Increased angiogenic factors during avascular retina prior to neovascularization in ROP model

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Abstract

Objective—To determine expressions of vascular endothelial growth factor (VEGF), pigment epithelial derived factor (PEDF) and respective receptors in retinas from a model of retinopathy of prematurity (ROP).

Method—Retinas isolated from the 50/10 oxygen-induced retinopathy model of ROP (50/10 OIR) and from room air raised pups (RA) at birth, age 14 days (time of persistent avascular retina in 50/10 OIR and complete retinal vascularization in RA), and age 18 days (time of intravitreous neovascularization in 50/10 OIR) were analyzed for mRNA of $VEGF_{164}$, PEDF, VEGF receptor (R)-1 and -2, PEDF receptor, and neuropilin-1 and -2 by real-time polymerase chain reaction (PCR).

Results—Compared to RA, fold changes in expression of $VEGF_{164}$, neuropilin-1 and -2 were significantly increased at day 14 while avascular retina persistent in 50/10 OIR and at 18 days during intravitreous neovascularization. There was also a trend for increased fold change in VEGFR2 at day 14 and a significant increase at day 18 in 50/10 OIR. PEDF receptor was significantly increased at day 14 in 50/10 OIR compared to RA.

Conclusions—Increased expression of $VEGF_{164}$, and angiogenic receptors provides evidence that over-expressed VEGF may disorder developmental angiogenesis and contribute to peripheral avascular retina and intravitreous neovascularization.

Clinical Relevance—Disordered angiogenesis through increased VEGF signaling may contribute to avascular retina prior to the development of stage 3 ROP.

Introduction

Interactions between neural and vascular growth factors and receptors are recognized as important in retinal vascular development and in disease[1]. For example, the vascular endothelial growth factor (VEGF) family and signaling pathway is important in retinal vascular development[2,3] and in neuronal survival[4,5]. The neuropilins, originally described as receptors for proteins involved in neuronal axon guidance, can form complexes with VEGF receptors and act as coreceptors for VEGF mediated angiogenic signaling[1]. Pigment epithelial derived factor (PEDF) is a known neurotrophic factor[6] and is also involved in retinal vascular development[7]. One way PEDF was reported to inhibit pathologic angiogenesis is by downregulating VEGF expression and interfering with its binding to

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VEGF receptor 2 (VEGFR2)[8], the receptor believed most involved with angiogenic processes[9].

Since the VEGF signaling pathway and PEDF are important in physiologic and pathologic angiogenesis[2,3,7,8], we wished to determine the expression of these factors and their respective receptors in a model that is relevant to ROP of modern-day. We previously studied the effects of oxygen stresses on the expression of VEGF isoforms and reported that $VEGF_{164}$ was the most prevalent isoform and was increased by repeated fluctuations in oxygen[10], a known risk factor for modern-day severe ROP[11,12]. Therefore, in this report, we used the same 50/10 oxygen induced retinopathy model of ROP (50/10 OIR) developed by John Penn[13], which exposes rat pups to repeated fluctuations in oxygen. The model reproducibly and consistently develops peripheral avascular retina, similar to zone II ROP, followed by intravitreous neovascularization at the junction of vascular and avascular retina, similar to zone II, stage 3 ROP. Previous studies of ROP have used oxygen-induced retinopathy models that expose animals to extreme constant hyperoxia to create vaso-obliteration of the central retinal capillaries. The most know of these is the mouse model developed by Smith and D'Amore[14]. During the obliterative phase, VEGF mRNA expression was downregulated in conjunction with arrest and loss of developing retinal vasculature[15]. The newly formed capillaries can be protected from death by giving growth factors[16,17,18] or nutrients[19,20] prior to hyperoxia. Upon return to room air, a relative retinal hypoxia triggers signaling of VEGF and angiogenesis with capillary budding into the vitreous [21], and this proliferative phase can be prevented by administering agents to inhibit angiogenic factors, such as VEGF.[22]

We used a relevant model of zone II stage 3 ROP, the 50/10 OIR model to measure the expression of mRNAs of $VEGF_{164}$, PEDF and their respective receptors. We proposed that the expression of angiogenic factors would be downregulated when avascular retina persisted in the model at day 14 compared to room air when the retina was fully vascularized and that angiogenic factors would be upregulated compared to room air during intravitreous neovascularization at day 18.

Methods

All animal studies complied with the University of North Carolina's Institute for Laboratory Animal Research (Guide for the Care and Use of Laboratory Animals) and the ARVO Statement for the Use of Animals in Ophthalmic and Visual Research.

Rat 50/10 OIR Model

We used a model of zone II, stage 3 severe ROP, the rat 50/10 OIR model[13]. The model causes rat arterial oxygen levels[13] that replicate transcutaneous oxygen extremes of modern-day human preterm infants who develop severe ROP[12]. Within 4 hours of birth at postnatal age 0 (p0), litters of 12–14 newborn Sprague-Dawley rat pups with their mothers (Charles River, Wilmington, MA) were placed into an Oxycycler incubator (Biospherix, New York, NY) to cycle inspired oxygen between 50% oxygen and 10% oxygen every 24 hours until p14. After 7 cycles of oxygen fluctuations, pups were then placed into room air for 4 days. Oxygen levels were monitored daily and recalibrated as needed. Carbon dioxide in the cage was also monitored daily and flushed from the system by maintaining sufficient gas-flow and by adding soda lime. The model reproducibly develops vascular tortuosity at p12, a peripheral avascular zone of retina at p14 (Figure 1) when retinal vascular development is complete in room air development, then intravitreous neovascularization at p18 (Figure 2)[23].

Previously, we showed that compared to rats raised in room air at the same time points after birth, those raised in an environment with oxygen cycling had increased areas of avascular

retina[10]. At p14 after seven cycles of fluctuations in oxygen, the percent avascular to total retinal area was 33% in the 50/10 OIR model, whereas room air raised pups had fully vascularized retinas in which the inner capillary plexus covered the rat retina to the ora serrata.

