**Impaired nephrogenesis in oxygen-induced retinopathy in neonatal rats**

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Impaired nephrogenesis in oxygen-induced retinopathy in neonatal rats

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Running Head: Oxygen-induced retinopathy due to impaired renal development
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Abstract

Objectives: Preterm neonates are born during ongoing nephrogenesis and are commonly exposed to factors in a hyperoxic environment that may impair renal development. Oxidative stress has also been implicated in the development of retinopathy of prematurity (ROP). The rat model of oxygen-induced retinopathy (OIR) is the most clinically relevant model of ROP because the biologic features of OIR closely resembles those of ROP in preterm infants. We aimed to determine impaired
renal development in a rat model of OIR. Methods: Newborn Sprague–Dawley rats were kept in either a normoxic (room air, 21% O₂) or controlled hyperoxic (80% O₂) environment from birth to postnatal day 12 (P12). All pups were then raised in room air from P12 to P19. Results: Our results indicate that the hyperoxic environment led to significant high urinary excretion of 8-OHdG, a marker of oxidative DNA damage, and reduction in nephrogenic zone width in pups raised in OIR group at P5. Additionally, glomerular counts were significantly reduced in the OIR group by 20%, and avascular area and neovascular changes in the retina were observed only in the OIR pups at P19. The mRNA levels of vascular endothelial growth factor A (VEGF-A) and platelet derived growth factor β (PDGF-β), essential angiogenic cytokines for glomerulogenesis, in the renal cortex were significantly lower at P5 and significantly higher at P19 in the OIR group than in controls. Conclusion: In the rat model of OIR, nephrogenesis was impaired and its mechanism was similar to ROP, which was caused by VEGF-A.
Keywords: nephrogenesis; neonatal hyperoxia; preterm infant; retinopathy of prematurity; vascular endothelial growth factor A

INTRODUCTION

Preterm neonates are often born while the renal development is still ongoing because nephrogenesis is not normally completed until 34–36 weeks of gestation(1); thus, renal development continues after birth in preterm neonates. However, glomerular abnormalities and reduced glomerular formation observed in this population indicate potentially impaired postnatal nephrogenesis(1, 2). Hypertension(3, 4), reduced kidney size(5, 6), and impaired renal function(7, 8) occur in preterm born children and adults, highlighting the long-term consequences of preterm birth on renal health.

The cause of impaired renal development following preterm birth and the mechanisms by which this may predispose to adult renal disease are largely unknown; however, exposure to O₂ in the extrauterine environment may be a contributing factor(9-11). Upon birth, infants are exposed to O₂ concentrations far exceeding the intrauterine levels(12), causing oxidative stress(13, 14) that they are particularly susceptible to due to their low antioxidant levels(15, 16).

Oxidative stress has been implicated in numerous commonly occurring
diseases of prematurity including retinopathy of prematurity (ROP)(17). ROP incidence and severity are directly proportional to the degree of prematurity as the retina is incompletely vascularized in preterm infants(18, 19). The rat model of oxygen-induced retinopathy (OIR) is the most clinically relevant model of ROP as the biologic features of OIR and severe ROP are similar in preterm infants. Importantly, studies using the OIR model propose altered vascular endothelial growth factor A (VEGF-A) signaling as a mechanism(20, 21), wherein renal damage in preterm infant probably results from altered microangiogenesis caused by reactive O$_2$ and VEGF-A. Herein, we determined the role of O$_2$ as a definitive risk for ROP and renal injury using a rat OIR model. We specifically assessed VEGF-A expression in the developing kidney and its influence on the number of nephrons.

MATERIALS AND METHODS

Animals

All studies were approved by the Juntendo University Animal Care Facility. The treatment and care of animals were in accordance with the principles of the Juntendo University Animal Care Facility. Sprague–Dawley rat pups (Nihon SLC, Shizuoka, Japan) were naturally born at term and maintained either in 80% O$_2$ (mixture of
medical grade 100% O\textsubscript{2} and room air; Oxycycler ProOx 110; Biosherix, Lacona, NY, USA) or room air from postnatal day (P) 0 to P12.

