

A novel model of retinopathy of prematurity in normobaric hyperoxic conditions

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Abstract

• **AIM:** To examine changes in retinal vasculature after treatment with different oxygen concentrations from common retinopathy of prematurity (ROP) models and to determine a novel and practical ROP model.

• **METHODS:** A sample of 14 newborn Sprague–Dawley rats was used. The study group ($n=7$) was exposed to 95% oxygen for 4h per day followed by normoxic laboratory conditions for 20h. This cycle was repeated for 14d. The control group ($n=7$) was subjected to normobaric normoxic conditions. On postnatal day 14 (P14), the two groups were placed in room air for 7d. On P21, the two groups were examined using indirect ophthalmoscopy. All eyes were enucleated for immunofluorescence (IF) staining of the vasculature of retinas and analysis of vascular endothelial growth factor (VEGF), hypoxia inducible factor -1 alpha (HIF -1 α), placental growth factor (PLGF) in vitreous humor, and then the rats were sacrificed by decapitation. All procedures were repeated using another litter of 14 pups.

• **RESULTS:** In the study group and under normobaric hyperoxic conditions, retinal neovascularization and peripheral avascular retina were determined in 85% of the rats through indirect ophthalmoscopic examination. Also

IF staining of retina of the study group showed retarded peripheral vascular growth. The difference between the two groups for VEGF, HIF-1 α and PLGF concentrations of vitreous humor was statistically significant ($P=0.003, 0.007, 0.027$ respectively).

• **CONCLUSION:** Fluctuating oxygen concentrations are primarily responsible for retinal neovascularization. Our new ROP model is practical and applicable for all retinal neovascularization studies, considering the laboratory procedures.

• **KEYWORDS:** retinopathy of prematurity; vascular endothelial growth factor; hypoxia inducible factor-1 alpha; placental growth factor; funduscopy; retina staining

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INTRODUCTION

Retinopathy of prematurity (ROP) is characterized by preretinal neovascularization and local ischemia. Although it was shown that preventable causes of childhood blindness such as ROP seemed to be less common in the last two decades in the Netherlands^[1], it is still the most common preventable cause of blindness and impaired vision among preterm infants in developing countries^[2-3]. In 1942, Terry^[4] first described ROP as a disease of prematurity, and the pathogenic role of oxygen was researched in subsequent studies^[5-6].

The pathogenesis of ROP is mainly based on prematurely delivery and fluctuation of oxygen concentrations, especially relative hyperoxia. Prematurely delivery results in decrease of some cytokines, playing an important role on vasculogenesis. Preterm infants usually need supplemental oxygen due to their immature lungs. Relatively high oxygen concentrations compared to in-utero life result in depression of angiogenic mediators. Especially the decrease in vascular endothelial growth factor (VEGF) concentrations halts vasculogenesis. However, the developing retina needs more nutrients and oxygen. This time, ineffective vasculogenesis results in hypoxia. Hypoxia causes an increase in VEGF, resulting in uncontrolled vascular growth into the vitreous and over

surface of retina. At last, retinal detachment and visual loss occurs. Cryotherapy, transpupillary laser treatment, lens-sparing vitrectomy were used for treatment. There are also some candidate interventions for treatment and prevention; such as insulin-like growth factor-1 (IGF-1), anti-VEGF antibodies^[7].

The role of relative hyperoxia in the pathogenesis formed by higher oxygen support has been studied using animal models. Smith *et al*^[8] described vaso-obliterative and vaso-proliferative phases of retinopathy with 75% oxygen support [75 oxygen induced retinopathy (OIR) model] in newborn mice. Another commonly used animal model is the 50/10 OIR model, which was first developed by Penn *et al*^[9]. Penn *et al*^[9] performed experiments to determine a predictable neovascular response with different oxygen concentrations and showed that the 50/10 OIR model simulates human ROP pathogenesis.