Dissection of Retinal Tissue for mRNA Analyses

Pups were euthanized using pentobarbital (80 mg/kg intraperitoneal injection), and retinas were isolated using a modification of Chan-Ling[24]. Retinas were dissected, without ora serratas, from at least 5 separate pups for each time point in room air or the 50/10 OIR model. Separate litters were used for each time point and condition to assure adequate litter size and consistency of features in the 50/10 OIR model. Replicate experiments were performed for each condition and time point. Tissue was frozen in RNAlater (Applied Biosystems, CA) until analysis.

Real time PCR

Retinas were homogenized in lysis buffer and total RNA extracted by RNeasy Kit (Qiagen, CA). DNA-free Kit (Applied Biosystems, CA) was used to remove DNA contamination and RNA quantity was determined. 1µg of RNA was reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, CA). $VEGF_{164}$ was chosen, because it is the most prevalent of VEGF isoforms associated with intravitreous neovascularization in the 50/10 OIR model, is upregulated by repeated oxygen fluctuations[10], and it is also a ligand for neuropilin-1, which acts as a coreceptor with VEGFR2[1]. Primers for: $VEGF_{164}$: 5′GCACATAGGAGAGATGAGCT 3′, 5′GCTCACAGTGATTTT CTGGC3′, Fam TGCAG CATAGCAGATGTGAATGCAGACC Tamara; VEGFR1: 5′CCACCTCCATGTTTGAAGAC3′, 5′AGTCCAGGTGAATC GCTTCA3′, Fam TACCAGCAGTCTGCTGACCTCCCC Tamara; VEGFR2: 5′CTCCATCTTTTGGTGGGATG3′, 5′GCTGGTCTGGTTGGAGCCT3′, Fam AGGCCACAGACTCCCTGCTT TTACTG3 Tamara; PEDF: 5′CCAACTCTTTGCAGGACATG3′, 5′TCACAGGTTTGCCGGTAATC3′, Fam ACAGTCCTTGTTTGAGTCCCCTGAC Tamara; neuropilin-1 (NP-1): 5′GGAGCTACTGGGCTGTGAAG3′, 5′ATGTGGGGAAGTCTGA3′, Fam CACCCAATGGGAACCTA Tamara; neuropilin-2 (NP-2): 5′ACACAAGGAGCCATTTCCAG3′, 5′CGGATCCTGATGAAACGAGT3′, Fam CAAAGGGGGAAGATTGGATGTamara; PEDF receptor: 5′CCAACTCTTTGCAGGACATC3′, 5′TCACAGGTTTGCCGGTAATC3′, Fam ACAGTCCTTGTTTGAGTCCCCTGAC Tamara[25] were made by UNC's Oligonucleotide Core facility (http://www.med.unc.edu/olioli/index.htm). cDNA was mixed 1:1 with Taqman Universal Master Mix (Applied Biosystems, CA) and primers. Triplicate samples were cycled in the AB 7500 PCR System. The 7500 System Software calculates cycle threshold automatically for each well. Rat β-actin, which we found to be stably expressed in retinas under these oxygen stresses, was used as the control gene and was amplified with forward and reverse primers TGCCTGACGGTCAGGTCA and CAGGAAGGAAGGCTGGAAG, respectively. Duplicate reactions with a total volume of 16 µL were run for each sample and control using the AB 7500 PCR System. The 7500 System Software calculates cycle threshold automatically for each well. Cycle threshold values were normalized to β-actin and expressed as a fold increase over p0 value for each growth factor or receptor, thus permitting comparisons between room air and 50/10 OIR model for each growth factor or receptor.

Statistical Analysis

For real time PCR analysis, samples from room air and 50/10 OIR model were normalized to β-actin for each ligand or receptor group to allow comparisons of fold differences within each group. Student t-tests were used to analyze fold changes in expression of each ligand or receptor mRNA between room air and 50/10 OIR at postnatal day (p)14 (avascular retina) and p18 (intravitreous neovascularization).

Results

Expression of VEGF, VEGF receptors 1 and 2, neuropilins, PEDF and PEDF receptor in room air

In room air, the retinal extent is fully covered by retinal vessels at p14. $VEGF_{164}$ mRNA expression increased 3-fold between p0 and p14 (Figure 3). The fold changes in expression differed between p0 and p14 for each of the 4 angiogenic receptors and coreceptors involved in VEGF signaling (VEGFR1, VEGFR2, neuropilin -1, neuropilin -2). Whereas VEGFR2 increased 5 fold (Figure 4), VEGFR1 increased approximately 42 fold (Figure 5). There was little change in expression of neuropilin -1 (Figure 6), and neuropilin -2 increased approximately 5-fold (Figure 7).

PEDF and PEDFR expression increased about 5- and 4-fold, respectively, between p0 and p14 (Figures 8 and 9).

Expression of VEGF, VEGF receptors 1 and 2, neuropilins, PEDF and PEDF receptor at p14 during persistent avascular retina in the 50/10 OIR model

The 50/10 OIR model exposes animals to repeated fluctuations in hyperoxia and hypoxia and causes delayed retinal vascularization and retinal apoptosis[26], both believed to contribute to peripheral avascular retina at p14, when room air raised pups have fully vascularized retinas. Since VEGF is important in developmental angiogenesis[2,3], and the 50/10 OIR model had incomplete retinal vascularization compared to room air counterparts[10], we anticipated that the expression of mRNAs of $VEGF_{164}$, VEGF receptors and coreceptors would be lower at p14 in the 50/10 OIR model compared to those in room air. However, we found that $VEGF_{164}$ expression increased with an almost 10-fold change between p0 and p14 in the 50/10 OIR model and this represented a 3-fold increase in expression compared to room air (Figure 3). This finding also contrasts with reports using the mouse oxygen-induced retinopathy model, in which VEGF expression was lower than room air during the phase of hyperoxia induced vaso-obliteration[15].

Compared to p0, all receptors had increased expression at p14 in the 50/10 OIR model and all except for VEGFR1 had increased fold change in expression compared to room air values (Figures 4 through 7). The fold change in expression of neuropilin-1 and -2 were significantly increased in 50/10 OIR compared to room air, whereas there was a trend for increased fold expression of VEGFR2 in the 50/10 OIR model. The fold changes in PEDF and PEDFR mRNAs increased between p0 and p14, and there was a significant increase in fold expression of PEDFR mRNA in 50/10 OIR compared to room air (Figure 9). There was little fold difference in PEDF mRNA between room air and the 50/10 OIR model at p14 (Figure 8).

