Neonatal hyperoxia: effects on nephrogenesis and long-term glomerular structure

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Sutherland MR, O’Reilly M, Kenna K, Ong K, Harding R, Sozo F, Black MJ. Neonatal hyperoxia: effects on nephrogenesis and long-term glomerular structure. Am J Physiol Renal Physiol 304: F1308–F1316, 2013. First published February 20, 2013; doi:10.1152/ajprenal.00172.2012.—Preterm neonates are born while nephrogenesis is ongoing and are commonly exposed to factors in the extraterine environment that may impair renal development. Supplemental oxygen therapy exposes the preterm infant to a hyperoxic environment that may induce oxidative stress. Our aim was to determine the immediate and long-term effects of exposure to hyperoxia, during the period of postnatal nephrogenesis, on renal development. Newborn mice (C57BL/6dJ) were kept in a normoxic (room air, 21% oxygen) or a controlled hyperoxic (65% oxygen) environment from birth to postnatal day 7 (P7d). From P7d, animals were maintained in room air until early adulthood at postnatal day 56d (P56d) or middle age (10 mo; P10mo). Pups were assessed for glomerular maturity and renal corpuscle cross-sectional area at P7d (control n = 14; hyperoxic n = 14). Nephron number and renal corpuscle size were determined stereologically at P56d (control n = 14; hyperoxic n = 14) and P10mo (control n = 10; hyperoxic n = 10). At P7d, there was no effect of hyperoxia on glomerular size or maturity. In early adulthood (P56d), body weights, relative kidney weights and volumes, and nephron number were not different between groups, but the renal corpuscles were significantly enlarged. This was no longer evident at P10mo, with relative kidney weights and volumes, nephron number, and renal corpuscle size not different between groups. Furthermore, hyperoxia exposure did not significantly accelerate glomerulosclerosis in middle age. Hence, our findings show no overt long-term deleterious effects of early life hyperoxia on glomerular structure.

nephrogenesis; renal disease; preterm birth; supplemental oxygen therapy

NPHROGENESIS (the formation of nephrons in the kidney) is usually complete by ~34–36 wk of gestation (11, 29). Hence, preterm infants are often born at a time when nephrogenesis is ongoing; in these infants, nephrogenesis continues after birth (8, 29). It is apparent that renal development may be impaired after preterm birth, with studies demonstrating accelerated postnatal renal maturation (29) and reduced formation of glomeruli (25). Of particular concern, glomerular abnormalities are often present in the outer renal cortex of preterm infants, where the most recently formed glomeruli are located (8, 25, 29, 30). In preterm infants, the number of morphologically abnormal glomeruli within the kidneys is variable, with a high proportion of glomerular abnormalities in some kidneys, whereas other kidneys appear to be unaffected (29). Although the cause of the glomerular abnormalities is unknown, the abnormal glomeruli are located only within the outer renal cortex, and are in an immature stage of development (29), suggesting that it is the nephrons that are formed in the extraterine environment that are “at risk.” Therefore, it may be factors associated with the postnatal care of the infant that are leading to the abnormalities.

Supplemental oxygen therapy is common practice in the care of preterm neonates, and high levels of oxygen in the neonatal period may adversely impact upon nephrogenesis. It has been shown in explants of rat kidneys that low-oxygen concentrations (1–3%) are optimal for renal vascular and tubular development (31). The intrauterine environment is relatively hypoxic (5, 24), with arterial oxygen saturation rising immediately after birth (14, 23). Preterm neonates at the time of birth are therefore abruptly and prematurely exposed to high-oxygen concentrations relative to the intrauterine environment. It is now recognized that even brief exposure to high-oxygen concentrations can lead to oxidative stress (33, 34); free radicals are reported to cause cellular injury and cell death when the antioxidant capacity of the neonate is overwhelmed (17). Preterm infants are particularly vulnerable as they have low concentrations of antioxidants (7, 15). In this regard, common complications of prematurity such as bronchopulmonary dysplasia, retinopathy of prematurity, necrotizing enterocolitis, patent ductus arteriosus, and periventricular leukomalacia are all strongly linked to oxidative stress (26).

