Published in final edited form as:

J Hypertens. 2016 October ; 34(10): 1988–1997. doi:10.1097/HJH.0000000000001028.

Effects of preterm birth and ventilation on glomerular capillary growth in the neonatal lamb kidney

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Abstract

Objectives—Preterm birth is linked to the development of hypertension later in life. This may relate to impaired glomerular capillary growth following preterm birth. The aim of this study was to determine the effects of preterm birth, and/or ventilation, on glomerular capillary growth in the neonatal lamb kidney.

Methods—Four experimental groups were analysed: 1) Preterm lambs delivered at 130d gestation (term=147d) and mechanically ventilated for 3 days (Preterm ventilated: n=9); 2) 133d gestational controls (Gestational control: n=5); 3) Term controls, unassisted breathing for 3 days (Term control: n=8); and 4) Term lambs ventilated for 3 days (Term ventilated: n=5). In perfusion-fixed kidneys, total nephron number and average total capillary length and surface area per renal corpuscle were stereologically assessed and total renal filtration surface area (TRFSA) calculated.

Results—In comparison to term controls, preterm lambs had significantly reduced glomerular capillary length, surface area and TRFSA, indicative of a low renal functional capacity. Term ventilated lambs exhibited significantly reduced glomerular capillary length and surface area compared to term controls, indicating that ventilation impairs glomerular capillary growth independently of preterm birth.

Conclusion—Impaired glomerular capillary growth and subsequent reduced TRFSA following preterm birth may mediate the increased predisposition to hypertension later in life.

Keywords

Preterm birth; ventilation; hypertension; renal development; nephrogenesis; angiogenesis

INTRODUCTION

Over recent decades there have been many epidemiological studies linking preterm birth (birth prior to 37 weeks gestation) with the development of hypertension later in life [1, 2];

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DISCLOSURE STATEMENT

None

increases in systolic blood pressure of 0.31 to 0.5 mmHg for each week less than term at birth have been reported [3, 4]. The cause of the elevated blood pressure in subjects born preterm may relate to impaired growth of glomerular capillaries in the neonatal period, and this would adversely impact total renal filtration surface area (TRFSA). A reduced TRFSA is linked to sodium retention, and a subsequent elevation in blood pressure [5, 6].

When birth comes early, the developmentally immature kidneys are prematurely exposed to the hemodynamic transition that occurs at birth; this includes a marked rise in arterial blood pressure and renal blood flow [7, 8]. In addition, the immature kidneys are abruptly exposed to high oxygen (O₂) concentrations relative to the intrauterine environment through ventilation and supplemental oxygen therapy (21–100% O₂ is utilised[9]), with arterial blood oxygen saturation rapidly increasing after birth [10]. These high O₂ concentrations are known to impact hypoxia inducible factor (HIF)-1 α levels and subsequently downregulate the expression of vascular endothelial growth factor (VEGF) [11, 12] which is essential for glomerular capillary growth and development [13, 14]. Impaired vascularisation in other organ systems following preterm birth is related to oxygen exposure [15].

Mechanical ventilation is often required to facilitate survival after preterm birth; 60–70% of infants born at 22–28 weeks gestation have been reported to be intubated and ventilated after birth [16] and a proportion of infants born moderately preterm or near-term also require mechanical ventilation following respiratory distress [17]. In this study we hypothesized that preterm birth and mechanical ventilation impair glomerular capillary growth in newborn infants. Utilising a sheep model of moderate preterm birth, the aim of this study was to examine the effect of moderate preterm birth and/or mechanical ventilation on glomerular capillary length, surface area, and TRFSA.

METHODS

Animals

Studies were conducted using mixed-breed lambs of known delivery dates. Moderately preterm lambs were delivered via caesarean at 130d gestation (term = 147d), and ventilated for 3 days after birth (Preterm ventilated: n=9). Age-matched gestational (fetal) controls were delivered by caesarean and euthanised at 133d gestation (Gestational control: n=5). Term lambs were delivered vaginally following spontaneous labour, and they were either non-ventilated (Term control: n=8) or ventilated for 3 days after birth (Term ventilated: n=5). All animals, except the fetal controls, were euthanised at postnatal day three (approximately 72 hours after birth).