Expression of VEGF, VEGF receptors 1 and 2, neuropilins, PEDF and PEDF receptor at p18 during intravitreous neovascularization in 50/10 OIR model

In the 50/10 OIR model, pups are moved to a relatively hyperoxic environment (from 10% to 21% oxygen; i.e., room air) at day 14. At day 18, intravitreous neovascularization develops at the junctions of vascular and avascular retina. At p18, in association with the

intravitreous neovascularization, we found greater fold changes in expression of $VEGF_{164}$, VEGFR2, neuropilin -1, and neuropilin -2 compared to room air values (Figures 4–7). However, the fold change in expression of VEGFR1 was similar to that in room air (Figure 5).

Since PEDF is an angiogenic inhibitor, we anticipated that PEDF and PEDF receptor would be reduced at p18 compared to room air. At p18, however, the fold change in expression of PEDF and PEDF receptor were not significantly greater than those at room air (Figures 8 and 9).

Discussion

We found increased fold expression of mRNAs of $VEGF_{164}$, VEGFR2, and the neuropilins at p18 in association with intravitreous neovascularization and compared to room air. This was anticipated because $VEGF_{164}$, VEGFR2 and the neuropilins are involved in angiogenic processes. However, we also found that when avascular retina persisted in the 50/10 OIR model, the fold change in expression of mRNAs of $VEGF_{164}$, both neuropilins, and a strong trend for VEGFR2 was greater than that in room air raised rats in which retinal vascularization was complete at p14. Furthermore, the fold expression of VEGFR1, believed to limit the amount of VEGF ligand present to signal through VEGFR2 during development, was not significantly elevated between the 50/10 OIR model and room air. These were unexpected findings based on data from the mouse model of oxygen-induced retinopathy in which VEGF was downregulated during vaso-obliteration[15] and upregulated during angiogenesis[22].

Both VEGF and PEDF are involved in pathologic angiogenesis and physiologic developmental retinal angiogenesis, and are regulated by oxygen tension[2,7,27,28,3]. VEGF expression is upregulated by hypoxia[27], and PEDF by hyperoxia[28]. PEDF is a potent angiogenic inhibitor[28], whereas VEGF is an agonist[27]. VEGFR2 is believed to be important in angiogenesis, whereas VEGFR1 is thought to bind and limit the amount of VEGF ligand that can signal through VEGFR2[9], thus permitting ordered angiogenesis. It is increasingly recognized that the neuropilins are also important in angiogenic processes[1]. Neuropilin-1 was initially described as a receptor for the semaphorins, and was involved in axonal guidance in the central nervous system. Akula et al. reported associations between VEGF, semaphorin 3A, vascular tortuosity, and electrophysiologic sensitivity parameters of rods and the post-receptor retina in the 50/10 OIR model suggesting that the post-receptor retina mediates vascular abnormalities in the model[29]. We now report that the neuropilins are upregulated in association with avascular retina and intravitreous neovascularization. Both neuropilins can function as receptors for a number of VEGF family members including VEGF isoforms, $VEGF_{164/165}$ and $VEGF_{145}$, placental growth factor-1, and VEGFB[30]. Less is known about PEDF receptor signaling[25]. However, counter to the anticipated role of PEDF as an angiogenic inhibitor, PEDF knockout mice were reported to develop larger areas of vaso-obliteration following hyperoxia than wild type littermates[31]. We found increased fold expression of PEDFR at p14 during persistent avascular retina in the 50/10 OIR compared to room air. This finding may have importance in the development of avascular retina, because PEDF is an inhibitor of angiogenesis[28].

We used the 50/10 OIR model, which causes rat arterial oxygen levels[13] to be similar to transcutaneous oxygen levels measured in preterm infants who develop severe ROP[12] and uses repeated oxygen fluctuations, a risk for ROP[12,11], rather than constant oxygen used in other models[28,14,32,33]. As in zone II Stage 3 ROP, in which fluorescein angiograms demonstrated reduced peripheral retinal vascularization and perfusion with minimal central nonperfusion (Lepore D., ARVO meeting, 2008, Abstract), the rat 50/10 OIR model also has

peripheral avascular retina and minimal central obliteration[13]. The 50/10 OIR model reproducibly and consistently develops vessel tortuosity (analogous to plus disease)[34,35], avascular retina at p14 (analogous to human zone II ROP)[13,35], and subsequently intravitreous neovascularization at the junctions of vascular and avascular retina (analogous to stage 3 ROP)[36]. Other models of oxygen-induced retinopathy including the mouse model, use higher oxygen levels than that currently used in neonatal intensive care units in the US today and creates central rather than peripheral avascular retina.

There are several reasons we propose for our findings. At p14, VEGF expression may be increased as a result of previous exposure to 10% oxygen. We used real-time PCR to measure expression, because it is the most quantitative method available. The high mRNA is in alignment with later development of intravitreous neovascularization at p18, but it does not explain why blood vessel growth occurs into the vitreous rather than into the retina. Loss of the physiologic gradient of VEGF in the retina may be a reason for the growth of blood vessels into the vitreous. However, Geisen et al. measured vitreous VEGF and retinal VEGF in the 50/10 OIR model and found that the vitreous levels were 1/10 that of the retina at p18[37], failing to provide support for a gradient of VEGF toward the vitreous and away from the retina. It is also possible that at p14, the model is moving toward a vasoproliferative phase. However, several papers have found that inhibition of VEGF bioactivity using either a neutralizing antibody[37] or a VEGF receptor 2 kinase inhibitor[38] reduced angiogenesis into the vitreous (intravitreous neovascularization), but did not interfere with angiogenesis into the retina (intraretinal vascularization). Excessive signaling through VEGFR2 has been shown to interfere with developmental angiogenesis in other models[39]. It is possible that increased signaling through VEGF receptors may disorder and interfere with developmental angiogenesis. Such a finding might delay normal vascularization and contribute to peripheral avascular retina. However, since VEGF is also a neuronal survival factor[4,5], there is concern about possible systemic adverse effects of anti-VEGF agents that are absorbed into the blood stream of the developing preterm infant.

Although the measurement of mRNA with real-time PCR provides the most quantitative data of expression, it does not always correspond to the concentration of translated proteins or activated proteins involved in signaling pathways. Further study in these areas is warranted.

