygen-induced retinopathy (OIR) [16-26]. Infants who are at the highest risk for severe ROP experience numerous episodes of IH during the first week of life, a critical time of retinal development in preterm infants [7-9,12-15]. These repeated, although brief, episodes of IH experienced by ELGANs occur in response to a variety of morbidities including, but not limited to, chronic lung disease (CLD), patent ductus arteriosus (PDA), apnea of prematurity (AOP)/bradycardia, sepsis, and intraventricular hemorrhage (IVH).

Repetitive IH episodes are major determinants in the impairment of retinal endothelial function leading to many ocular diseases, including ROP [25]. This is due to the sensitivity of the immature retina to fluctuations in inspired oxygen. The retina is one of the highest oxygen-consuming tissues of the body, exceeding even that of the brain [27-30]. Hypoxia triggers processes involved in angiogenesis through transcriptional activation of vascular endothelial growth factor (VEGF) via binding of hypoxia inducible factor (HIF)α to the VEGF gene promoter [31-33]. During re-oxygenation following an IH episode, or IHR, elevations in VEGF, a vascular permeability factor, causes retinal vessels to become leaky. Restitution of blood flow through severely injured, leaky microvasculature and hemorrhage, results in aberrant angiogenesis, cell death, and in extreme cases, retinal detachment and blindness. The role of retinal astrocytes in the development of the superficial retinal vasculature is well-described [34-37]. In normal retinal development, retinal astrocytes emerge from the optic nerve head preceding the retinal vasculature and form a scaffold or template. They produce VEGF to control and promote retinal endothelial cell (EC) proliferation, migration and vascular patterning [36-38]. Therefore, any disturbances in the astrocytic template strongly influence VEGF production and normal vascular patterning. Studies have shown that vascular pathology in the retina is regulated to a large extent by astrocytes and astrocyte-derived VEGF is essential for hypoxia-induced neovascularization [35].

The multifactorial pathogenesis of ROP may preclude the use of a single pharmacologic agent that targets the complex interactions of growth factors, oxidative stress, inflammatory responses, and membrane disruption. One possible strategy is identification of the exact timing for the onset of retinal damage in order to establish a “critical” window of opportunity for effective therapies. The neonatal IH/IHR rat model produces oxygen-induced retinopathy (OIR) with many characteristics consistent with severe ROP. This model has established that: a) clustering IH episodes produces a more severe form of OIR than scattered IH episodes [21]; b) the immature rat retina cannot sustain numerous IH events without irreversible damage [19]; and c) reactive oxygen species (ROS) accumulation during IHR contributes to the severity of OIR [19]. These findings suggest that the mechanisms associated with IHR are more injurious to the retina, and is likely due to resumption of blood flow through damaged vessels, a form of reperfusion injury. While these previous studies have contributed to advances in the knowledge regarding the role of neonatal IH in retinal damage, knowledge gaps exist including: a) the exact timing for the onset of IH-induced retinal damage; b) the primary site(s) of the damage; and c) the role of retinal astrocytes in IH-induced retinal damage.

Conflict of Interest: No conflicts declared.

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The current study aimed to close these gaps in knowledge and test the hypothesis that chronic exposure of the immature retina to neonatal IH induces early manifestations of retinal damage that can be utilized as key time points for strategic pharmacologic intervention. To prove our hypothesis, we conducted a series of time course experiments to examine retinal integrity at three developmental stages of the rat retina: 1) Birth (P0). The rat retina at birth is developmentally comparable to the human preterm retina which is avascular and highly susceptible to retinopathy [39]. Eye opening in rats which concurs with maturation of the neural retinal circuitry, occurs at around P14; 2) During neonatal IH exposure (P0-P14). During this period, resumption of oxygen between each IH event is accomplished with hyperoxia; and 3) During re-oxygenation in room air (P24-P21). This period is consistent with reperfusion injury. These experiments are continuations of our previous work [16-21] and were designed to provide a model for pre-clinical drug development and delivery systems that target IH-induced retinopathy in preterm infants.

Material and Methods
All experiments were approved by the State University of New York, Downstate Medical Center Institutional Animal Care and Use Committee, Brooklyn, NY. Animals were managed according to the ARVO Statement for the Use of Animals in Ophthalmic and Visual Research. Animals were treated humanely, according to the guidelines outlined by the United States Department of Agriculture and the Guide for the Care and Use of Laboratory Animals.