These two animal models require advanced technological laboratory units. In this study, we aimed to determine the practical and applicable ROP animal models, especially oxygen treatment with shorter durations daily and totally. The supplemental therapeutic oxygen for prethreshold retinopathy of prematurity (STOP-ROP) study showed no change in progression of prethreshold ROP to proliferative disease when they increased oxygen saturation to 96% -99% from conventional 89%-94% for 2wk and more^[7]. So we designed our study with higher oxygen concentrations for 2wk to mimic and investigate this clinical use.

MATERIALS AND METHODS

This study was undertaken on Sprague-Dawley inbred rats (Gulhane Military Medical Academy Research and Development Center Laboratory of Animal Health Department), with the approval of the Animal Care and Use Committee at Gulhane Military Medical Academy. We certify that all applicable institutional and governmental regulations concerning the ethical use of animals were followed during this research. All the procedures on animals in this study were performed by adhering to the tenets of the ARVO Statement for the use of Animals in Ophthalmic and Vision Research.

Totally 14 newborn Sprague-Dawley inbred rats of the same litter were divided into two groups. The study group ($n=7$) comprised newborn rats within 4h of birth that were placed in an oxygen-chamber in which they were exposed to 95% oxygen for 4h per day followed by normoxic laboratory conditions for 20h (Figure 1). This cycle was repeated for 14d. This oxygen exposure was delivered using an oxygen tube connected to a chamber and the desired oxygen levels were maintained and verified by an oxygen sensor (Teledyne Analytical Instruments, California, USA). The control group ($n=7$) was subjected to normobaric normoxic conditions. On postnatal day 14 (P14), the two groups were placed in room

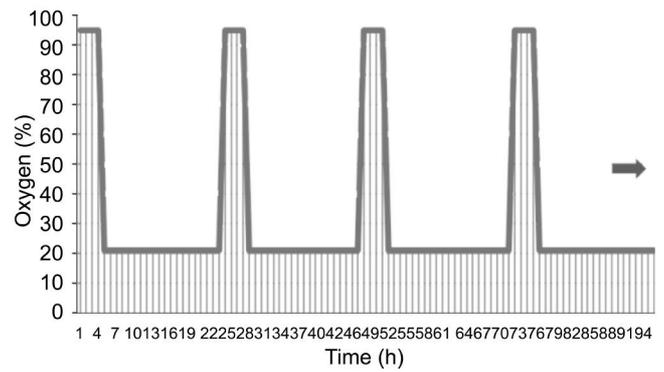


Figure 1 Schematic illustration of oxygen exposure protocol
The rats in the study group were placed in incubators within 4h of birth; after 4h under 95% oxygen support, they were placed in room air for 20h. On P14, the rats were returned to room air for a week to allow neovascularization to occur.

air for 7d. On P21 each rat was sacrificed, and the eyes were enucleated. For each pup, one globe was used for enzyme-linked immunosorbent assay (ELISA) analysis, and the other was used for retina immunofluorescence (IF) staining, as it was not possible to perform both of these analyses on the same globe due to the fixation procedure. The same sampling procedures were repeated using another litter size of 14 pups. As a result, a total number of 28 pups were used during this experiment; consisting of 14 pups in control group and 14 pups in study group.

Fundoscopy Analysis On P21, all the rats were anesthetized by intraperitoneal injection of ketamine (90 mg/kg) and xylazine (10 mg/kg). Then, the two groups were examined using indirect ophthalmoscopy and fundus photographs (Canon CR-1 Mark II Digital Retinal Camera Systems; Canon Inc. Tokyo, Japan) for both litters.

Enzyme-linked Immunosorbent Assay Analysis The eyes from each rat were enucleated under ketamine anesthesia. The cornea, aqueous humor and crystalline lens were removed by perilimbal incision. A pair of vitreous humour was pooled for each rat in 250 µL phosphate-buffered saline (PBS) to assure the effective volume for ELISA. The vitreous humor samples were removed and were kept at -80°C (liquid nitrogen) for VEGF, hypoxia inducible factor-1 alpha (HIF-1α) and placental growth factor (PLGF) analysis. The rats were sacrificed by decapitation.