In summary, we found that during avascular retina, expression of $VEGF_{164}$ and its receptors and coreceptors were increased in 50/10 OIR model compared to room air when retinal vascularization was complete. We propose that overexpression of VEGF may interfere with ongoing retinal vascular development and contribute to avascular retina. We also found that VEGF was increased during the period of intravitreous neovascularization. In addition, increased expression of PEDF receptor in 50/10 OIR model compared to room air may have importance in contributing to avascular retina.

Acknowledgments

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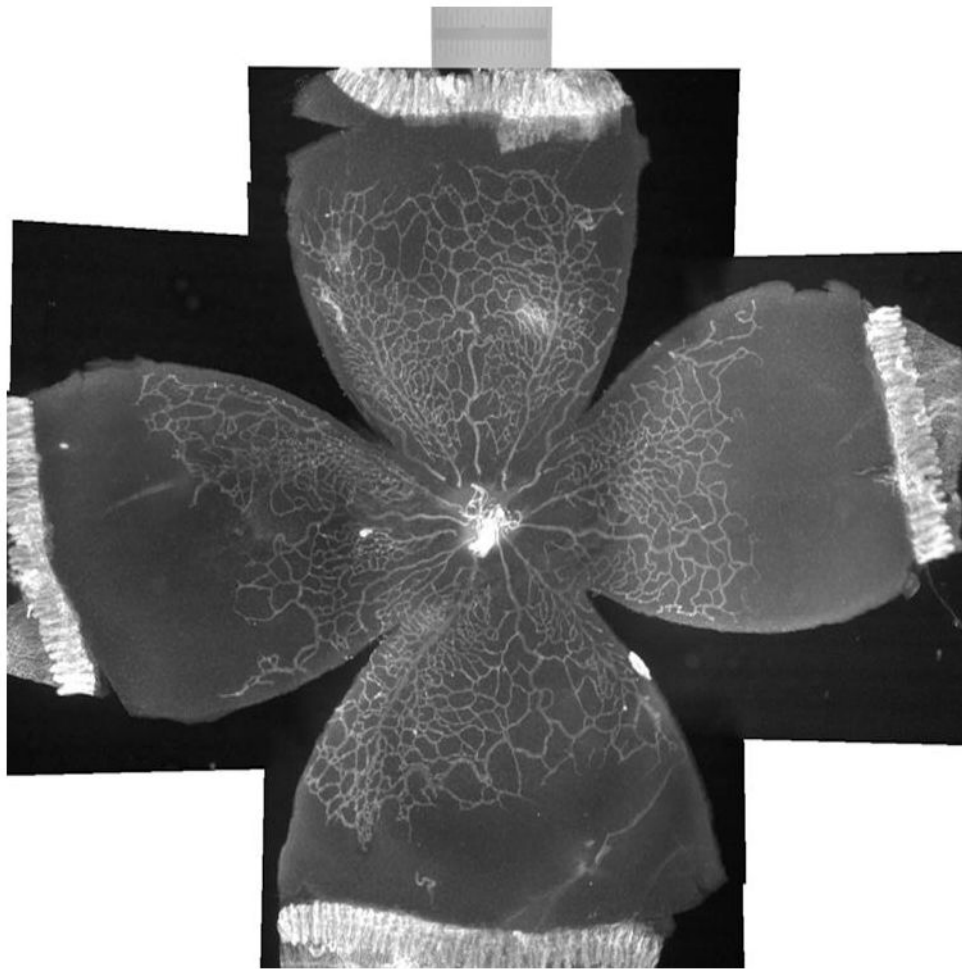
Figure 1.
Lectin-stained retinal flatmount from postnatal day 14 rat pup in the 50/10 OIR model
following 7 cycles of oxygen fluctuations every 24 hours between 50% and 10% oxygen.
Peripheral avascular retina persists, whereas room air counterparts have fully vascularized
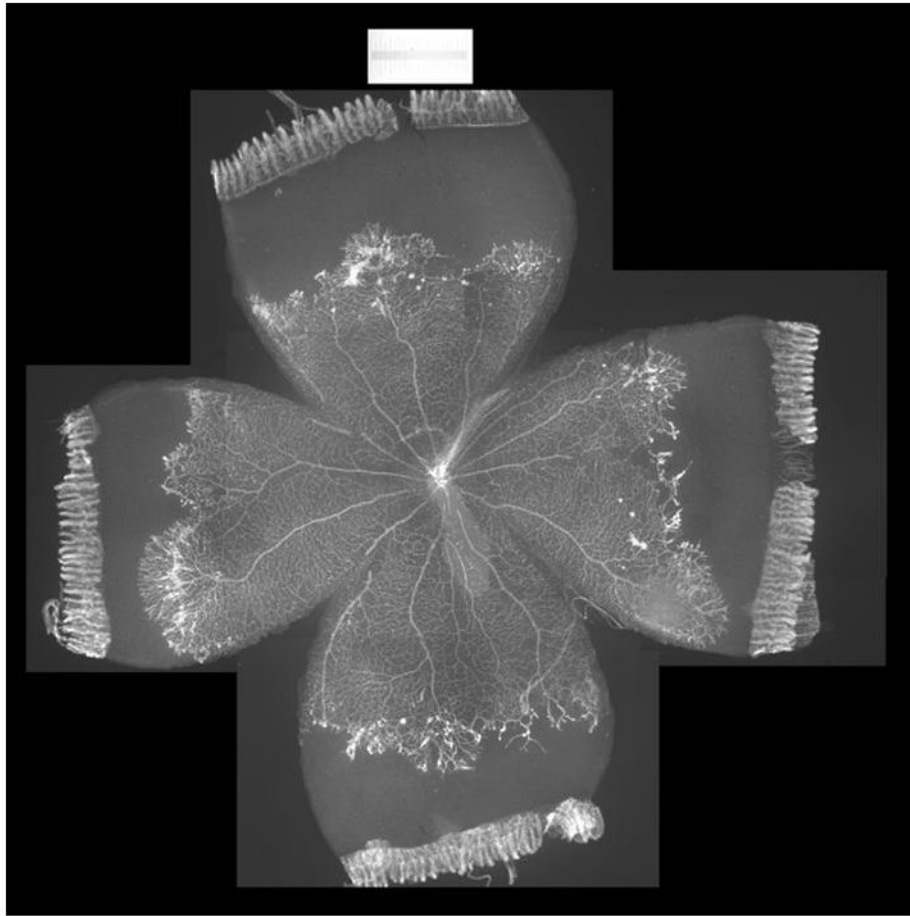retina.