Detailed descriptions of the animal care procedures have been published previously [18, 19]. Briefly, preterm lambs were delivered by caesarean section, intubated, and administered 2.5 ml of Survanta (NDC 0074-1040-08; Ross Products Division, Abbott Laboratories, Columbus, OH). During resuscitation, preterm lambs received intermittent mandatory ventilation (IMV), with warmed and humidified 100% oxygen (Bird VIP ventilator, model 15215; Bird Products, Palm Springs, CA). Lambs were kept prone in a veterinary sling mounted on a radiantly heated bed. Respiratory rate was 60 breaths/minute, inspiratory time 0.3 s, positive end-expiratory pressure (PEEP) was set at 8cm H₂O, and peak inspiratory

pressure (PIP) was adjusted to reach a target PaCO₂ of 45–60 mm Hg and pH 7.25–7.35. Target expiratory tidal volume was 5–7 ml/kg/breath. At 5–10 minutes of age, additional Survanta (2.5 ml) was administered, and the concentration of inspired O₂ was then decreased to a target PaO₂ of 60 – 80 mm Hg [18]. The term ventilated group were delivered naturally, and received IMV as described above (except that they were not administered surfactant) [19]. Lambs were administered intravenous buprenorphine (0.01 mg/kg; Buprenex, Recritt and Coleman Pharmaceuticals, Richmond, VA) and either pentobarbital (3–5 mg/kg; Vet Lab, Lenexa, KS) or phenobarbital (10 mg/kg; Wyeth Laboratories, Philadelphia, PA) as required for sedation [18, 19]. Preterm and term ventilated animals were continuously monitored over the 72 hours for temperature, heart rate, blood pressure, FiO₂, and urine output. Arterial blood was sampled hourly to measure PaCO₂, PaO₂, blood glucose, and blood pH. At the end of the experimental period the lambs were euthanised by intravenous infusion of pentobarbital sodium solution (60mg/kg). All protocols adhered to the American Physiological Society/National Institute of Health guidelines for the humane use of animals for research, and were approved by the Institutional Animal Care and Use Committee at the University of Utah Health Sciences Center.

Tissue processing

At necropsy, the left kidney of each lamb was excised and perfusion-fixed via the renal artery using 10% buffered formalin. Fixed kidneys were cleaned of connective tissue, cut into quarters, and weighed. Two opposing quarters were sliced using a razor blade device into 2 mm thick slices. From these, every fourth slice was selected (beginning from a random starting point). Each slice was then further cut into equal pieces, with 10–12 pieces then sampled using a smooth fractionator approach [20] and embedded in glycolmethacrylate. Glycolmethacrylate blocks were exhaustively sectioned at 20 µm and every 10th and 11th section were collected and stained with haematoxylin and eosin [21]. In the remaining 2 mm slices, at a position selected at random, an approximately 2 mm wide strip of tissue extending from the outer cortex to the medulla was cut. Each strip of tissue was then cut equally into three cubes of tissue representing the inner, mid and outer renal cortex. Four cubes of cortex per each of the three regions (12 per kidney) were post-fixed in osmium tetroxide before being embedded in epon araldite [22]. Epon araldite blocks were sectioned at 1 µm and stained with toluidine blue. Researchers were blinded to the experimental groups during all kidney analyses.

Stereological estimation of kidney volume, nephron number, renal corpuscle volume and glomerular tuft volume

Glycolmethacrylate sections were used in the stereological estimation of kidney volume, nephron number, renal corpuscle volume and glomerular tuft volume. Every 10th section was viewed using a microfiche reader and an orthogonal grid (2cm X 2cm) superimposed over the projected image. Kidney volume was then estimated using the Cavalieri principle [21, 23] with the following equation:

$$V_{kid}(mm^3) = 2 * 4 * 2 * 10 * t * a(p)_{volume\ count\ grid} * Ps$$

Where, 2 is the inverse sampling fraction of the whole kidney, 4 is the inverse sampling fraction of kidney slices (1/4 pieces chosen for further sampling), 2 is the inverse sampling fraction of kidney slices chosen to be embedded, 10 is the inverse sampling fraction of kidney sections collected from each block (every 10th section was collected), t is the section thickness (0.02 mm), a(p) is the area associated with each intersecting point on the orthogonal grid, and Ps is the number of intersecting grid points counted for each kidney.