Vitreous VEGF (R&D Systems, Inc. Minneapolis, MN, USA), HIF-1α and PLGF (Cusabio Wuhan, China) levels were analyzed using 7 pairs of vitreous samples per each group, by commercially available ELISA kits. The intra-assay precision of three different levels (high, normal, low) of VEGF was 3.7%, 5.6% and 2.2%, respectively; while they were stated as <8% for HIF-1α and PLGF. The results were read on an ELISA plate reader (model ELx 800 bioelisa microplate reader; Biokit, Spain).

Immunofluorescence Staining Enucleated globes were

fixed in 4% paraformaldehyde (PFA) for 15min at room temperature (RT). The dissected retinas were flat mounted and treated overnight at 4°C in permeabilization buffer [1% BSA (Roche Fraction V) and 0.5% Triton X-100 (Sigma)]. After washing, the retinas were incubated with Isolectin GS-IB4 from Griffonia simplicifolia, Alexa Fluor® 488 conjugate in Pblec solution (1 mmol/L MgCl₂, 1 mmol/L CaCl₂, 0.1 mmol/L MnCl₂, 1% Triton X-100 in PBS) for 2h at RT. The retinas were washed 6 times, for 10min in PBS, fixed briefly for 5min in 4% PFA, washed twice in PBS and mounted in fluorescent mounting medium (DAKO, Carpinteria, CA, USA) [10]. Low and high magnification images were acquired using fluorescent (Olympus BX50) microscope.

Statistical Analysis The test results were given as mean and standard deviation. Mann-Whitney *U* analysis was used to compare the two groups. Statistical calculations were performed using a commercially available SPSS 15.0 program, and a value of *P*<0.05 was considered significant in all statistical analyses.

RESULTS

On P21, fundoscopic examinations were conducted on all rats in the two groups of both litters by indirect ophthalmoscopy. Vaso-oblivation and vascular tortuosity were determined on the central retina, as it was seen in disease. The study group had peripheral avascularity and intravitreal neovascularization and ridge-like formations at vascular avascular retinal junctions. The control animals had fully vascularized retinas. Fundus photographs of central and peripheral retina were taken (Figure 2).

VEGF, HIF-1α and PLGF levels for the study and control groups were shown in Table 1. The difference between the control and study groups was statistically significant (*P*=0.003, 0.007, 0.027 respectively). Study group had significantly higher vitreal VEGF levels compared to the control group. HIF-1α and PLGF levels in vitreous humor of the study group were also found to be elevated.

IF stained retina specimens were examined under a fluorescent microscope. The control group retina specimens were fully vascularized including the peripheral retina. IF staining of retina of the study group showed some vascular abnormalities (tortuous vessels, retarded peripheral vascular growth, and possible hemorrhage) (Figure 3). There were avascular areas especially on peripheral retina in the study group. Ridge-like formations were seen in junctions of vascular and avascular parts. Also, there were potential remnants of the hyaloid vasculature. Increased tortuosity of the retinal vessels was another sign indicating that ROP model was created in the study group.

DISCUSSION

Retinal blood vessel development depends on a physiologic

Table 1 VEGF, HIF-1α and PLGF levels for the two groups

Parameters	n=7 for each group		
	Control	Study	<i>P</i>
VEGF (pg/mL)	55.57±16.83	239.57±155.07	0.003
HIF-1α (pg/mL)	8.88±3.86	14.95±2.19	0.007
PLGF (pg/mL)	57.13±34.51	103.38±40.28	0.027

VEGF: Vascular endothelial growth factor; HIF-1α: Hypoxia inducible factor-1 alpha; PLGF: Placental growth factor.

hypoxic uterine environment with a partial pressure of dissolved arterial oxygen (PaO₂) of 30 mm Hg. This physiologic hypoxia stimulates growth factor production, such as VEGF and IGF-1, resulting in retinal vascular development. PaO₂ rises to 55-80 mm Hg as a result of premature birth [11]. This early post-natal hyperoxic environment is believed to play a role in the retardation of angiogenesis by reducing the stimulation for growth factors [12].