Experimental Design
Certified infection-free, timed-pregnant Sprague Dawley rats were purchased from Charles River Laboratories (Wilmington, MA) at 18 days gestation. The animals were housed in an animal facility with a 12-hour-day/12-hour-night cycle and provided standard laboratory diet and water ad libitum until delivery. Within 2-4 hours of birth, newborn rat pups delivering on the same day were pooled and randomly assigned to expanded litters of 18 pups/litter. The expanded litter size was used to simulate relative postnatal malnutrition of ELGANs who are at increased risk for severe ROP. The pups were assigned to either: 1) IH from P0 to P14, or allowed to recover in RA until P21 (IHR); or 2) room air (RA). These served as controls. On day 0, newborn rat pups were euthanized for retinal status, immediately post birth. Rat pups were euthanized on days 1, 3, 5, 7, 10, 12, 14 (during IH); and 16, 18, and 21 (during IHR) for retinal and astrocyte integrity and retinal growth factor levels. To determine retinal integrity, the retinal vasculature was stained using fluorescein-dextran, ADPase, GS-lectin and H&E. Retinal astrocytes were stained for glial fibrillary acidic protein (GFAP) reactivity. Retinal growth factors (VEGF; soluble VEGFR-1, or sVEGFR-1; insulin-like growth factor-I or IGF-I; and hypoxia inducible factor-1a or HIF-1α) Six pups were euthanized in each RA or IH group at each experimental day. Therefore, one litter of 18 pups was used for 3 days for a total of 4 litters in RA and 4 litters in IH.

Intermittent Hypoxia (IH) Profile
Animals randomized to IH were placed with the dams in specialized oxygen chambers (BioSpherix, New York) attached to an oxy-cycler (10"H x 22"W x 19"D, 55 pounds). The animal chambers housed two rat cages, were optimized for gas efficiency, and provided adequate ventilation for the animals in a controlled atmosphere with minimal gas usage. Oxygen content inside the chamber was continuously monitored and recorded on a Dell Computer. Carbon dioxide in the chamber was monitored and removed from the atmosphere by placing soda lime within the chamber. The IH profile consisted of an initial exposure of hyperoxia (50% O2) for 30 minutes followed by three brief, 1-minute, clustered hypoxic events (12% O2), with a 10-minute recovery in 50% O2 between each IH event (Figure 1). Re-oxygenation in 50% O2 followed each clustered IH event for 2.5 hours for a total of 8 clustering IH episodes per day for 14 days, as previously described (16-21). Animals were either euthanized during IH (P1, P3, P5, P7, P10, P12, or P14) or placed in RA for re-oxygenation and euthanized (P16, P18, or P21). Oxygen saturation was confirmed on a sentinel unanesthetized rat pup from each group using the MouseOx Pulse Oximeter and WinDaq Waveform Browser software (STARR Life Sciences Corp., Oakmont PA) before and after IH exposure.

Retinal Flatmounts
Eyes were enucleated and placed in 4% paraformaldehyde on ice for 120 minutes. The corneas, lens, vitreous, and sclera were removed, and the retinas were cut in quadrants, and flattened. Retinal flatmounts for ADPase staining was immersed in 4% PFA overnight at 4°C. Following several washes in Travis maleate buffer (pH 7.2) and incubation in ADPase, retinas were stained with ammonium sulfide, washed, and mounted on slides with phosphate buffered saline (PBS)/glycerin. For GS-lectin and GFAP staining, retinal flatmounts were fixed in methanol for 20 minutes, followed by permeabilization and blocking in PermBlock (PBS+0.3% Triton X-100+0.2% bovine serum albumin) in 5% goat serum for 1 hour. After washing in PBS/Triton X-100 (TXPBS), flatmounts were then incubated with rabbit GFAP primary antibody (Cell Signaling Technologies, Danvers, MA) overnight at 4°C. Following several washes with TXPBS, the flatmounts were incubated with Alexa Fluor 488 goat anti-rabbit fluorescent secondary antibodies, and Alexa Fluor 594 Isolcetin B4 (ThermoFisher Sci/Life Technologies, Grand Island, NY) overnight at 4°C. The flatmounts were washed with TXPBS and mounted on slides with prolong anti-fade fluorescent mounting media. Images were captured at 20X magnification using an Olympus BX53 microscope, DP72 digital camera, and CellSens imaging software attached to a Dell Precision T3500 computer (Olympus America Inc.).

Fluorescein-Dextran Perfusion
For fluorescein-dextran staining of the retina, rats were deeply anesthetized with intraperitoneal sodium pentobarbital (40 mg/kg IP) and a median sternotomy was performed. The left ventricle was perfused with 1 ml of 4% phosphate buffered formaldehyde containing 50 mg of 2 x 10^6 molecular weight fluorescein-dextran (Sigma, St. Louis, MO). The eyes were enucleated and placed in 4% paraformaldehyde for at least 2 hours prior to removal of the retina [20,21].