**Experimental groups**

In the OIR group, pups were exposed to daily cycles of 80% O\textsubscript{2} (20.5 h), ambient air (0.5 h), and a progressive return to 80% O\textsubscript{2} (3 h) in an Oxycycler from P0 to P12 ($n = 1$ litter). Control pups were kept with their dams in room air [normoxia group (control); $n = 1$ litter]. Pups in both groups were then raised in room air (21% O\textsubscript{2}) from P12 to P19. All litters were equalized to $n = 13$ at P0; there was no difference in the survival of pups between groups in all periods. In all groups, five randomly selected pups were assessed at P5; the remainder of pups were grown until P19 ($n = 8$/group). At P5 and P19, kidneys were collected for analysis following euthanasia, and body and kidney weights were recorded. Furthermore, eyes were collected for analysis of retina vascularization at P19. Spot urine samples were collected using bladder puncture at P5 and P19.

**Histomorphometry**

Harvested kidneys were fixed in 10% formalin, embedded in paraffin, and cut into sections starting from the central region of the kidney across the full coronal plane. Slides were stained with hematoxylin and eosin (H&E) and examined by optical
For microscopy. For each section, three microphotographs from the anterior, posterior, and mediolateral regions of the kidney, each including the full thickness of the cortex, were obtained with a digital camera at a magnification of 400× (DS-L3; Nikon, Japan). The width of the nephrogenic zone indicating the area of growth in the outer renal cortex was determined in P5 pups. Five measurements of each parameter were recorded in each of the three fields of view (15 measurements per kidney), which were then averaged to determine the mean width per kidney. To determine the change in the number of nephrons, glomeruli in the whole section starting from the central region of the kidney across the full coronal plane were counted in P19 pups.

**Processing of the retina**

Both eyes from each pup at P19 were enucleated, and each right eye was fixed in 4% paraformaldehyde in cacodylate buffer (0.1 M, pH 7.2) for 48 h. The corneas, lenses, and scleras were removed under a microscope; the retinas were stained with ADPase. The digital images of flat-mounted retinas were captured using a Nikon D100 camera (Nikon, Tokyo, Japan). The total retinal area (TRA) and peripheral avascular area (AVA) were measured using an imaging software (Definiens XD, Definiens, Germany). The peripheral AVA was expressed as the percentage of TRA (%AVA)(22).

**Measurement of urinary 8-OHdG**
Spot urine samples were collected from each pup by bladder puncture at P5 and P19. They were then stored at −20°C until the assay. They were diluted 10 times with water. The concentration of 8-OHdG was determined using a commercially available competitive enzyme linked immune-sorbent assay (ELISA) kit (New 8-OHdG check; Japan Institute for the Control of Aging, Shizuoka, Japan). The specificity of the assay was established, and the determination range was 0.5–200 ng/ml. Urinary 8-OHdG excretion was expressed as a creatinine ratio. When 8-OHdG was undetectable, it was considered to be zero.

**RT-PCR for VEGF-A and PDGF-β**

TaqTMan probe-based quantitative RT-PCR was performed to detect changes in the expression of VEGF-A and PDGF-β in the renal cortex. Briefly, cDNA was synthesized from renal tissue RNA (ReverTraAce qPCR RT Master Mix; Toyobo, Osaka, Japan) and was amplified using primers and probes for VEGF-A, PDGF-β and β-actin from TaqManGene Expression Assays (Applied Biosystems, Foster City, CA, USA). The products were analyzed using a 7500 Fast Real-Time PCR system (Applied Biosystems) with default settings. The expression of VEGF-A and PDGF-β was normalized to that of β-actin using the relative comparative CT (ΔΔCT) method.

**Statistical analysis**
Data are expressed as means ± standard error of the mean (SEM). Comparisons among groups were performed using the Mann–Whitney $U$ test, and a $p$-value of $<0.05$ was considered to be statistically significant.