In the kidney, oxidative stress is associated with proximal tubule injury in human neonates (20, 36). It is likely that it may also lead to injury to the glomerular capillaries and thus may be the cause of the glomerular abnormalities in kidneys of preterm infants. Early life exposure to hyperoxia in rats (80% oxygen from postnatal days 3–10, a time when nephrogenesis is still ongoing) has been shown to lead to hypertension, microvascular rarefaction, vascular dysfunction, and a 25% reduction in nephron endowment in adulthood (38), strongly suggesting that hyperoxia during nephrogenesis adversely impacts on renal development.

Hence, the aim of the present study was to determine whether exposure to hyperoxia during postnatal nephrogenesis in the neonatal mouse leads to abnormal glomerular formation, size, and maturation. In mice, nephrogenesis begins during midgestation and continues until ~7 days after birth (3, 10). After exposing pups to moderately high-oxygen concentrations (65% O2) during the neonatal period, we examined the kidneys at three timepoints: immediately following hyperoxic exposure at postnatal day 7 (P7d), in early adulthood at P56d, and in “middle age” at 10 mo (P10mo). At P7d, we assessed the immediate consequences of neonatal hyperoxia on glomerular morphology, size, and maturation. At P56d and P10mo, nephron number and renal corpuscle size were stereologically measured.

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METHODS

Animals

Mice (C57BL/6j) were born naturally at term and exposed to hyperoxic gas (hyperoxia group, 65% O2), or room air (controls, 21% O2), from the time of birth (P0) until P7d. Pregnant dams in the hyperoxia group were exposed to 65% O2 from ~0.5 days before birth such that pups were born into the hyperoxic environment. O2 and CO2 concentrations in the mouse housing chambers were continuously monitored (Servox MiniMP 5200; Servomex) for the duration of the hyperoxia exposure period; CO2 concentrations did not rise throughout the exposure period. We used 65% O2 to avoid maternal and neonatal death through severe oxygen toxicity; this is a lower O2 concentration than has been used in other recent studies (32, 38). All studies were approved by the Monash University Animal Ethics Committee and the treatment and care of animals conformed to the National Health and Medical Research Council of Australia’s Code of Practice for the Care and Use of Animals for Scientific Purposes.

Experimental Groups

Nineteen control litters and 19 hyperoxia-exposed litters were analyzed in this study (7 short-term survival, 7 survival until early adulthood, and 5 survival until middle age in each group). In all groups, one male and one female pup, selected at random, were analyzed per litter.

Short-term survival (P7d) group. Hyperoxia-exposed pups were continuously exposed to hyperoxic gas (65% oxygen) from P0 to P7d (hyperoxia; male n = 7, female n = 7). Controls breathed room air (21% oxygen) from P0 to P7d (control; male n = 7, female n = 7). At P7d, the offspring underwent necropsy for collection of the kidneys. Pups were weighed at birth and at P7d.

Long-term survival groups (P56d and P10mo). Hyperoxia-exposed pups were continuously exposed to hyperoxic gas (65% oxygen) from P0 to P7d, and then raised in room air (21% oxygen) until either P56d (hyperoxia; male n = 7, female n = 7) or P10mo (hyperoxia; male n = 5, female n = 5). Controls were exposed to room air (21% oxygen) from P0 to either P56d (control; male n = 7, female n = 7) or P10mo (control; male n = 5, female n = 5). Offspring were weaned at P21d, and were weighed weekly from birth until P56d, and monthly from P3mo to P10mo, at which time they underwent necropsy for collection of the kidneys.

Tissue Processing

All kidneys collected at necropsy were cleaned of connective tissue and immersion-fixed in 10% buffered formalin. The entire right kidney from each animal in the short-term survival groups was paraffin-embedded and sectioned at 5 μm. Every 10th section was collected and stained with hematoxylin and eosin. The right kidney from each animal in the long-term survival groups was weighed, cut in half along the coronal axis, and embedded in glycolmethacrylate. Glycolmethacrylate blocks were serially sectioned at 20 μm, with every 10th and 11th section collected and stained with hematoxylin and eosin. During all analyses, researchers were blinded as to the sex and experimental grouping of each animal.