The pathogenesis of ROP is biphasic. The first phase (early phase: 30-32wk) is characterized by the cessation of vascular development due to a post-natal hyperoxic environment worsened by supplemental oxygen therapy in neonatal intensive care units. Delayed development of vasculature causes physiologic retinal hypoxia, which leads to the second phase (late phase: 32-34wk) characterized by vasoproliferation and neovascularization [13-14] (Figure 4). The aim of animal retinal neovascularization modeling studies is to mimic this pathogenic pathway.

Animal models of OIR aim to delay vascularization with oxygen supply and to stimulate growth factor production and neovascularization with relative hypoxic phase [12]. Different OIR models were determined in different animals. This model was first developed for kittens by Ashton *et al* [15] in 1954 and was then extended to other animal species, including rats [9], mice [8], beagle puppies [15] and zebrafish [16]. The mouse OIR model described by Smith *et al* [18] (75 OIR model) and the rat OIR model described by Penn *et al* [9] (50/10 OIR model) are the most common models for studying pathologic angiogenesis associated with ROP [12].

In 1953, Patz *et al* [6] first described "preretinal neovascularization" in rats due to extreme hyperoxia. Thereafter, many investigators, such as Ricci and Calogero [17], attempted to demonstrate a reliable rat ROP model. Ricci and Calogero [17] used a 80% oxygen/5d, followed by room air/5d protocol, Ventresca *et al* [18] used a 80% oxygen/10d, followed by room air/15d protocol and Reynaud and Dorey [19] used a 80% oxygen/11d, followed by room air/6d protocol. However, all of these investigations failed in the modeling, particularly in determining reliable neovascular pathology.

Over time, investigators have reflected on the role of therapeutic oxygen use and PaO₂ in the pathogenesis of ROP [20-21]. PaO₂ levels in premature infants can fluctuate rapidly. These severe hypoxia and hyperoxia periods are related to systemic diseases, such as patent ductus

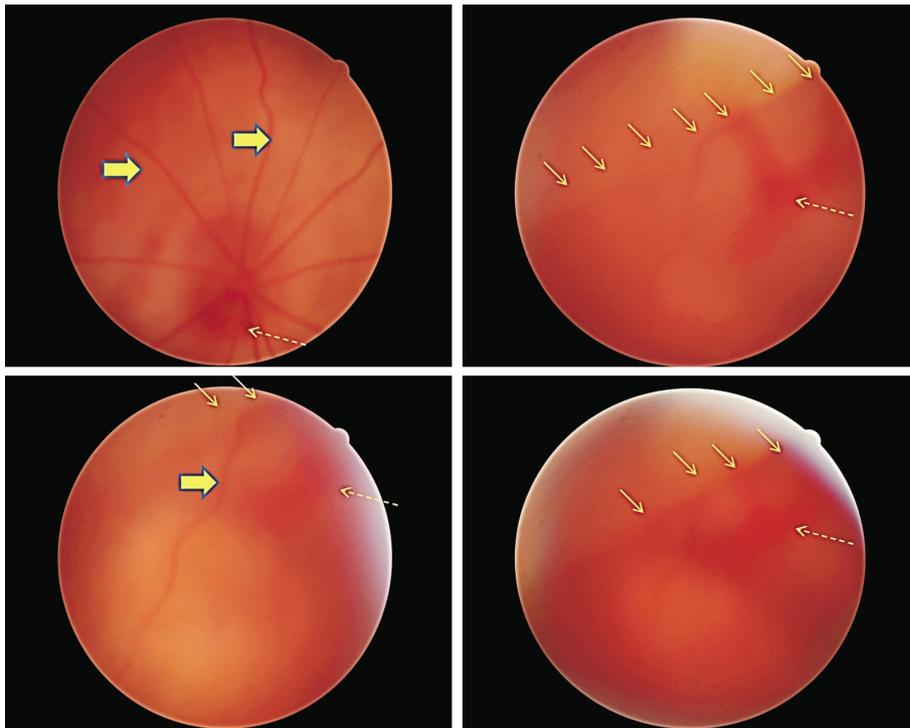


Figure 2 Fundus photographs of the study group Bold arrow: Vascular tortuosity. Thin arrow: Peripheral vascular-avascular retinal junction. Dashed arrow: Retinal and intravitreal neovascularization (retinal hemorrhage at optic nerve head).