Sample Collection & Processing
Eyes were enucleated and rinsed in ice-cold phosphate buffered saline (PBS, pH 7.4) on ice. Enucleation was performed with the use of iris forceps and scissors for separation of the eyes from the surrounding connective tissue, nerves, and muscle. The retinas were then excised under a dissecting microscope and placed in sterile Lysing Matrix D 2.0 mL tubes containing 1.4 mm ceramic spheres (MP Biomedicals, Santa Ana, CA, USA) and 1.0 mL PBS prior to snap-freezing in liquid nitrogen. Samples were stored at -80°C until analysis. All samples were analyzed on the same day. On the day of analyses, the tubes were allowed to defrost on ice and placed in a high-speed FastPrep-24 instrument (MP Biomedicals, Santa Ana, CA, USA), which utilizes a unique,
optimized motion to efficiently homogenize biological samples within 40 seconds via multidirectional simultaneous beating of the Lysing Matrix ceramic beads on the tissue. The homogenates were then centrifuged at 4°C at 10,000 rpm for 20 minutes. The supernatant was filtered, and the filtrate was used for the assays.

**Assay of Angiogenesis Biomarkers**

HIF$_{1\alpha}$ levels in retinal homogenates were assayed using commercially-available rat enzyme-linked immunoassay (ELISA) kits from MyBioSource (San Diego, CA, USA). VEGF, sVEGFR-1, and IGF-I levels in retinal homogenates were assayed using commercially-available quantikine ELISA kits from R & D Systems (Minneapolis, MN, USA). All assays were conducted according to the manufacturer’s protocol. All data were standardized using total cellular protein levels.

**Total Cellular Protein Levels**

On the day of assays an aliquot (10 μL) of the retinal homogenates was utilized for total cellular protein levels using the Bradford method (Bio-Rad, Hercules, CA USA) with bovine serum albumin as a standard.

**H&E Staining**

To determine the extent of endothelial cells (ECs) migrating into the vitreous, and the integrity of the retinal layers, whole eyes were fixed in-situ in 10% neutral-buffered formalin. The eyes were excised, marked for orientation, and sent to Histowiz, Inc. (Brooklyn, NY) for standard processing, embedding and H&E staining. Measurements of retinal layer thickness, as well as individual measurements of the nerve fiber layer/ganglion cell layer (NFL/GCL), inner plexiform layer (IPL), inner nuclear layer (INL), outer plexiform layer (OPL), outer nuclear layer (ONL), and photoreceptor (PR) layer were carried out on H&E stained sections using the count and measure tool of CellSens software (Olympus America, Inc., Center Valley, PA). Four eyes were examined at each experimental time. Measurements were carried out on three areas on both sides of the optic disk, approximately 100 μm apart, for a total of 24 measurements per eye.

**Integrity of Retinal Vasculature**

Measurements of the retinal vasculature and avascular area were conducted using the count and measure tool of the CellSens software (Olympus America Inc.). Four eyes were examined at each experimental time. Measurements were done on each of the 4 quadrants of each eye. Each quadrant was divided into 3 parts, corresponding to 12 clock hours. The perimeter of the vasculature around the optic disk was determined using the freehand polygon tool to draw a circle around the outer edge of the vasculature for a total of 4 measurements per group. The distance from the optic disk to the edge of the vascular bed (vascular zone), and the distance from edge of the vascular bed to the ciliary body (avascular zone) was measured using the arbitrary line tool at each clock hour for a total of 48 measurements each.

**Statistical Analysis**

To determine differences in morphometric measurements between the RA and IH groups, unpaired t-test was used for normally-distributed data and Mann-Whitney U tests was used for non-normally distributed data, following Levene’s test for normality, where applicable. For comparison among the age groups (angiogenesis biomarkers), a test for normality was first conducted using the Bartlett’s test. Normally distributed data were analyzed using two-way analysis of variance (ANOVA) with Bonferroni post-hoc tests. Non-normally distributed data were analyzed using Kruskall Wallis test with Dunn’s multiple comparison test. Data are presented as mean±SD and a p-value of <0.05 was considered as statistically significant, using SPSS version 16.0 (SPSS Inc., Chicago, IL, USA). Graphs were prepared using GraphPad Prizm version 7.03 (GraphPad, San Diego, CA, USA).

**Results**

**Retinal Vasculature at Birth**

At birth, the retina was predominantly avascular with vessels extending 771.1±45.3 μm from the optic disk and occupying a perimeter of 6674.8±113 μm around the optic disk. At the periphery, there were no vessels and the width of the avascular zone was 2086.9±36.1 μm, with hyaloid vessels extending from the optic disk to the ciliary body (Figure 1, panels B, E, H-J). In comparison, the astrocytes populated a 2 mm area around the optic disk with a perimeter of 4821.0±386.2 μm extending to 826.2±72.8 μm from the optic disk, confirming their emergence from the optic nerve head prior to the vasculature (Figure 1, panels C and F). The NFL/GCL and IPL were well defined, but consisted mostly of progenitor cells, and the outer OPL was not defined (Figure 1, panel G). Fluorescein-dextran perfusion showed a predominance of hyaloid vessels (a transient network of arteries that provide nourishment to the developing retina) extending to the ciliary body (Figure 1, panel H-I). These images confirm the immaturity of the rat retina at birth, consistent with a preterm infant at 24-26 weeks gestation.