Figure 2.
Lectin-stained retinal flatmount from postnatal day 18 rat pup in 50/10 OIR (7 cycles of oxygen fluctuations every 24 hours between 50% and 10%, followed by 4 days in room air). Intravitreous neovascularization exists at the border of vascular and avascular retina.
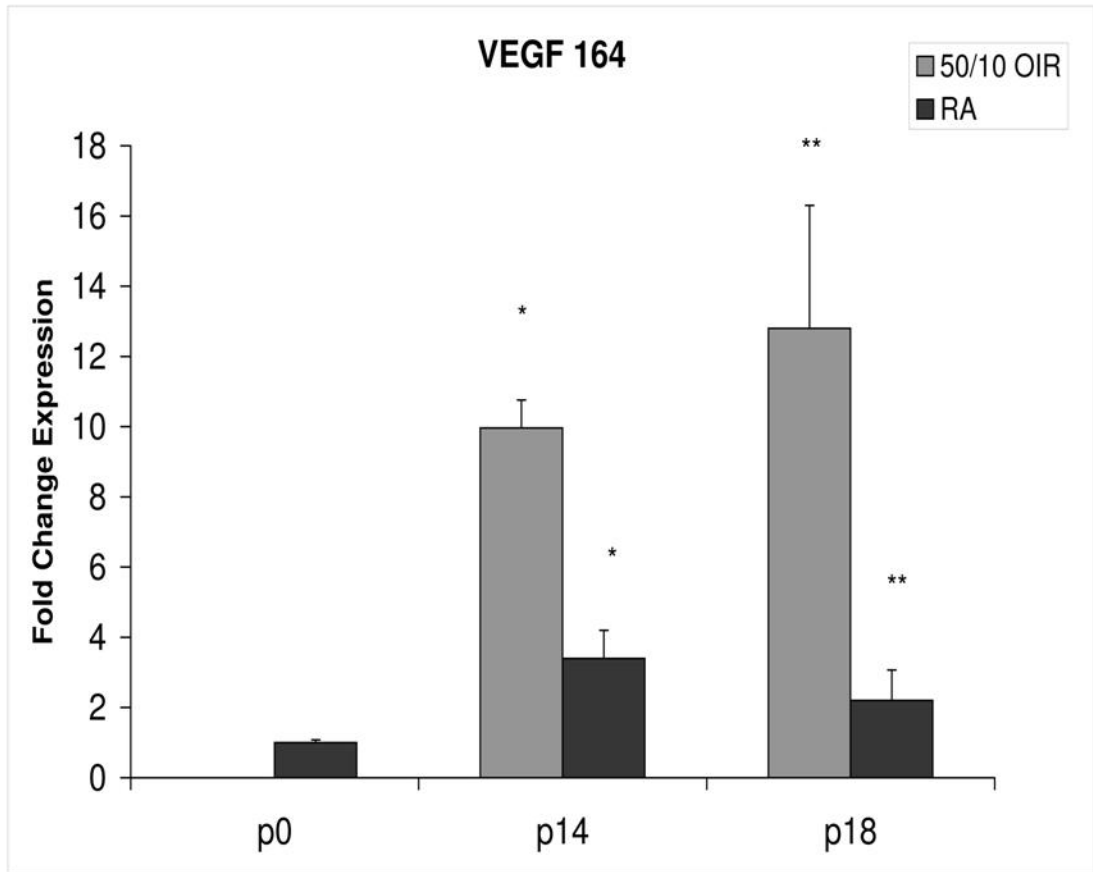
Figure 3.
Fold change in $VEGF_{164}$ mRNA expression, relative to β-actin, at p0, p14 and p18 room air
(RA) and 50/10 OIR. Significantly increased fold expression in $VEGF_{164}$ mRNA was