One intact pair of sections from each block were used in the estimation of nephron number, renal corpuscle volume and glomerular tuft volume using the physical disector/fractionator approach [21, 24, 25]. Total nephron (glomerular) number in the kidney (N.glom.kid) was determined using the following equations:

$$N.glom.kid = 2 * 4 * 2 * 10 * \frac{Ps}{Pf} * \frac{t}{2fa} * Q^-$$

$$fa = \frac{(Pkid * a(p) \text{ physical disector grid})}{(Pf * a(p) \text{ volume count grid})}$$

Where the numbers represent the inverse tissue sampling fractions (described above), Ps/Pf is the inverse sampling fraction of sections used for the physical disector (points counted during the volume analysis: Pf is the is the number of intersecting grid points counted for the sections chosen for analysis, and Ps the total number of intersecting grid points counted for each kidney), 1/2fa is the fraction of the total section area used to count glomeruli, Q⁻ is the total number of glomeruli counted, Pkid is the total number of points counted per kidney during the physical disector analysis, a(p) is the area associated with each intersecting point on the orthogonal grids.

Mean renal corpuscle (glomerular tuft plus Bowman's space and capsule) volume (V_{corp}) and mean glomerular tuft volume (V_{glom}) per kidney were estimated using the following equations:

$$V_{corp} \text{ or } V_{glom} (mm^3) = \frac{\text{volume density of the renal corpuscles (V.v.corp) or glomeruli (V.v.glom)}}{\text{numerical density of nephrons in the kidney (Ndensity)}}$$

$$\text{Where, } V.v.corp \text{ or } V.v.glom = \frac{\text{total number of points overlying the renal corpuscles or glomeruli}}{\text{total number of points intersecting the kidney (Pkid)}}$$

$$N_{density} = \frac{N.glom.kid}{Vkid}$$

In each kidney, the presence or absence of morphologically abnormal glomeruli (exhibiting an enlarged Bowman's space and shrunken glomerular tuft) was also recorded [21].

Estimation of glomerular capillary length, surface area, and total renal filtration surface area

Two glomeruli per epon araldite section (8 glomeruli per inner, mid, and outer cortex; 24 per kidney) were chosen for analysis using an unbiased method [26]. The glomeruli selected were photographed using an 100x oil-immersion lens (Image Pro Plus, v. 6.0), viewed at a total magnification of 1700x, and imaging software (Adobe Photoshop CS5 Extended, v. 12.0.4) was used to trace the inner margin of the Bowman's capsule, and the boundary of the capillary loops within the glomerular tuft. A 15 mm × 15 mm orthogonal grid was superimposed over the glomerular tracings. The number of grid points overlaying the renal

corpuscle (P_{corp}), the number of capillary profiles within each glomerulus (Q -), the number of capillary boundaries that intersect with the horizontal and vertical lines of the superimposed grid (I_{cap}), and the area associated with each grid point $a(p)$ were recorded [22, 24].

The following equation was utilised to calculate renal corpuscle cross-sectional area:

$$\text{Renal corpuscle cross-sectional area (mm}^2\text{)} = P_{corp} * a(p)$$

Average capillary length per renal corpuscle was determined using the following equation:

$$\text{Capillary length (mm)} = L_{vcap, corp} * V_{corp}$$

Where the capillary length density per corpuscle and mean renal corpuscle volume were calculated by:

$$L_{vcap, corp} (\text{mm/mm}^3) = \frac{(2 * Q -)}{(P_{corp} * a(p))}$$

$$V_{corp} (\text{mm}^3) = \frac{V.v.crop.kid}{N_{density}}$$

Where, 2 is a constant that accounts for the capillaries being isotropic.

The average capillary surface area per renal corpuscle was determined by the following equation:

$$\text{Capillary surface area (mm}^2\text{)} = S_{vcap, corp} * V_{corp}$$

Where the capillary surface area density per renal corpuscle and mean renal corpuscle volume were calculated by:

$$S_{vcap, corp} (\text{mm}^2/\text{mm}^3) = \frac{(2 * I_{cap})}{(P_{corp} * a * d)}$$

$$V_{corp} (\text{mm}^3) = \frac{V.v.crop.kid}{N_{density}}$$

Where, k is a constant accounting for the number of lines associated with a grid point ($k=2$ for an orthogonal grid), and d is the distance between gridlines divided by the total magnification.

The total renal filtration surface area (TRFSA) per kidney was then determined by multiplying the average surface area of capillaries per renal corpuscle by the total number of nephrons in that kidney [22, 24].