Short-Term Survival Group (at P7d)

Renal corpuscle cross-sectional area and glomerular maturity. Hematoxylin and eosin-stained paraffin sections of kidneys from the short-term survival groups (P7d) were used in the assessment of the cross-sectional area and maturity of renal corpuscles; the renal corpuscle comprises the Bowman’s capsule, Bowman’s space, and glomerular tuft. Every 10th section from each kidney was systematically sampled at ×1,500 magnification, at a step length of 1 mm × 1 mm in the x- and y-axis. At each field of view, two glomeruli were randomly chosen for assessment, as described previously (29). The cross-sectional area of each renal corpuscle was determined by tracing the perimeter of the Bowman’s capsule using image analysis software (Image Pro Plus, v6.0 for Windows; Media Cybernetics); the average renal corpuscle cross-sectional area was then calculated per kidney (29).

For each field of view, using the uniform systematic sampling method described above, the stage of maturation of all glomeruli within each sampled field was recorded (29). Glomeruli were classified as stage 0 if they were still developing (at the vesicle, comma, or capillary loop stages of development). Stage 1 comprised immature fully formed glomeruli with at least half of the glomerular tuft lined with dark-staining epithelial cells and a densely cellular glomerular tuft. Stage 2 glomeruli presented less than half of the glomerular circumference lined with darkly stained cells, and stage 3 glomeruli (most mature) had no dark-staining layer of cells surrounding the glomerular tuft, and a more open glomerular tuft (29).

Cellular proliferation within glomeruli. Paraffin-embedded kidney sections were immunohistochemically stained with an antibody against the Ki67 antigen, as a marker of cellular proliferation. Sections were dewaxed and rehydrated before antigen retrieval in 0.1 M sodium citrate (pH 6) in a microwave oven (on high power) for 20 min. Endogenous peroxidases were blocked using 3% hydrogen peroxide, and nonspecific binding was blocked using 5% normal goat serum. Sections were incubated overnight at 4°C with a rabbit anti-human Ki67 monoclonal antibody (1:150; RM-9106-S0, Thermo Scientific). The following day, sections were washed with phosphate-buffered saline with 0.1% Tween-20 before incubation with streptavidin-horseradish peroxidase for 30 min at room temperature. Nuclei stained for Ki67 were visualized using 3,3′-diaminobenzidine; sections were counterstained with hematoxylin. Specific staining of Ki67 was confirmed by omission of the primary antibody.

A series of nonoverlapping microphotographs were taken of each kidney section at ×200 magnification. In each field of view, each glomerulus was assigned a number from 1 to n. Two glomeruli (G1 and G2) were chosen for assessment; a random number generator was used to determine the first glomerulus (G1), and the equation G2 = G1 + (n/2) was used to determine the second glomerulus (G2). In the event that the equation produced a G2 value higher than n, the equation G2 = G1 + (n/2) was used (29). The percentage of glomerular proliferation was assessed by counting the total cell number in the number of Ki67-positive cells within the glomerular tufts, and the average percentage of proliferating cells per glomerulus was determined for each kidney.

Long-Term Survival Groups (at P56d and P10mo of Age)

Kidney volume, renal corpuscle volume, and nephron number. Hematoxylin and eosin-stained glycolmethacrylate sections of kidneys from the long-term survival groups (P56d and P10mo) were used in the stereological estimation of kidne volume, nephron number, and renal corpuscle volume. Kidney volume was estimated using the Cavalieri principle (9, 28). Three pairs of intact 10th and 11th sections from each kidney (each section showing 2 faces of kidney tissue, as the kidneys were cut in half before embedding) were used in the estimation of nephron number. The number of glomeruli (and thereby nephrons) was estimated using an unbiased physical dissector/fracti onator technique (1, 19, 28). Renal corpuscle volume was also stereologically measured (1, 19, 28); glomerular tuft volume was not used as a measure of glomerular size due to the likely collapse of capillaries following tissue-processing procedures (1). These techniques have been previously described in detail (28).