**Biomarkers of Retinal Angiogenesis**

Figure 2 represents the levels of key biomarkers of angiogenesis on day 0 and weekly at P7, P14 (IH) and P21 (IHR). During the IH experiments, animals on days 1, 3 and 5 were not assessed due to insufficient retinal tissue samples. This was not the case for day 0 because left-over pups were used once the group litter sizes were achieved. In the RA controls, HIF$_{1\alpha}$ levels peaked at P14 (the time of eye opening in rats), and declined at P21. A similar pattern emerged in IH, but the levels were much higher compared to P0 and to the RA controls (panel A). Retinal VEGF mirrored HIF$_{1\alpha}$ peaking at P14. However, the levels did not achieve statistical significance until P14 and remained elevated in the IH groups at P21 (panel B). sVEGFR-1 (the endogenous VEGF regulator), also peaked at P14, but was significantly reduced in the IH groups (panel C). Similarly, IGF-I levels were reduced with IH although the peak was noted at P7 in the RA controls (panel D).

**Retinal Vascularization**

Images for retinal flatmounts from P1-P5 rat pups are not shown, but by day 1 in RA, the vessels extended to 1035.1±28.9 μm from the optic disk (264 μm), and populated a perimeter of 7626.7±172 μm around the optic disk (952 μm). The avascular area decreased to 1438.3±65.6 μm (649 μm). In IH, there was a significant reduction in vessels extension (724.4±28.4, p<0.001) and perimeter (6244.5±489.7, p<0.001) and increased avascular zone (1650.6±67.9, p<0.05). Day 3 vessels in RA extended to 1798.9±47.7 μm, with a perimeter of 11028.4±115.2 μm and avascular zone of 1203.8±35.4 μm. In IH, the vessels extended 1614.9±82.4 μm, with a perimeter of 10218.0±47.7 μm (p<0.001), and avascular zone of 1480.0±65.3 μm (p<0.002), with a persistence of hyaloid vessels and the appearance of vascular anastomoses. Figure 3 represents the ADPase-stained retinal vasculature from P7 to P14. At P7 in RA, the vessels extended 3211.6±47.4 μm and the avascular zone was reduced to 232.6±19.5 μm (panel A). The first evidence of retinal hemorrhage occurred...
Figure 1. Representative retinal flatmounts showing retinal status in newborn rats at 2 hours of birth. ADPase stained flatmounts are shown in Figure 1A (optic disc) and Figure 1D (periphery) at 4X magnification, scale bar is 200 μM. GS-Lectin stained flatmounts are shown in Figure 1B (optic disc) and Figure 1E (periphery) at 4X magnification, scale bar is 200 μM. GFAP stained flatmounts are shown in Figure 1C (optic disc) and Figure 1F (periphery) at 4X magnification, scale bar is 200 μM. H&E stained retinal layers are shown in Figure 1G at 20X magnification, scale bar is 50 μM (vitreous fluid, VF; NFL/GCL, nerve fiber layer/ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; ONL, outer nuclear layer; PR, photoreceptor layer; and Ch, choroid). Fluorescein-dextran-stained flatmounts are shown in Figures 1H (optic disk, 4X magnification), 1I (periphery, 4X magnification), and 1J (20X magnification).

Figure 2. Effects of neonatal intermittent hypoxia (IH) on HIF-1α (A), VEGF (B), soluble VEGFR-1 (C), and IGF-I (D) levels in retinal homogenates. Data were standardized using total cellular protein levels. The open bar represents the room air (RA) groups and the solid bar represents the IH groups. Data are expressed as mean ±SD (n=4 samples/group). *p<0.05; **p<0.01 vs. P0; ***p<0.01 vs IH; ‡p<0.01 vs RA.
Figure 3. Representative retinal flatmounts of ADPase stained retinas from neonatal rats on postnatal day 7 to day 14. Room air (RA) control retinas are represented in panels A to C. IH exposed retinas are represented in panels D to F. Day 7 shows appearance of hemorrhage (arrow), day 10 shows capillary dropout (arrow), and day 14 shows punctate hemorrhages around the optic disk (arrows). Images are 4X magnification, scale bar is 200 μM.