Glomerular localization of VEGF and HIF-1 α

VEGF and HIF-1 α protein localisation in glomeruli was examined using immunohistochemistry (5 kidneys per group). Five μm paraffin-embedded sections were de-

waxed and antigen retrieval performed using Target Retrieval Solution (Dako, Carpinteria, CA, USA). Non-specific proteins were blocked using a protein blocking agent (Dako, Carpinteria, CA, USA). The primary antibodies were applied to the sections and allowed to bind overnight; the VEGF primary antibody (rabbit monoclonal; Abcam, anti-VEGF, Ab52917, Cambridge, UK) was applied at a dilution of 1:50 and the HIF-1 α primary antibody (mouse monoclonal Abcam, anti-HIF1-alpha, Ab463, Cambridge, UK) was applied at a dilution of 1:100. Biotinylated secondary antibodies (goat anti-rabbit or goat anti-mouse, respectively) were subsequently applied (Dako, ready to use Envision+, Glostrup, Denmark) and binding sites identified using diaminobenzidine (Dako, Glostrup, Denmark). The sections were then counterstained with haematoxylin. The primary antibody was omitted from the negative controls.

The immunolocalisation of VEGF was semi-quantitated in 50 glomeruli per kidney using a grading system from 0 to 4; glomeruli with no VEGF immunostaining were assigned a score of 0 and glomeruli with high VEGF immunostaining were assigned a score of 4. The renal cortex was systematically sampled so that glomeruli from the inner cortex through to the outer cortex were examined. At the time of analysis, it was recorded as to where the glomeruli were located in the inner, middle or outer renal cortex.

The HIF-1 α immunostaining was localised to cellular nuclei within the glomeruli. The number of positive immuno-labelled nuclei and non-immuno-labelled nuclei were counted in 50 glomeruli per kidney and the proportion of positive staining nuclei per glomerulus determined. Glomeruli were sampled from the inner to the outer renal cortex, using a systematic sampling approach; at the time of analysis the location of each sampled glomerulus within the renal cortex (inner, middle and outer) was recorded.

Statistical analysis

Data were analysed using GraphPad Prism (v.5.03 for Windows; GraphPad Software, CA, USA), with data presented as the mean $\pm$ SEM. Body and kidney weights, nephron number and density, glomerular tuft volume, and TRFSA were compared between groups using a one-way analysis of variance (ANOVA), followed by a Bonferroni post-hoc test. Glomerular capillary length and surface area were analysed in pairs of groups using a two-way ANOVA, with the factors group (p_G), region of cortex (p_R), and their interaction ($p_{G \times R}$). In the ventilated animals, twelve-hourly averages were taken for each recorded physiological parameter, and data were assessed using a two-way repeated measures ANOVA with the factors group (p_G), time (p_T), and their interaction ($p_{G \times T}$). Linear regression analyses were utilised to examine the relationships between glomerular capillary length, surface area, nephron number, TRFSA, and physiological parameters; this was followed by an analysis of covariance (ANCOVA) to compare the slope of the regression lines between groups. Statistical significance was accepted at $p < 0.05$.

RESULTS

Arterial blood gases and cardiovascular function in ventilated animals

FiO₂ was significantly higher in preterm ventilated animals compared to term ventilated, particularly within the first 12 hours of life; there was no statistically significant difference between groups in PaO₂ or PaCO₂ over the 72 hours (Figure 1A–C). Preterm ventilated lambs had significantly lower systolic blood pressure (SBP; Figure 1D) and pH (Figure 1H), but diastolic blood pressure, heart rate, blood glucose and urine output were not different to the term ventilated animals (Figure 1). No animals were hypotensive (SBP < 45 mm Hg) at any time point.

Body weight and kidney weight

Body weight was significantly greater in term animals (control and ventilated groups) compared to the preterm ventilated; there was no significant difference between the weight of gestational controls at birth and the preterm ventilated group at postnatal day 3 (Figure 2A). Absolute kidney weight was significantly greater in the term control and term ventilated groups compared to the preterm and gestational control animals (Figure 2B), whereas kidney weight relative to body weight (Figure 2C) was significantly lower in the gestational controls compared to all other preterm and term groups.

Nephron number and kidney volume

During the assessment of nephron number, no glomeruli with overt abnormalities were observed. As shown in Figure 3A, total nephron number was not significantly different between the four groups ($p=0.09$). Kidney volume averaged $7,954 \pm 927 \text{ mm}^3$ in the gestational control, $8,559 \pm 716 \text{ mm}^3$ in the preterm ventilated, $15,597 \pm 2568 \text{ mm}^3$ in the term ventilated, and $14,455 \pm 585 \text{ mm}^3$ in the term control groups; kidney volume was significantly greater in both the term groups compared to the gestational control and preterm ventilated groups ($p<0.001$).