Cellular proliferation within glomeruli. Ki67 immunohistochemistry was used to identify proliferating cells, using the same protocol as described above in the P7d kidneys, was undertaken in paraffin-embedded kidney sections at P56d. Glomerulosclerosis. Glomerulosclerosis was assessed in paraffin sections of kidneys from animals at P10mo. Sections were stained
with periodic acid Schiff’s to highlight the extracellular matrix within glomeruli. All glomeruli within a longitudinal section taken through the center of the kidney were scored for evidence and severity of glomerulosclerosis, with a grading range from 0 (no sclerosis) to 4 (severely sclerotic) (37).

**Statistical Analysis**

Data were analyzed using GraphPad Prism (v5.03 for Windows; GraphPad Software), with data presented as means ± SE. Data at each age (P7d, P56d, and P10mo) were analyzed using a two-way ANOVA, with the factors sex (pS), hyperoxic gas exposure (pH), and their interaction (pSxH). To identify differences between individual groups, a Bonferroni post hoc test was conducted following the two-way ANOVA. The growth trajectory of animals from birth to P56d and from P3mo to P10mo was analyzed using a two-way ANOVA with repeated measures, with the factors postnatal age (pA), pH, and their interaction (pSxH); this was followed by a Bonferroni post hoc test. Body weights at the three endpoints, P7d, P56d, and P10mo, were further assessed using an unpaired two-tailed Student’s t-test. At P56d and P10mo, linear regression analyses were also undertaken to determine correlations between kidney volume and body weight, and between nephron number and kidney weight and volume. An analysis of covariance was subsequently applied to determine whether there were any significant differences in the slope and y-intercept of the linear regression lines between the control and hyperoxia-exposed groups. Statistical significance was accepted at the level of P < 0.05.

**RESULTS**

**In the Neonate at P7d**

**Body weight.** There was no significant difference between experimental groups, or between sexes, in birth weights. Body weights at P7d (Fig. 1) were significantly lower (P = 0.046) in the hyperoxia-exposed animals (males: 3.0 ± 0.2 g, females: 3.0 ± 0.2 g) compared with controls (males: 4.0 ± 0.1 g, females: 3.9 ± 0.1 g), with no effect of sex on body weight.

**Renal corpuscle cross-sectional area.** The mean cross-sectional area of renal corpuscles at P7d was 1,540 ± 62 μm² in the control group (males: 1,517 ± 46 μm², females: 1,567 ± 130 μm²), and 1,530 ± 59 μm² in the hyperoxia-exposed group (males: 1,545 ± 97 μm², females: 1,515 ± 75 μm²). There was no significant difference in renal corpuscle cross-sectional area between sexes or between hyperoxia-exposed animals and the controls.

**Renal morphology.** There was no observable difference in renal cortical morphology between control and hyperoxia-exposed animals (Fig. 2), and there was no evidence of morphological glomerular abnormalities in the kidneys of hyperoxia-exposed mice. There was evidence of ongoing nephrogenesis in all kidneys examined at P7d, with glomeruli in stage 0 of development (most immature stage) present in the outer renal cortex of all control and hyperoxia-exposed animals (Fig. 2).

**Glomerular maturity.** The percentage of glomeruli at each maturational stage is shown in Fig. 3. There was no significant effect of sex or hyperoxia exposure on glomerular maturity. In all kidneys, the majority of glomeruli (~64% per kidney) were at stage 1 (immature) of development, 19% were at stage 2 (intermediate), and an average of 8% were at both stage 0 (developing) and stage 3 (mature).