**RESULTS**

*Hyperoxia does not impact kidney weight in P19 pups*

Body weight was significantly lower in the OIR group than in the control group at P19 (36.1 ± 0.5 and 41.0 ± 0.9 g, respectively, $p < 0.01$). There were no significant differences in kidney weight between groups (0.22 ± 0.01 g and 0.23 ± 0.01 g, respectively, $p = 0.75$). There were no important adverse events in each experimental group.

*Hyperoxia increases oxidative stress in P5 pup*

Urinary excretion of 8-OHDG creatinine ratio significantly increased in the OIR group at P5 (330.0 ± 58.9 ng/mg and 59.3 ± 19.3 ng/mg, respectively, $p < 0.01$) (Fig. 1). At P19, urinary excretion of 8-OHDG did not significantly differ between the two groups (133.5 ± 24.1 ng/mg, 153.7 ± 44.0 ng/mg, respectively, $p = 0.90$) (Fig. 1).

*Hyperoxia decreases nephrogenic zone width and glomeruli number*
Hyperoxia exposure led to a significant reduction in nephrogenic zone width at P5 (Fig. 1) in the OIR group; the average nephrogenic zone width was 160.01 ± 3.84 µm and 124.2 ± 3.30 µm in the control and OIR groups, respectively, indicating a significant reduction of 21% in pups exposed to hyperoxic conditions ($p = 0.01$). In addition, the assessment of P19 pups showed that the number of glomeruli per section was 308–414 in the control group (average, 340.25) and 181-320 in the OIR group (average, 272.29) (Fig. 2), indicating a significant reduction in the number of glomeruli in response to hyperoxia exposure (20%, $p < 0.01$) despite no changes in kidney weight.

**Hyperoxia-induced retinal avascularity at P19**

The retinas of OIR pups exhibited neovascular changes, as detected by increased ADPase staining of flat-mounted retinas of pups exposed to hyperoxic conditions (Fig. 3,4). In addition, the further assessment of TRA and AVA to quantify avascularity in these specimens revealed that %AVA was 13.75 ± 15.10% in the OIR group, whereas AVA was not detected in any control pup.

**Hyperoxia-induced and time-dependent alterations in VEGF-A and PDGF-βmRNA**

VEGF-A mRNA levels in the renal cortex were significantly lower in the OIR group than in the control group at P5 (0.60 ± 0.01 and 0.69 ± 0.02, respectively, relative ratio to one randomly selected control rat, $p < 0.01$). PDGF-β mRNA levels in the renal...
cortex were also significantly lower in the OIR group than in the control group at P5 (0.66 ± 0.02 and 0.86 ± 0.03, respectively, relative ratio to one randomly selected control rat, \( p < 0.01 \)). In contrast, VEGF-A mRNA levels in the renal cortex were significantly higher in the OIR group than in the control group at P19 (1.432 ± 0.22 and 0.86 ± 0.13, respectively, relative ratio to one randomly selected control rat, \( p = 0.0004 \)) (Fig. 5). PDGF-B mRNA levels in the renal cortex were also significantly higher in the OIR group than in the control group at P19 (1.14 ± 0.07 and 0.90 ± 0.04, respectively, relative ratio to one randomly selected control rat, \( p = 0.02 \)) (Fig. 5).

**DISCUSSION**

Here, using the OIR model, newborn rat pups were exposed to daily cycles of 80% \( O_2 \) from P0 to P12 during a time interval of ongoing nephrogenesis and retinal vascularization. Our findings provided further support that both renal and retinal changes might share a common mechanism. Our results clearly demonstrated significant hyperoxia-mediated increase in oxidative stress and reductions in nephrogenic zone width and VEGF-A mRNA expression in the kidney at P5. Further, after returning to room air at P12, retinal avascular changes and decreases in the number of glomeruli, we detected an increase in VEGF-A mRNA expression in the
kidney seven days later at P19 at a time when nephrogenesis and retinal vascularization were finished.