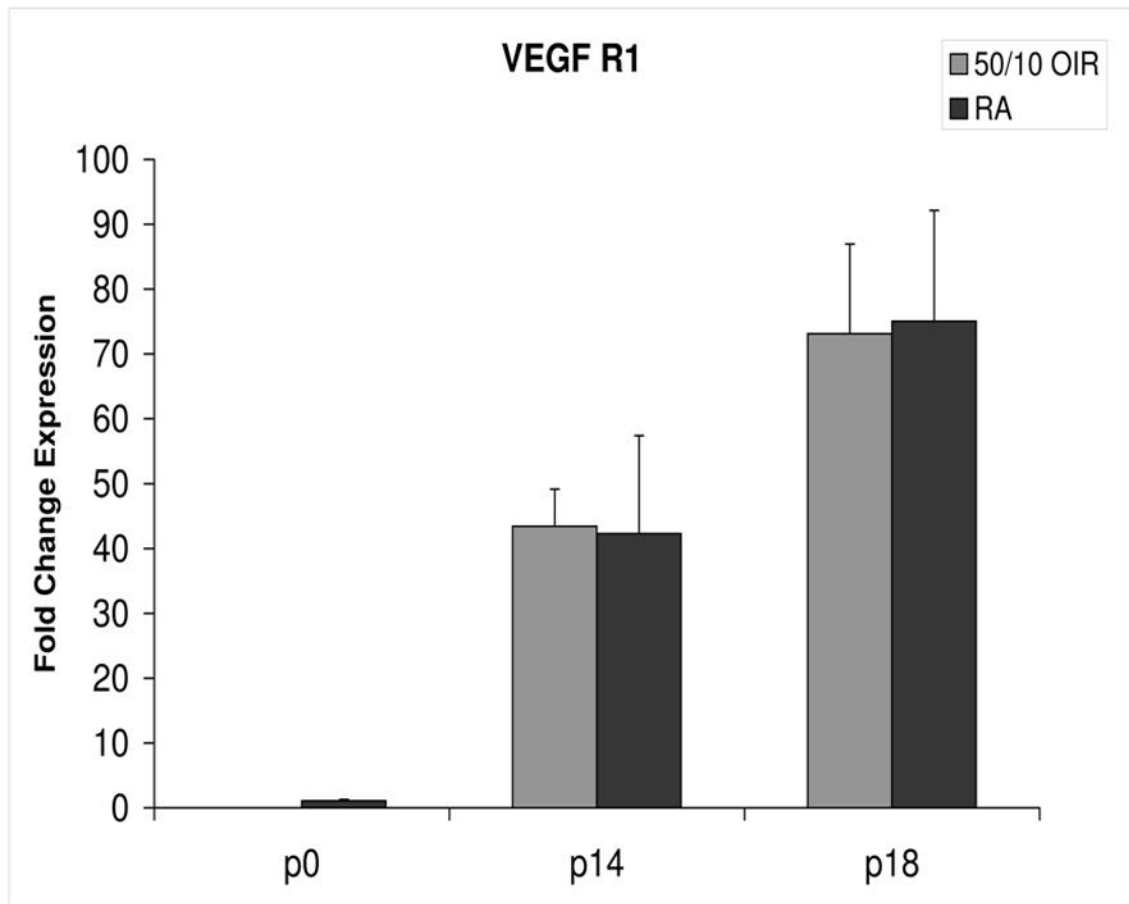present in 50/10 OIR compared to RA at p14 (*p=0.0004) and p18 (**p=0.02). Student t-
tests.
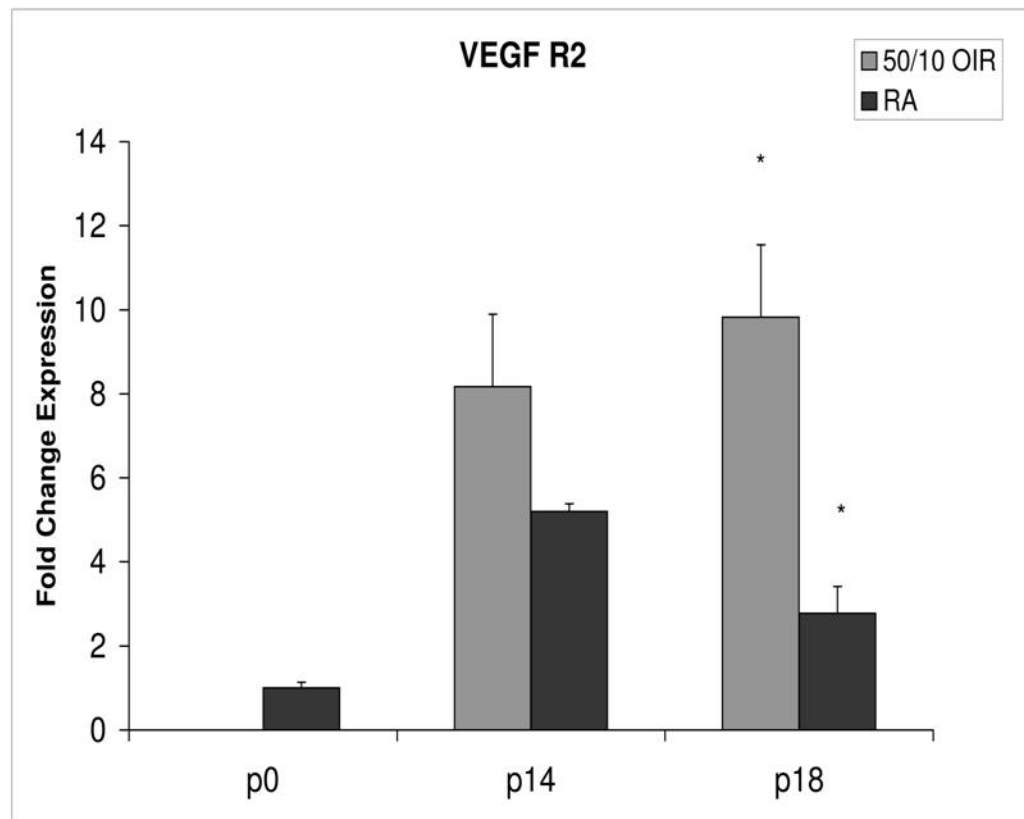
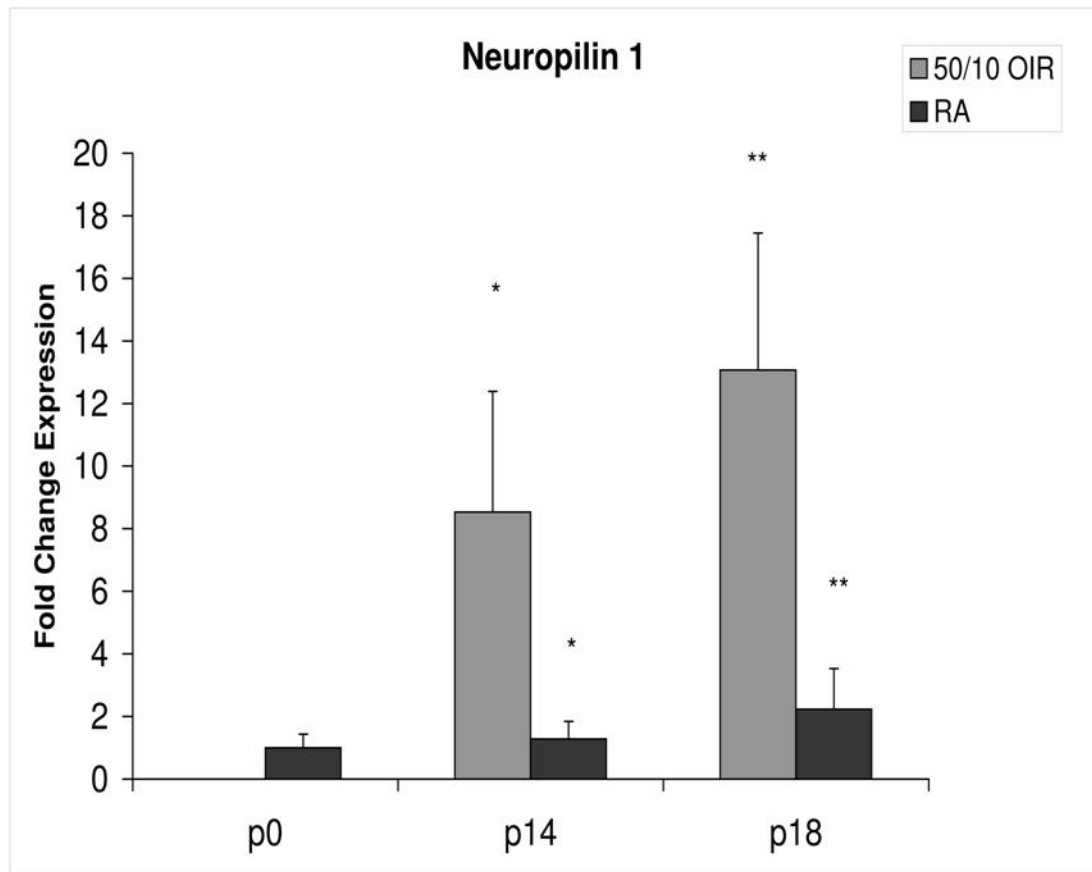**Figure 4.**
Fold change in expression of VEGFR2 mRNA, relative to β-actin, at p0, p14 and p18 room air (RA) and 50/10 OIR. Significantly increased fold expression in VEGFR2 mRNA was present in 50/10 OIR compared to RA at p18 (*p=0.004). A trend toward increased fold expression of VEGFR2 in 50/10 OIR was noted at p14 (p=0.06). Student t-tests.