**Cellular proliferation.** The percentage of proliferating cells (positive for Ki67 staining) in the glomerulus in the P7d kidneys averaged 23.1 ± 1.2% in controls (23.2 ± 1.6% males, 21.8 ± 1.4% females) and 21.9 ± 1.5% in hyperoxia-exposed animals (24.1 ± 2.4% males, 20.3 ± 1.8% females). There was no significant difference in the percentage of proliferating cells between experimental groups or between sexes.

Fig. 1. Growth trajectory of male (A) and female (B) mice from birth postnatal day 0 (P0d) to middle age (P10mo). Controls are shown by black lines and the hyperoxia-exposed by gray lines. Body weights significantly increased with increasing postnatal age (pA). Neonatal exposure to hyperoxia significantly reduced body weight at P7d and P10mo in both males and females, but there was no difference between groups at P56d. There was a significant overall effect of hyperoxia exposure (pH) on the growth trajectory from P3mo to P10mo in males, but not in females. *P < 0.05 control vs. hyperoxia.

Fig. 2. Representative photomicrographs of renal cortical morphology in neonatal (P7d) and adult (P56d) mice. Developing glomeruli (stage 0) were present in the outer renal cortex of all kidneys examined in the control (C) and hyperoxia-exposed (H) groups at P7d (arrows). In adult kidneys at P56d, there was no discernible difference in renal morphology between control (C) and hyperoxia-exposed (D) animals. There was no evidence of morphologically abnormal glomeruli in the outer renal cortex of hyperoxia-exposed kidneys. Scale bar = 50 μm.
In Early Adulthood at P56d and Middle Age at P10mo

Body weight. Body weight significantly increased from 1.3 ± 0.0 g at birth to an average of 25.0 ± 0.4 g in males and 19.1 ± 0.3 g in females at P56d, and 36.8 ± 1.4 g in males and 29.6 ± 1.6 g in females at P10mo (Fig. 1). The postnatal increase in body weights of females followed the same trajectory as males until P28d; from this time point males were significantly heavier than females (P < 0.001). Body weights did not differ significantly between the control and hyperoxia-exposed groups at P56d; however, at P10mo the hyperoxia-exposed animals were significantly lighter than the controls (Fig. 1).

Kidney weight and volume. Kidney weight and volume significantly increased from P56d to P10mo in male control and hyperoxia-exposed groups, but there was no significant difference between females at P10mo compared with P56d. At both P56d and P10mo, absolute right kidney weights (Fig. 4, A and D) and volumes (Fig. 4, C and F) were significantly smaller in females compared with males, with no effect of hyperoxia on kidney size. A strong trend (P = 0.06) was evident for an increased kidney weight relative to body weight in females compared with males at P56d, but no difference between sexes was observed at P10mo (Fig. 4, B and E). In both the control and hyperoxia-exposed groups at P56d, there was a significant positive linear correlation between kidney weight and body weight (control: r² = 0.84, P = 0.0002; hyperoxia: r² = 0.67, P = 0.004), as well as kidney volume and body weight (control: r² = 0.72, P < 0.0001; hyperoxia: r² = 0.56, P = 0.002); no significant associations were observed at P10mo.

Nephron number and density. At P56d, nephron number (Fig. 5A) ranged from 8,191 to 12,860 in the control animals (mean: 10,140 ± 433) and from 7,212 to 10,980 in the hyperoxia-exposed animals (mean: 9,425 ± 327); there was no significant effect of sex or early life hyperoxia exposure on nephron number. Similarly, at P10mo nephron number ranged from 7,714 to 12,375 in controls (mean: 9,455 ± 383), and from 6,419 to 11,148 in hyperoxia-exposed animals (mean: 8,934 ± 437), with no difference between sexes or treatment groups (Fig. 5C). There was no change in nephron number between P56d and P10mo. Nephron density (number of nephrons per mm³ of kidney tissue) tended to be greater in females compared with males at P56d (P = 0.054) and this was significant at P10mo (P < 0.0001; Fig. 5, B and D); between P56d and P10mo there was a significant reduction in nephron density in males and females in both control and hyperoxia-exposed groups. There was no correlation between nephron number and kidney weight, kidney volume, or body weight at either P56d or P10mo.