Previously, Yzydorczyk et al. (10) showed that nephron endowment was reduced by 25% in 25–35-week-old rats exposed to hyperoxia during postnatal nephrogenesis (rat, in 80% O\textsubscript{2} from P3 to P10). While they reported decreases in glomeruli number, it was not clear whether hyperoxic exposure in the neonatal period directly impaired nephrogenesis as the endpoints were assessed in adult rats. Popescu et al. (23) reported reduced nephrogenic zone width and decreased glomerular size in P5 pups exposed to hyperoxia during postnatal nephrogenesis (rat, in 80% O\textsubscript{2} from P3 to P10). Although they found reductions in nephrogenic zone width in pups assessed at P10, these changes did not extend to the glomerular number after the completion of nephrogenesis. Exposure to hyperoxia during postnatal nephrogenesis (mouse, in 65% O\textsubscript{2} from P0 to P7) did not lead to any overt adverse effects on renal development (32). Only in early adulthood, glomeruli were enlarged in hyperoxia-exposed kidneys.

Based on current and previous findings, hyperoxic exposure during postnatal nephrogenesis is clearly associated with renal damage. However, the degree of damage appears to vary across studies, and the involved mechanisms remain unclear. Different O\textsubscript{2} levels used across different studies and individual variabilities in oxidative stress
induced by the same O$_2$ levels within a study may be the reasons underlying variations in renal damage.

Herein, glomerular number decrease was observed at the end of renal development, which has not been demonstrated before. Several current models of ROP recreate fluctuations in O$_2$ tension, which is recognized as a risk factor for severe ROP(24, 25). Also, rat pups were exposed to daily cycles of 80% O$_2$ (20.5 h), ambient air (0.5 h), and a progressive return to 80% O$_2$ (3 h)(22, 26). Our methods may have induced higher renal damage of neonatal rats than previously employed methods.

The unmonitored use of supplemental O$_2$ is considered the main causal factor in the ROP development(27, 28). In the 1950s, the restriction of high O$_2$ levels to preterm infants resulted in a decline in the incidence of ROP(29). In animals, normal retinal vascularization occurs postnatally, in contrast to humans in whom it is a prenatal process; thus, ROP is induced in animals a few weeks after birth. The rat model of OIR is the most clinically relevant model of ROP as its biologic features closely resemble those of severe ROP in preterm infants.

In animals, ROP develops in two phases. Phase I comprises exposure to hyperoxia with subsequent downregulation of VEGF-A. In animal models, the degree of hyperoxia is significantly higher than in humans and is associated with
vaso-oblitration in the central retina in mice and the peripheral retina in rats; the latter site has also been shown to be affected in humans(30). Our findings of avascular area in P19 pup retinas are in agreement with these observations. Phase II of ROP involves the exposure of animals to room air, in which stage hypoxia-induced pathological angiogenesis occurs, similar to that observed in children with ROP. During the proliferative vasculopathy of ROP, VEGF-A expression was stronger than in the normally developing retina, and the astrocytes that normally express VEGF degenerated(31). We detected neovascular changes of retina in P19 pups. Because a previous study showed that both phase I avascular retina and phase II neovascularization peaked at P19 in ROP models(32), we have assessed retina and kidney at P19.

In the developing kidney, podocytes and renal tubular epithelial cells express VEGF-A(33). Gene deletion studies in mice have shown that VEGF-A and its cognate receptor, VEGFR-2, are essential for vasculogenesis(34, 35). Glomerular endothelial cells express VEGFR-2 as they migrate into the vascular cleft. The role of VEGF-A in kidney development was studied in vivo by blocking endogenous VEGF-A activity with an anti-VEGF-A antibody in newborn mice; vessel formation in the superficial renal cortex was disturbed, and nephrogenic zones and the number of developing
nephrons were significantly decreased in these mice (36, 37). Further, Eremina et al. (38) used the Cre-loxP system to develop transgenic mice with three different expression levels, i.e., doses, of VEGF-A within the podocytes based on the allele copy number. The results demonstrated an exquisite dosage sensitivity for VEGF-A in the developing glomerulus.