Figure 5.
Fold change in expression of VEGFR1 mRNA, relative to β-actin, at p0, p14 and p18 room air (RA) and 50/10 OIR. No significant differences were found at p14 (p=0.47) or p18 (p=0.97). Student t-tests.

Figure 6.
Fold change in expression of neuropilin-1 (NP-1) mRNA, relative to β-actin, at p0, p14 and p18 room air (RA) and 50/10 OIR. Significantly increased fold expression in NP-1 mRNA was present in 50/10 OIR compared to RA at p14 (*p=0.042) and p18 (**p=0.027). Student t-tests.
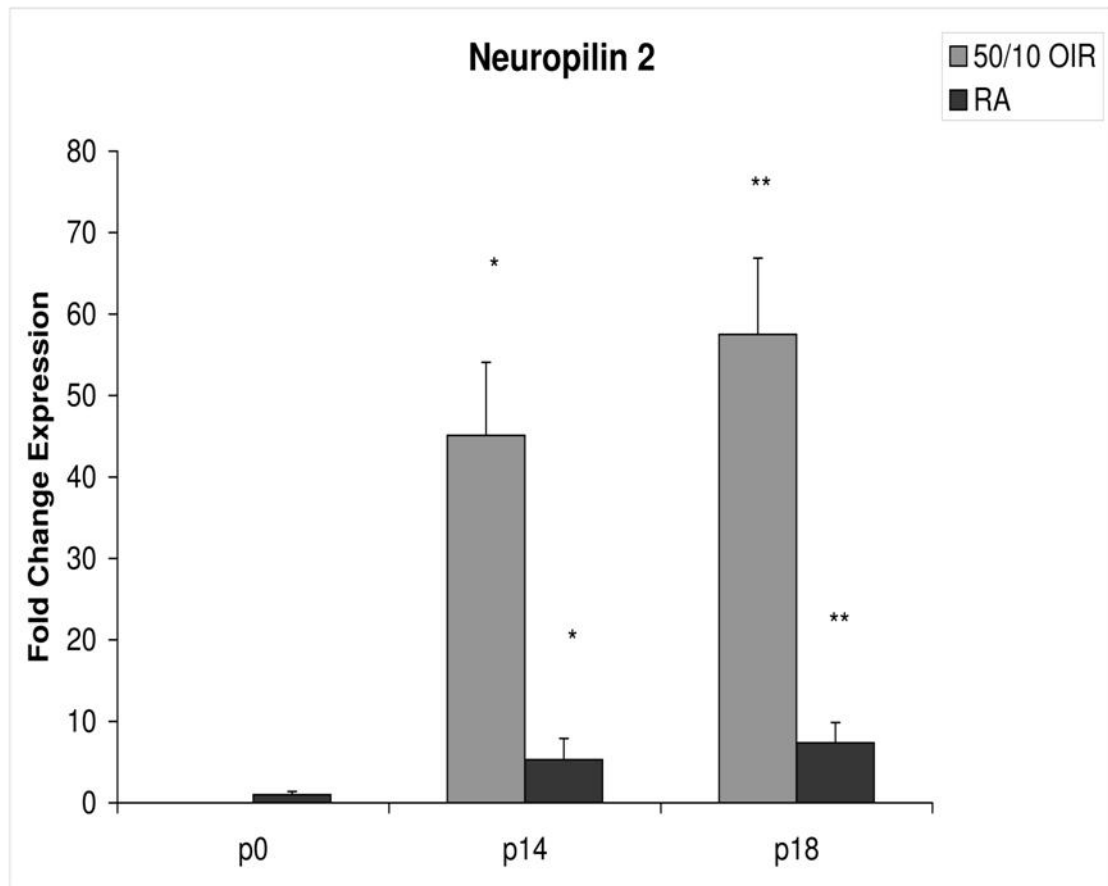
Figure 7.
Fold change in expression of neuropilin-2 (NP-2) mRNA, relative to β-actin, at p0, p14 and p18 room air (RA) and 50/10 OIR. Significantly increased fold expression