Renal corpuscle volume. At P56d, renal corpuscle volume was significantly greater (P = 0.03) in hyperoxia-exposed kidneys (range: 1.5 – 3.9 × 10⁻⁴ mm³; mean: 2.6 ± 1.9 × 10⁻⁴ mm³) compared with controls (range: 1.6 – 3.0 × 10⁻⁴ mm³; mean: 2.1 ± 1.1 × 10⁻⁴ mm³); there was no difference between males and females (Fig. 6A). There was a significant inverse linear correlation between renal corpuscle volume and nephron number, within both the control (r² = 0.57, P = 0.002) and hyperoxia-exposed (r² = 0.51, P = 0.004) groups (Fig. 6B). There was also a trend (P = 0.08) for a difference in the slopes of the regression lines between the control and hyperoxia groups, such that for a decline of 1,000 nephrons, renal corpuscle volume was increased by 0.20 ± 0.00 mm³ in control animals, and 0.41 ± 0.12 mm³ in hyperoxia-exposed animals.
Renal corpuscle volume also increased significantly in both control and hyperoxia-exposed animals between P56d and P10mo; there was on average a 64.1% increase in renal corpuscle volume in control animals with increasing age (p < 0.0001), and a 30.9% increase in the hyperoxia-exposed animals (p = 0.01). At P10mo, renal corpuscle volume did not differ between experimental groups or between sexes (Fig. 6C). Furthermore, there was no correlation between renal corpuscle volume and nephron number at P10mo (Fig. 6D).

**Renal morphology and glomerulosclerosis.** There was no discernible difference in renal cortical morphology between control and hyperoxia-exposed animals at P56d, with no evidence of glomerular abnormalities (Fig. 2). There was little evidence of any cell proliferation in glomeruli at P56d, with only an occasional cell staining positive for Ki67 in any of the experimental groups.

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**Fig. 4.** Kidney weight (A, D), kidney weight-to-body weight ratio (B, E), and kidney volume (C, F) in males (black) and females (gray) in control and hyperoxia-exposed groups at P56d (A–C) and P10mo (D–F). There was a significant effect of sex (pS) on kidney weight (A, D) and kidney volume (C, F) at both time points, with a greater kidney size evident in the males. There was no effect of hyperoxia exposure (pH) on any parameter of kidney size.

**Fig. 5.** Nephron number (A, C) and nephron density (B, D) in males (black) and females (gray) in control and hyperoxia-exposed groups at P56d (A–B) and P10mo (C–D). There was no effect of sex (pS) or hyperoxia exposure (pH) on nephron number (A, C). Nephron density was significantly greater in females compared with males (pS) at P10mo, with no effect of hyperoxia at P56d or P10mo (B, D).
kidneys examined, with no difference between the control and hyperoxia-exposed kidneys. At P10mo, glomeruli with glomerulosclerosis (classified as stage 1–4) were present in all control and hyperoxia-exposed male and female kidneys. There was no difference between treatment groups in the percentage of normal (stage 0) glomeruli (control: 23.0 ± 3.7%, hyperoxia: 22.5 ± 4.9%) or the percentage of glomeruli at each stage of glomerulosclerosis (Fig. 7). Control females tended to have an increased percentage of glomeruli with stage 1 glomerulosclerosis (P = 0.052; Fig. 7A), but there were no significant differences observed between groups.

DISCUSSION

In this study, exposure to hyperoxic gas during the period of postnatal nephrogenesis did not lead to any overt adverse effects on renal development, with no detectable evidence of abnormal renal morphology, or alterations in glomerular size or maturation in the early postnatal period. In early adulthood, kidney size and nephron number were normal but glomeruli were enlarged in the hyperoxia-exposed kidneys. However, in the long-term, renal and glomerular structure appeared normal with no differences in nephron number, glomerular size, or degree of glomerulosclerosis between the hyperoxia-exposed and control kidneys in middle-aged mice.