Glomerular tuft and renal corpuscle volumes

There was no significant difference in mean glomerular tuft volume between the four groups ($p=0.96$; Figure 3B). Average renal corpuscle volume, however, was significantly larger in the term control animals compared to the preterm ventilated group (Figure 3C).

Glomerular capillary length and surface area

Representative images of glomeruli from each group are shown in Figure 4. There was a strong trend for a longer average capillary length per glomerulus in the preterm ventilated lambs compared to the age-matched gestational controls ($p=0.053$; Figure 5A). Capillary length was significantly reduced in the preterm group compared to the term controls; however, there was no difference between the preterm and term ventilated animals. Term ventilated lambs had markedly reduced average length of capillaries per glomerulus compared to the term controls (Figure 5A).

Compared to the age-matched gestational controls, preterm lambs had significantly increased average capillary surface area (Figure 5B). Capillary surface area was significantly

lower in the preterm group compared to term controls, but was not different to the term ventilated animals. Term ventilated lambs had a significantly reduced average surface area of capillaries per glomerulus compared to the non-ventilated term controls (Figure 5B).

There was no significant difference between the three regions of cortex with respect to glomerular capillary length or surface area, in any of the four groups.

There was a significant positive correlation between average glomerular capillary length and glomerular capillary surface area per kidney in both the preterm ventilated ($r^2=0.63$, $p=0.01$) and term ventilated ($r^2=0.78$, $p=0.048$) groups, however this relationship was not statistically significant in the gestational control ($r^2=0.55$, $p=0.15$) or term control animals ($r^2=0.32$, $p=0.15$). There were no significant differences in the slope of the regression lines between groups (ANCOVA analysis), therefore all animals were pooled which revealed a strong positive correlation between average capillary length and surface area (Figure 5C). Overall, there was no correlation between nephron number and average capillary length or surface area (data not shown). There was also no significant correlation between average glomerular capillary length or surface area and any physiological parameters (data not shown).

Total renal filtration surface area

Total renal filtration surface area (TRFSA) averaged $27,136 \pm 4,504 \text{ mm}^2$ in the gestational controls, $32,249 \pm 2,537 \text{ mm}^2$ in the preterm ventilated, $37,403 \pm 7,899 \text{ mm}^2$ in the term ventilated, and $47,782 \pm 3,515 \text{ mm}^2$ in the term control groups. TRFSA in the term control lambs was significantly greater than in both the gestational control and preterm ventilated groups (Figure 5D). There was no significant correlation between TRFSA and any physiological parameters (data not shown).

Glomerular localisation of VEGF and HIF-1 α

VEGF immuno-labelling was clearly observed in the vascular endothelium in all kidney sections. In all kidneys there was variable VEGF localisation in the glomeruli, with high immuno-labelling (score of 4) in some glomeruli and no detectable immuno-labelling in other glomeruli (score of 0). The variability in VEGF immuno-localisation occurred throughout the cortex, with no difference in the proportion of glomeruli with high or low immuno-labelling in the inner, middle and outer regions of the renal cortex. Overall, there was no detectable difference in VEGF immuno-labelling in the glomeruli from the four treatment groups with an average grade of 2 in VEGF immuno-labelling per glomerulus in the kidneys from the 4 treatment groups (Figure 6).

HIF-1 α immuno-localisation, in the nuclei of glomerular cells, was observed in the glomeruli of all treatment groups. There was no detectable difference in the proportion of positively labelled nuclei per glomerulus between the 4 groups. The number of positively labelled nuclei within the glomeruli ranged from 15 to 20% in the kidneys from all treatment groups.

DISCUSSION

Using an ovine model, we have demonstrated that both preterm birth, and ventilation, are associated with altered glomerular capillary growth. This study carefully modelled the early postnatal clinical treatment of respiratory distress in an infant born moderately preterm; additionally, the impact of mechanical ventilation in a more mature (term) kidney was assessed. Overall, we found that nephron number did not differ between groups; however, TRFSA was significantly reduced in the preterm ventilated animals compared to term controls, indicative of reduced renal functional capacity. Of concern, we have additionally shown that glomerular capillary length and surface area were significantly reduced in the term ventilated animals compared to term controls. These findings suggest that preterm birth (in association with ventilation), as well as ventilation alone, impair glomerular capillary growth in the developing kidney.