Figure 4. Representative retinal flatmounts of ADPase stained retinas from neonatal rats on postnatal days 16 to 21. RA control retinas are represented in panels A to C. IH exposed retinas are represented in panels D to I. IH exposed retinas show increasing occurrences of hemorrhage at the optic disk and the periphery as well as tortuous and dilated vessels, and vascular overgrowth at the periphery (arrows). Figures are 4X magnification, scale bar is 200 μM.

at 7 days of exposure to IH (panel D, arrow). IH prevented vascular outgrowth with vessels extending 2378.7±82.1 μm (p<0.01), and a widened avascular zone (1202.0±73.3 μm, p<0.001). The retinal vasculature appeared mature by P10 with vessels reaching the ciliary bodies. The vessels extended 3473.2±28.9 μm with preservation of the normal avascular zone between the peripheral retina and the ciliary body (panel B). In contrast, IH resulted in capillary dropout around the optic disk, vascular tufts, tortuous vessels, vascular overgrowth at the periphery, and worsening vascular abnormalities (panels E, arrow). In some quadrants, vessel outgrowth was curtailed compared to RA, resulting in a mean vascular extension of 3089.3±122.1 μm (p<0.01), and avascular zone of 530.1±178.9 μm (p<0.01). By P14, IH caused vessels to bypass the normal avascular zone and grow into the ciliary body (panel F). There were several punctate hemorrhages around the optic disk (arrows), vascular loops, and tortuous vessels at the periphery. These characteristics (hemorrhage, vascular tortuosity, vascular overgrowth, and vascular abnormalities/loops) worsened during IHR at P16 through P21 (Figure 4, panels D-I, arrows).
**Fluorescein-Dextran Images**

Fluorescein-dextran was used to confirm and supplement the findings of ADPase (Figures 5 and 6). Images for retinal flatmounts from rat pups at P1-P5 are not shown. In normoxia, progressive regression of the hyaloid vasculature began by P1 and was completed at P10. In contrast, IH caused a persistence of hyaloid vessels. Consistent with ADPase-stained flatmounts, fluorescein-dextran images showed many characteristics consistent with severe ROP, including vascular tortuosity, enlargement, and leakiness, as well as tufts and disorganization in IH (Figure 5, panels D-F). During IHR, the damage persisted and worsened (Figure 6, panels D-F), compared to RA controls (Figure 6, panels A-C).

**Figure 5.** Representative fluorescein-dextran stained retinal flatmounts from neonatal rats on postnatal days 7 to 14. RA control retinas are represented in panels A to C and IH-exposed retinas are represented in panels D to F. Retinas from RA controls show normal vasculature. Retinas from IH-exposed rats show leaky vessels, vascular tufts, tortuous vessels, vascular disorganization and neovascularization (arrows). Images are 4X magnification.

**Figure 6.** Representative fluorescein-dextran stained retinal flatmounts from neonatal rats on postnatal days 16 to 21. RA control retinas are represented in panels A to C and IH-exposed retinas are represented in panels D to F. Retinas from RA controls show normal vasculature. Retinas from IH-exposed rats show leaky vessels, vascular tufts, tortuous vessels, vascular disorganization and neovascularization (arrows). Images are 4X magnification.
Astrocytic Template

Astrocytes are found only in the NFL/GCL, are GFAP positive, and account for 10% of the total retinal glial population. They exist proximal to the inner retinal vasculature providing guidance cues and structure. Retinal flatmounts for rat pups at P1-P5 are not shown, however, P1 the astrocyte perimeter increased in RA to 9171.5±100.3 μm and extended to 1261.9±67.2 μm. In contrast, IH caused a larger perimeter of 10320.9±196.3 μm and longer extension of 1307.87±76.6 μm. By P3, the astrocyte template extended to 2148.2±48.5 μm (RA) and 2956.1±124.5 μm (IH) to reach the ciliary body by P7 (RA: 3574.8±52.4 μm; IH: 3943.2±81.2 μm, p<0.002). GFAP staining to visualize astrocytes showed faint staining in RA controls, whereas IH induced strong GFAP staining suggesting reactive gliosis (characterized as increased GFAP expression in response to injury) as early as day 3 and persisted to day 14 (Figure 7, panels D-F). This was also associated with disruption and disorganization of the astrocytic template starting at day 7 (Figure 7, panel G), P10 (Figure 7, panel H) and P14 (Figure 7, panel I). During IHR, astrocyte disorganization worsened as did GFAP hypertrophy, and activated Müller cell end-feet at the optic disk and periphery (Figure 8, panels D-I, arrows).

Figure 7. Representative retinal flatmounts of GFAP stained astrocytes from neonatal rats on postnatal days 7 to 14. RA control retinas are represented in panels A to C. IH exposed retinas are represented in panels D to I. Retinas from IH-exposed rats show increased GFAP expression at the optic disk, abundant vascular network, disturbances in the astrocyte template, and activated Müller cell end feet (arrows). Images A-F are 4X magnification (scale bar is 200 μM) and images G-I are 20X magnification (scale bar is 50 μM).