To our knowledge, this is the first report on VEGF-A in the renal cortex of hyperoxia exposure rat model. Herein, the decrease in VEGF-A expression in the renal cortex at P5 and decreased glomeruli number at P19 were similar to phase I of ROP. Furthermore, the increase in VEGF-A expression in the renal cortex at P19 was similar to phase II of ROP. These findings suggest that the mechanism of impaired nephrogenesis induced by hyperoxia is similar to the mechanism of ROP that includes avascular and neovascular changes, and impaired nephrogenesis can be observed in OIR pups at P19. However, in this study, we did not evaluate neovascular changes by increase in VEGF-A.

In conclusion, renal impairment due to the hyperoxic environment during nephrogenesis and the pathology of OIR in this model recapitulated ROP observed in preterm infants. Our findings suggest that the clinical care of preterm infants with a focus in decreasing ROP incidence should provide protection from the development of
renal impairment.
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Disclosure

No conflicts of interest, financial or otherwise, are declared by the authors.

Author contribution

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Figure Captions

8-OHdG: 8-hydroxy-2'-deoxyguanosine, OIR: oxygen-induced retinopathy, SEM: standard error of the mean, VEGF-A: vascular endothelial growth factor A, PDGF-β: platelet derived growth factor β

Figure 1. Urinary 8-OHdG excretion was expressed as a creatinine ratio at P5 and P19

(a) There was a significant effect of hyperoxia exposure on urinary 8-OHdG excretion at P5 in the OIR group (dark gray bar) compared with the control group (light gray bar).

Results are expressed as the mean ± SEM; n = 5 per group, * p = 0.01. (b) At P19, there were no significant differences between the two groups. Results are expressed as the mean ± SEM; n = 7 per group.

Figure 2. Comparison of nephrogenic zone width at P5

There was a significant effect of hyperoxia exposure on nephrogenic zone width (µm) at P5 in the OIR group (dark gray bar) compared to the control group (light gray bar).

Results are expressed as the mean ± SEM. n = 5 per group, * p = 0.01.
Figure 3. Comparison of glomerular counts at P19

At P19, there was a significant effect of hyperoxia exposure on glomerular counts; counts were reduced by 20%. Results are expressed as the mean ± SEM. \( n = 8 \) per group, * \( p < 0.01 \).

Figure 4. Adenosine diphosphatase staining of flat-mounted retinas of OIR pups at P19

Figure 5. VEGF-A and PDGF-\( \beta \) mRNA expression levels in the renal cortex

(a) VEGF-A mRNA expression levels in the renal cortex were significantly lower in the OIR group (dark gray bar) than in the control group (light gray bar) at P5. \( n = 5 \) per group, * \( p < 0.01 \). (b) VEGF-A mRNA expression levels in the renal cortex were significantly higher in the OIR group (dark gray bar) than in the control group (light gray bar) at P19. \( n = 8 \) per group, * \( p < 0.01 \). (c) PDGF-\( \beta \) mRNA expression levels in the renal cortex were significantly lower in the OIR group (dark gray bar) than in the control group (light gray bar) at P5. \( n = 5 \) per group, * \( p < 0.01 \). (d) PDGF-\( \beta \) mRNA expression levels in the renal cortex were significantly higher in the OIR group (dark gray bar) than in the control group (light gray bar) at P19. \( n = 8 \) per group, * \( p = 0.02 \).
For Peer Review

P5d urinary 8-OHdG/Cre (ng/mg)

control  |  OIR
---|---

P19d urinary 8-OHdG/Cre (ng/mg)

control  |  OIR
---|---

*
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**a)**

![Graph showing VEGF-A mRNA expression](control vs OIR)

**b)**

![Graph showing VEGF-A mRNA expression](control vs OIR)

**c)**

![Graph showing PDGF-β mRNA expression](control vs OIR)

**d)**

![Graph showing PDGF-β mRNA expression](control vs OIR)