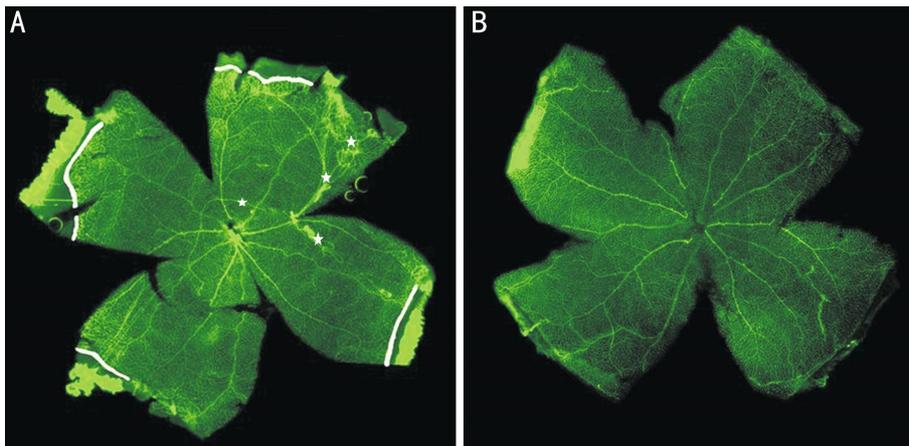


Figure 3 The difference between retina vasculature of the two groups A: Sample retinal quadrants from a 21-day pup from study group, showing avascular area in the peripheral retina (white line: demarcation line of vascularized and avascularized area) and neovascularization can be seen well. Neovascular tufts extending into vitreous were marked with an asterisk; B: Sample retinal quadrants from a 21-day pup, showing normal retinal vasculature from control group.

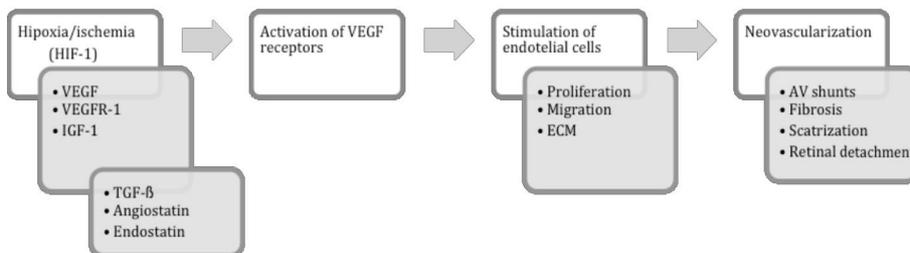


Figure 4 Retinal neovascularization pathway VEGF: Vascular endothelial growth factor; VEGFR-1: VEGF receptor-1; IGF-1: Insulin-like growth factor-1; TGF-β: Transforming growth factor-β; HIF-1: Hypoxia induced factor-1; ECM: Extracellular matrix.

arteriosus [22], or to therapeutic oxygen use in intensive care units [20]. The causal connection between PaO₂ fluctuation and the development of ROP is seen in both animal [9,23] and clinical studies [24-25].

Penn *et al* [26] attempted mimicking arterial fluctuation with systematically varied oxygen protocols. At the end of the trial, 66% of the rats that were exposed to a cycle of 80% oxygen for 12h followed by 40% oxygen for the next 12h

developed preretinal neovascularization. Although variable oxygen promotes retinal neovascularization, PaO₂ in healthy animals with normal lung function cannot mimic premature infants suffering from acute pulmonary distress.