Figure 8. Representative retinal flatmounts of GFAP stained astrocytes from neonatal rats on postnatal days 16 to 21. RA control retinas are represented in panels A to C. IH exposed retinas are represented in panels D to I. Retinas from IH-exposed rats show increased GFAP expression, abundant disorganized vascular network, disturbances in the astrocyte template, and activated Müller cell end feet (arrows). Images A-D are 4X magnification (scale bar is 200 μM) and images E-I are 20X magnification (scale bar is 50 μM).
Retinal Layers
Images for retinal layers at P1 and P5 are not shown. The OPL was also nonexistent at P1 and P3, but was visible from P7 onward in both RA and IH groups (Figure 9, panel A-D). In contrast, histological sections form IH-exposed rats showed retinal endothelial cells penetrating the inner limiting membrane (ILM) to violate the vitreous fluid (VF), as early as P1 and worsening at P3 (Figure 9, panel E). Vitreous condensation (*), widening of the NFL/GCL layer, breach of the ILM, and extraretinal neovascularization can result in vitreous traction or pulling forward causing retinal distortions. The formation of retinal folds or rosettes and PR degeneration appeared by day 10. Hemorrhage in the choroidal and ONL, retinal folds and PR degeneration were more evident during IHR (Figure 10, panels D-F, arrows).

Figure 9. Representative H&E stained retinas from neonatal rats on postnatal days 3 to P14. RA control retinas are represented in panels A-D and IH exposed retinas are represented in panels E-H. Exposure to IH caused early retinal neovascularization by day 3. Image E shows retinal endothelial cells penetrating the inner limiting membrane and migrating into the vitreous fluid (arrow). Vitreous condensation and traction (*) pulls the retina away from the choroid. Images A-D are 20X magnification (scale bar is 50 μM), and images E-H are 40X magnification (scale bar is 50 μM).

Figure 10. Representative H&E stained retinas from neonatal rats on postnatal days 16 to P21. RA control retinas are represented in panels A-C and IH exposed retinas are represented in panels D-F. Retinas from animals recovering from IH (IHR) showed widening of the NFL/GCL, endothelial cells migrating into the vitreous fluid (arrow), hemorrhage in the ONL and choroid, and rosettes (arrows). Images are 20X magnification (scale bar is 50 μM).
Retinal Thickness
Figure 11 shows that exposure to IH increased the overall retinal thickness (panel B) between P10 and P12. There was a crossover point (red circle) of permanent retinal thickness elevations suggesting a point of no return. Panel C represents the NFL/GCL layer which increased substantially as early as day 3 (represented in Figure 9, panel E) and remained elevated throughout the time course of IH events. This finding confirms that retinal neovascularization occurs very early during neonatal IH. The effects of IH on the other retinal layers are represented in Table 1. IPL thickness increased from day 5 with moderate adjustments on days 10, 16 and 18, but remained increased at day 21; INL thickness increased at day 1, but the major crossover point was observed on day 10 and remained elevated from days 12 to 21; the OPL was non-existent until day 7, increased on day 12, and did not appreciably change until days 18 and 21; the ONL was generally thicker from P0 to P5 in all groups and remained elevated in the IH groups from P12 to P21; the PR layer remained consistently lower in all IH groups from P1-P18, but doubled in size at P21, compared to RA.