Hyperoxia Does Not Appear to Affect Nephrogenesis

Given the adverse effects of hyperoxia in other organ systems (26), it was considered likely that hyperoxic exposure in the neonate would impair nephrogenesis. We hypothesized that the development of glomeruli would be adversely affected as high-oxygen concentrations have been shown to impair vascular development, likely due to altered vascular endothelial growth factor (VEGF) expression (6, 16). Furthermore, an increase in oxygen concentration has previously been postulated to trigger the cessation of nephrogenesis in the mouse kidney, with a significant change in the expression profile of genes involved in nephrogenesis evident immediately following birth (2). Contrary to our hypothesis, however, we found no overt evidence to suggest that exposure to hyperoxia adversely impacts renal development in the early postnatal period (P7d), at a time when nephrogenesis is ongoing in the mouse; kidney morphology was normal in the hyperoxia-exposed kidneys, and glomerular size and maturation were not different compared with controls.

In accordance with our finding of no impairment of renal development at P7d, nephron numbers in early adulthood (P56d) and middle age (P10mo) were also unaffected by neonatal hyperoxia exposure. This is an interesting finding given that body weight was significantly reduced in the hyperoxia-exposed offspring at P7d; in general, the number of nephrons formed in the kidney directly correlates with body growth (21). Nephrogenesis in the mouse commences in midgestation and continues during the first 1–2 wk postnatally. In our study, since the hyperoxic insult was initiated at birth, it was only postnatal growth that was reduced. The short duration of the hyperoxic exposure may therefore account for the
absence of any significant reductions in nephron number in the hyperoxia-exposed kidneys.

Contrary to our findings, in a previous study by Yzydorczyk et al. (38) nephron endowment was reportedly reduced by 25% in 25- to 35-wk-old rats that were exposed to hyperoxia early in life, during the period of postnatal nephrogenesis. The disparity in results between studies may be explained by differences in the concentration of hyperoxic gas used, with 65% O2 in the current study, and 80% O2 being used in the previous study (38). Certainly, elevated concentrations of oxygen are known to cause increases in oxidative stress (4, 35). Hence, it is likely that deleterious effects of hyperoxia on nephrogenesis may manifest due to oxidative stress when oxygen concentrations become markedly elevated. Also, it is important to note that the difference between species in our study (mice) and the study by Yzydorczyk et al. (rats) may also have contributed to the differences in findings.

The results of previous studies that examined sex differences in the C57BL/6J mouse model, with regards to nephron endowment, are inconclusive (12, 18). In the present study, nephron number in adulthood was not different between the sexes; however, nephron density was greater in females (highly significant at 10 mo), which may be explained by their reduced kidney size compared with males and also implies a proportionately greater increase in tubular mass in males. Likewise, the decreasing nephron density between P56 and 10 mo indicates disproportionate glomerular and tubular growth with ageing. Importantly, over this 8-mo period there was a more rapid growth of the glomeruli in the control kidneys such that at 10 mo postnatally there was no difference between groups in renal corpuscle volume. In contrast, the rate of tubular growth (inferred from nephron density) was not different between groups. Overall, this suggests that there may be some form of hyperoxia-induced toxicity on glomeruli, which is not reflected by glomerulosclerosis or nephron loss. Further studies are required to confirm this.