Penn *et al*^[23] proposed that predictable oxygen variability would lead to predictable and reliable neovascular responses. They altered the exposure protocol to 50% oxygen followed by 10% oxygen per 24h; in measuring Δ FiO₂, 97% of the rats developed retinal neovascularization. This study demonstrated the frequently used model of ROP (50/10 OIR model) and indicated that fluctuation is more important than quantity of oxygen in pathogenesis. The model's primary difficulties involve the advanced laboratory technology required, such as atmosphere and oxygen-level controlled cages, 24h manpower and feeding.

A similar model firstly used in mice was developed by Smith *et al*^[8] and is referred to as the 75 OIR model. They exposed mice to 75% oxygen on post-natal day 7 for 5d followed by room air. The major disadvantage of this model is that obliteration of central retinal vessels in response to hyperoxia occurs more than for peripheral vessels.

Compared with current OIR models, our new ROP model is practical and applicable. Unlike previous studies, we induced retinopathy using a fluctuation of 95% to room air oxygen over periods of between 4h and 20h. We determined retinal neovascularization and peripheral avascular retina in 85% of rats.

In our study, IF staining results showed that there were some vascular abnormalities in study group such as tortuous vessels and especially avascular areas on peripheral retina. These results are compatible with 50/10 OIR model and the clinical findings in human ROP disease^[23].

In addition to immunofluorescence results, VEGF and HIF-1 α concentrations analyzed with ELISA method in the vitreous humor were significantly higher in study group, likely with the other studies^[27-28]. To our knowledge, this is the first study about vitreal PLGF concentrations in ROP models. Hypoxia, due to the insufficient vasculature in peripheral retina, causes an accumulation of HIF-1 α . HIF-1 α binds to a promoter of VEGF gene and increases the expression of VEGF. In hyperoxic conditions, a post-translational modification (hydroxylation) causes HIF-1 α to bind to von Hippel-Lindau protein, resulting in ubiquitination and degradation. However, in hypoxia these hydroxylases become less effective and HIF-1 α is no longer degraded and VEGF level increases^[29]. VEGF is the main peptide that induces the proliferation, migration, differentiation and maturation of endothelial cells *via* mainly on vascular endothelial growth factor receptor (VEGFR)-2.

PLGF is another important factor during retinal vascularization. PLGF and VEGF together binds to

VEGFR-1 and leads to angiogenesis^[29-30]. It is thought to be responsible in ROP development rather in first phase. In some studies, it is stated that high levels of PLGF is related with ROP development, while there are some other controversies. In our study, we also found that PLGF levels of study group were significantly higher. This may be responsible for the pathological angiogenesis during the development of retinal vasculature. That's why VEGF and PLGF were chosen as a target for therapeutic approaches in ROP such as bevacizumab treatment^[31-33]. HIF-1 α inhibitors were also evaluated in ROP therapy^[34].

Our data and previously published data indicate that fluctuation in oxygen concentrations is one of the most important factors for retinal neovascularization. Animal models remain important for understanding ROP mechanisms and for therapeutic applications. Our IF staining images, funduscopy photographs and ELISA results are consistent with the previous studies. However, in our model we created a less severe ROP model, pointing out a lower stage of ROP, compared to the other models. That's why area of central hypoperfusion can not be clearly seen on retina specimens. Neovascular tufts extending into vitreous and peripheral avascular area accompanied by no hypoperfusion on central retina indicate a lower stage of ROP. Additionally, using some different rat strains for ROP models can result in higher severity of neovascularization. We used Sprague-Dawley rats in this study. ROP models of higher severity can be created by using Brown Norway rat strains, since this strain is known to be related with more severe retinopathy. Another limitation of this study was that this model was not validated by using an anti-VEGF agent. An inhibitor might be used to support and validate our results. Using different rat strains, considering the new interventions on this model to prevent ROP, such as anti-VEGF therapy is our future work. So, this new method can be used as an applicable and practical animal model of ROP, and all these results can be compared with clinical use (supplemental oxygen therapy for preterm infants) in neonatal intensive care units.

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Conflicts of Interest: Ozgurtas T, None; Tekin S, None; Yesildal F, None; Karaca U, None; Aydin FN, None; Ugurlu MT, None; Ozler M, None; Durukan H, None.

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