Table 1: Retinal Layer Thickness

<table>
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<th>IPL (μM)</th>
<th>INL (μM)</th>
<th>OPL (μM)</th>
<th>ONL (μM)</th>
<th>PR (μM)</th>
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<tr>
<td>P0</td>
<td>19.6±1.0</td>
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<td>170.4±3.5**</td>
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<td>10.6±0.4</td>
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<td>76.2±2.1***</td>
<td>0</td>
<td>267.8±4.3**</td>
<td>0**</td>
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<tr>
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<td>P5-IH</td>
<td>53.0±2.2**</td>
<td>68.4±2.4***</td>
<td>0</td>
<td>187.5±3.6**</td>
<td>12.3±0.6**</td>
</tr>
<tr>
<td>P7-RA</td>
<td>37.2±1.6</td>
<td>71.8±1.8</td>
<td>12.9±1.1</td>
<td>79.6±1.8</td>
<td>9.9±0.24</td>
</tr>
<tr>
<td>P7-IH</td>
<td>48.1±1.0**</td>
<td>89.6±0.22**</td>
<td>10.3±0.44**</td>
<td>80.1±3.0</td>
<td>7.1±0.84</td>
</tr>
<tr>
<td>P10-RA</td>
<td>62.6±4.2</td>
<td>134.6±8.3</td>
<td>17.7±0.92</td>
<td>111.5±5.1**</td>
<td>22.7±1.2</td>
</tr>
<tr>
<td>P10-IH</td>
<td>65.6±2.5</td>
<td>131.9±2.6</td>
<td>17.6±0.7</td>
<td>99.4±3.7</td>
<td>11.0±0.7**</td>
</tr>
<tr>
<td>P12-RA</td>
<td>62.4±12.9</td>
<td>70.7±11.7</td>
<td>25.8±4.2</td>
<td>102.0±29.6**</td>
<td>41.2±9.0</td>
</tr>
<tr>
<td>P12-IH</td>
<td>96.4±11.8***</td>
<td>183.1±27.8***</td>
<td>41.0±8.7**</td>
<td>144.4±19.4**</td>
<td>33.3±6.9*</td>
</tr>
<tr>
<td>P14-RA</td>
<td>52.7±8.8</td>
<td>63.3±16.2</td>
<td>16.3±3.8</td>
<td>84.9±6.4</td>
<td>37.0±10.6</td>
</tr>
<tr>
<td>P14-IH</td>
<td>71.8±18.1***</td>
<td>100.3±16.8***</td>
<td>15.1±3.5</td>
<td>101.6±28.0**</td>
<td>32.3±5.9</td>
</tr>
<tr>
<td>P16-RA</td>
<td>61.9±13.1</td>
<td>61.4±7.2</td>
<td>17.4±5.1</td>
<td>89.7±9.1</td>
<td>40.6±4.6</td>
</tr>
<tr>
<td>P16-IH</td>
<td>62.1±5.8</td>
<td>127.3±28.1***</td>
<td>15.6±1.8</td>
<td>102.4±5.6**</td>
<td>19.3±3.3**</td>
</tr>
<tr>
<td>P18-RA</td>
<td>63.6±9.1</td>
<td>70.9±11.6</td>
<td>20.1±5.8</td>
<td>89.5±22.8</td>
<td>43.2±6.6</td>
</tr>
<tr>
<td>P18-IH</td>
<td>60.1±6.6</td>
<td>65.2±9.5</td>
<td>14.0±2.7*</td>
<td>73.7±6.5*</td>
<td>36.3±2.1**</td>
</tr>
<tr>
<td>P21-RA</td>
<td>48.4±6.1</td>
<td>70.8±5.7</td>
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<td>90.3±9.2</td>
<td>34.7±6.7</td>
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<tr>
<td>P21-IH</td>
<td>103.5±24.5**</td>
<td>99.5±34.4**</td>
<td>21.2±4.5*</td>
<td>151.3±17.5***</td>
<td>64.7±24.1**</td>
</tr>
</tbody>
</table>

IPL (inner plexiform layer), INL (inner nuclear layer), OPL (outer plexiform layer), ONL (outer nuclear layer), PR (photoreceptors). Data are mean±SD (*p<0.05, **p<0.01 vs. RA); n=24 measurements per group.
Discussion

The present study describes the time course of changes in the integrity of the retinal vasculature, retinal layers, and retinal astrocytic template in response to chronic neonatal IH during retinal development. Each preceding IH event contributes to limited recovery from the following event, thus setting the stage for a “point of no return” or irreparable damage with detrimental outcomes during IHR. To specifically address our objectives, the major findings are: 1) the first signs of retinal damage begin as early as day 3. This was evidenced by widening of the NFL/GCL layer, invasion of the VF by retinal ECs, vitreous condensation and traction, and vascular abnormalities preceded by increased expression of GFAP as early as 24 hours of neonatal IH; 2) Retinal damage worsens with IH progression and the point of no return or irreparable damage may occur during this period. Day 7 is characterized by hemorrhage and vascular leakiness, disorganized retinal astrocytic template, dysregulated retinal vasculature, and photoreceptor degeneration. By days 10-14, these characteristics worsen; 3) During IH vascular abnormalities such as anastomoses and vascular loops worsened along with retinal degeneration, retinal folds or rosettes. This period is characterized by inner retinal deterioration, severely compromised astrocyte integrity, and worsened photoreceptor damage; 4) the primary site of damage is the inner retina with NFL/GCL thickening. This is where astrocytes and Müller end-feet are located; 5) the astrocyte template is highly vulnerable to neonatal IH. IH severely disrupted the normal astrocytic structure and appearance. This may lead to compromised blood-retinal-barrier (BRB), retinal hemorrhage, neovascularization, and retinal detachment; and 6) chronic neonatal IH is associated with early and long-lasting elevations in retinal HIF$_	ext{1a}$ and VEGF. These abnormal levels are likely due to activated astrocytes and Müller cells, as previously demonstrated [35, 40, 41]. Together, these findings prove our hypothesis and provide a strong case for implementation of early identification and preventative strategies to curtail neonatal IH, at least within the first 3 days of life. In doing so, mechanisms associated with aberrant angiogenesis can be intercepted long before irreversible damage has ensued and may preclude the use of later invasive therapies. The use of pharmacologic interventions during early neonatal IH may prove to be more beneficial for preventing or suppressing severe ROP than rescue treatment when the disease has irreparably progressed.