**Induction Of Glomerular Hypertrophy In Early Adulthood, But Normalization By Middle Age**

An interesting finding of our study was the induction of glomerular hypertrophy in early adulthood as a result of early life exposure to hyperoxia; renal corpuscle volume (indicative of glomerular size) was significantly greater in the hyperoxia-exposed animals than in the controls at P56d. The cellular mechanisms leading to the glomerular enlargement in hyperoxia-exposed kidneys at P56d are unknown. Certainly, at P7d there was no detectable increase in the number of proliferating cells in developed glomeruli of hyperoxia-exposed kidneys, suggesting that the glomerular hypertrophy observed at P56d was not the result of an increased proliferation rate in the glomerular cells (or in a subset of glomerular cells) early in life. At P56d, there was negligible glomerular proliferation in all kidneys. It is possible that the glomerular enlargement observed in hyperoxia-exposed kidneys at P56d is due to increased glomerular capillary growth, which may occur following withdrawal of the hyperoxia as occurs with retinopathy of prematurity (27). There would be a relative reduction in oxygen concentration when oxygen levels are returned to normal. As a result, this relative hypoxia may stimulate the
expression of the angiogenic growth factor VEGF (22). Whether VEGF protein expression was increased in the hyperoxia-exposed glomeruli could not be addressed in our study due to difficulties in staining the fixed tissue. Given that there was no detectable increase in cellular proliferation within the glomeruli of the hyperoxia-exposed kidneys, the most likely explanation for the renal corpuscle enlargement is hypertrophy of the glomerular capillaries and conceivably this could be reversed at P10mo. In future studies, in addition to assessing glomerular capillary growth, it will be important to examine the cellular constitution of the glomeruli over time, including absolute and relative proportion of podocytes (important for the maturation, integrity, and function of the glomerular capillaries), endothelial cells, and interstitial cells.

Additionally, it would be beneficial to compare renal function (in particular glomerular filtration rate) at the different postnatal time points over the life course of the mouse, to establish whether the changes in glomerular size reflect differences in renal function. Glomerular hypertrophy is often associated with glomerular hyperfiltration and a subsequent decline in renal function; prolonged hyperfiltration can ultimately lead to the induction of glomerulosclerosis and loss of glomerular capillaries. Indeed, renal corpuscle volume has been shown to be increased in a large number of conditions such as unilateral renal agenesis, obesity-related glomerulopathy, hypertension, oligomeganephronia, diabetes mellitus, and focal segmental glomerulosclerosis (13, 21).

Given that glomerular hypertrophy is often an antecedent to renal disease, it was important in our study to follow a cohort of mice to a time point much later in life to determine whether glomerulosclerosis consequently developed. Unexpectedly, however, by 10 mo of age glomerular hypertrophy was no longer apparent in the hyperoxia-exposed kidneys, and there was no evidence to suggest that glomerular hypertrophy in early adulthood had led to accelerated sclerotic changes in the hyperoxia-exposed kidneys. Therefore, the glomerular hypertrophy we observed at P56d was transitory and normalized by later in life. Why this occurred is unclear, but may potentially relate to greater functional demands on the kidney in early adulthood compared with older age. In this regard, at P10mo the hyperoxia-exposed mice were significantly smaller compared with controls and body mass is a known determinant of renal corpuscle size (21). Hence, if additional demands were placed on the hyperoxia-exposed kidneys later in life, for instance by inducing obesity, the long-term outcome on glomerular structure may be quite different and pathological processes may ensue.

Additionally, there may have been an elevation in blood pressure in the hyperoxia-exposed mice in early adulthood relative to controls, which was normalized later in life; hypertension is often associated with an increase in glomerular size. Notably, in this regard, in the study by Yzydorczyk et al. (38), neonatal hyperoxia exposure led to hypertension in male and female rats when studied at 7–15 wk of age. Associated with the hypertension was evidence of vascular dysfunction and microvascular rarefaction (38). Whether blood pressure is elevated in our model, and if so, whether the elevation in blood pressure persists throughout life, is unknown; further studies are required to elucidate this.

In conclusion, in a neonatal mouse model with ongoing postnatal nephrogenesis, we showed that exposure to 65% oxygen during the early postnatal period does not cause immediate changes in renal structure, and nephron endowment is not affected. By early adulthood, there was significant glomerular enlargement in kidneys of hyperoxia-exposed mice but this was subsequently normalized, with no deleterious effects on glomerular structure in middle age. Our findings therefore suggest that the long-term renal health of the many infants born preterm (in whom nephrogenesis is ongoing after birth) may not be deleteriously affected by exposure to hyperoxic gas at or below 65% oxygen during the neonatal period. However, given that there are likely to be species differences in the renal response to hyperoxic gas, especially since room air alone may be considered to be hyperoxic to preterm infants, further studies are required to verify this.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

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