in NP-2 mRNA was present in 50/10 OIR compared to RA at p14 (*p=0.001) and p18 (**p=0.002). Student t-tests.
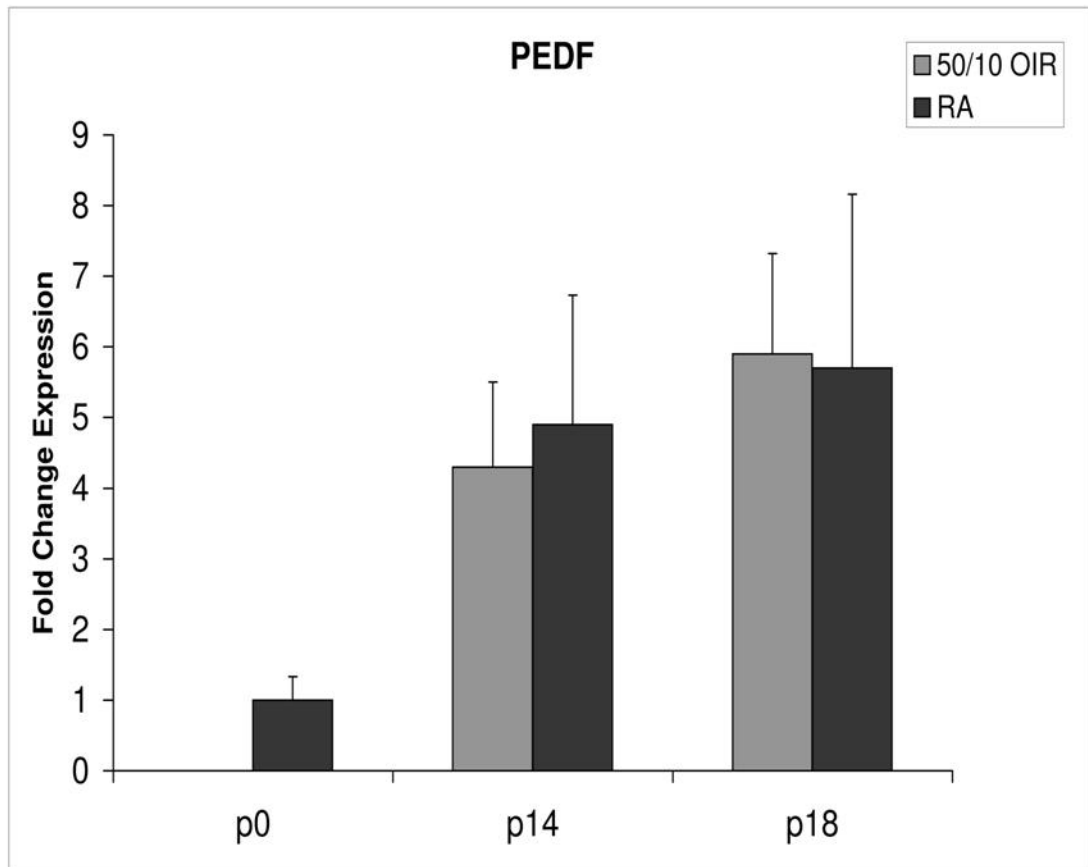
Figure 8.
Fold change in expression of PEDF mRNA, relative to β-actin, at p0, p14 and p18 room air (RA) and 50/10 OIR. No significant difference was found at p14 (p=0.47) or p18 (p=0.39). Student t-tests.
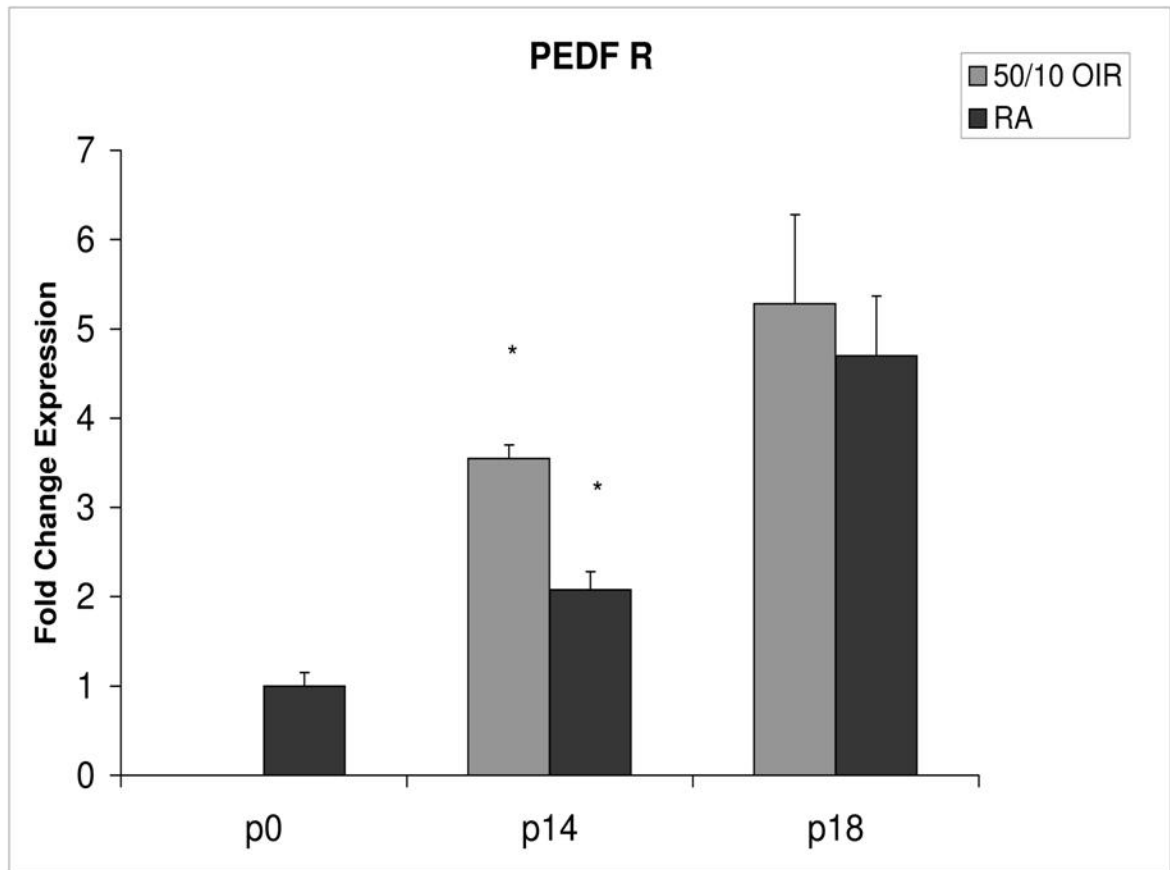
Figure 9.
Fold change in expression of PEDFR mRNA, relative to β-actin, at p0, p14 and p18 room air (RA) and 50/10 OIR. Significantly increased fold expression in PEDFR mRNA was present in 50/10 OIR compared to RA at p14 (*p=0.0006). No significant difference was found at p18 (p=0.32). Student t-tests.