The systematic analysis of the time course of retinal events related to neonatal IH permits the identification of key time points for the onset of aberrant retinal angiogenesis and allows the development of strategies which may be implemented at the most appropriate time in order to stabilize and/or prevent the development of pathological vessels and promote a normally functioning vascular network. The most remarkable finding in this investigation, and the event that may have preceded early onset of retinal damage, was evidence of astrocyte and Müller cell reactivity to neonatal IH. Unlike Müller cells which extend from the inner to outer limiting membranes with their end-feet residing in the NFL/GCL, astrocytes are only found in the NFL/GCL, and account for 10% of the glial population [40]. One of the best known biomarker for astrocytes and activated Müller cells is GFAP, the chief constituent in astrocytes and a major component of their intermediate filaments [40,41]. In the normal retina, astrocytes are generally more GFAP positive than Müller cells which are predominately vimentin reactive. After injury, Müller cells rapidly upregulate GFAP, but maintain their basic radial orientation within the retina [40]. Studies by Luna et al. [40] showed dramatic changes in astrocytes after retinal detachment, including upregulated GFAP, and loss of their stellate shape appearing irregular and “frayed”, thus supporting a key role for abnormal astrocytes in retinal injury and detachment. Other studies showed that astrocyte reactivity induced neuronal injury in the retina [42], was associated with retinal diseases such as glaucoma [43,44], and increased vascular permeability, infiltration of toxic compounds, and exacerbation of disease progression [45]. In a previous report, we demonstrated a 2.5-fold increase in retinal GFAP using proteomic analysis and validated by Western blot analysis in our OIR model [17]. We now show, for the first time, that progression of IH-induced retinal damage, evidenced by vascular permeability and hemorrhage; retinal folds and rosettes; and photoreceptor degeneration, is associated with major disturbances in the astrocytic template. In the retina, both astrocytes and Müller cells produce VEGF. Astrocyte and Müller cell reactivity, induced by IH, may also be responsible for the long-lasting elevations in HIF$_	ext{1a}$ and VEGF levels in our OIR model. These finding are of critical importance and indicate that preservation of the astrocytic template and prevention of astrocyte and Müller cell reactivity may be a key strategy for preserving retinal vascular integrity in neonatal IH within a defined therapeutic window. As new therapeutic approaches are being developed to preserve retinal integrity and function, this study suggests that strategies to preserve the astrocyte template should also be considered, given their significance in retinal vascular development and their intimate relationship with the retinal vasculature.

It was interesting to note the association between neonatal IH and the appearance of vascular loops, which appeared as early as day 3, and abnormalities in the retinal layers, particularly in the ONL during IHR, consistent with retinal folds or rosettes. Pioneering studies by Ashton N [46] found similar abnormalities in a preterm infant who died with retrolental fibroplasia, validating our OIR model. More recent studies reported the presence of similar rosettes and folds in the mouse model of retinal detachment [47] and the rat and canine models of OIR [48,49]. Retinal vascular loops are variants of the normal retinal vasculature and are associated with retinal artery occlusion, and vitreous and subretinal hemorrhages [50,51]. Retinal folds and rosettes in the ONL resulting in layer derangement suggest apoptosis and degeneration [52]. Studies have shown that retinal infolding and rosette formation was associated with partial retinal detachment, loss of retinal pigment epithelium and/or function (53-55). Another interesting finding is the absence of an OPL in the rat retina at birth which appears only on day 7. Studies mapping the time line of development of each layer in the human retina showed that the OPL is well-defined at 20 weeks gestation [56]. Two important synaptic interactions occur in the OPL, the splitting of the visual signal into two channels for light and dark objects and the instillation of pathways to create simultaneous contrast of visual objects [57]. Exposure to IH did not affect the timing of OPL development which was ostensibly similar to the RA controls, although the thickness was somewhat altered. Therefore, delayed OPL maturation in rats is likely reflective of postnatal eye opening and neural circuitry development. Examination of the overall retinal thickness, which may indicate excess vascular permeability and loss of BRB function, revealed a crossover point between P10 and P12. At this same time point, we noted severe retinal hemorrhage, retinal folding, vascular abnormalities, and changes in astrocyte appearance. It is tempting to speculate that this time point may indicate a modification in the reparative trajectory, leading to a point of no return.

In conclusion, given that drugs targeting the very vulnerable preterm newborn infant to prevent severe ROP is fraught with multiple barriers and challenges, the overarching goal of these descriptive studies was to identify strategic time points for drug
few days of life. Furthermore, early and timely use of pharmacologic interventions may prove to be more beneficial for preventing severe ROP than treatment during late stages of the disease. Strict monitoring and identification of neonates who experience a significant number of IH episodes during the first few days of life are warranted.

Acknowledgements
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