Conventional mechanical ventilation is in common use in the clinical treatment of preterm neonates [16, 17]. In this study, animals from the preterm and term ventilated groups were ventilated using intermittent mandatory ventilation (IMV); all animals were initially exposed to 100% O₂, then were weaned to lower oxygen concentrations. The reduction in FiO₂ occurred rapidly in the term ventilated animals (with more mature lung function), but the preterm ventilated animals had a longer exposure to significantly higher oxygen concentrations; however, there was no statistically significant difference in PaO₂ between groups. Systolic blood pressure was significantly lower in the preterm lambs compared to those born at term, as is expected due to the strong correlation between blood pressure and both gestational age and body weight [27]. A disadvantage of this study is that the physiological measures were not comparatively assessed in term control animals. Previous studies, however, in term and preterm lambs within the first 3–4 days of life show that all were within the expected range [18, 19, 28, 29].

In a study of kidney development in human neonates [26], we previously demonstrated that preterm birth results in significantly increased renal corpuscle volume (glomerular hypertrophy, possibly indicative of hyperfiltration) compared to age-matched gestational (fetal) controls. In the current study conducted in an ovine model, however, renal corpuscle and tuft volumes were not different between the preterm and gestational control groups. The disparity in findings may relate to the short duration between the time of birth and assessment (3 days) in the current study. Despite there being no overt changes in glomerular size, the preterm lambs did exhibit a significantly increased capillary surface area and a strong trend for increased capillary length compared to the gestational controls. The triggers for capillary growth are unknown, but likely relate to the sudden transition from the intrauterine to the extra-uterine environment whereby the immature vasculature needs to quickly adapt to the haemodynamic changes associated with being born (such as significant increases in renal blood flow [7, 8]).

We also previously reported the presence of glomeruli exhibiting shrunken glomerular tufts in the kidneys of human and baboon neonates born preterm [26, 30–32]. The abnormal glomeruli were only observed in the outer renal cortex, suggesting that it is only glomeruli that were newly formed that are at risk of impairment [33]. In the current study, however, no

abnormal glomeruli were observed. This finding is not surprising given that nephrogenesis in the sheep is completed by approximately 120 days gestation [34], prior to delivery of the preterm and gestational control animals at 130–133 days gestation in this study; we found no significant difference in nephron number between groups.

Although nephrogenesis was completed, it is possible that glomerular capillary development (glomerulogenesis) was continuing postnatally. In the human fetal kidney, research has shown that the maturation of glomerular endothelial cells is ongoing until approximately 35–39 weeks gestation [35], which follows the completion of nephrogenesis at 32–36 weeks [26, 36]. Indeed, the term control lambs exhibited significantly greater capillary length than the gestational controls which is suggestive of increased growth in relation to increasing age. As the term animals were examined at postnatal day 3, however, it is possible that these differences reflect only postnatal, rather than prenatal, growth. This is supported by the finding of equivalent glomerular capillary length in the preterm ventilated and term ventilated animals at postnatal day 3; it may be speculated that increased capillary growth (as observed in the term controls) may also occur in the preterm kidney in the absence of ventilation. Further studies are certainly required in order to fully describe the time-course of glomerular capillary growth in the developing kidney.

The preterm ventilated lambs had significantly reduced glomerular capillary length, surface area, and renal corpuscle volume compared to term controls (due to increased capillary lengthening and dilatation with increased gestational age in the term animals, and/or to impaired growth of the capillaries after birth in the preterm animals). Importantly, TRFSA was also significantly reduced in the preterm ventilated animals compared to term controls. An important indicator of renal functional capacity, decreased TRFSA implies that renal function was compromised after preterm birth. This is in accordance with the findings of many clinical studies showing that preterm neonates have a much lower glomerular filtration rate compared to neonates born at term [33, 37]. In this study, it was not possible to determine whether the lesser glomerular capillary length and surface area in the preterm kidney at postnatal day 3 represents a persistent alteration in glomerular structure. In the long-term, however, a low TRFSA would be expected to progressively lead to the development of glomerulosclerosis, nephron loss, hypertension and ultimately significant renal disease [5, 38].

The adverse effects of exposure to high oxygen levels in preterm neonates have been well described in other organ systems [15]; however, to date there have not been any studies that have investigated the effects of ventilation on glomerular vascularisation. Studies conducted previously in a rat model of neonatal hyperoxia exposure (80% O₂ P3-P10, a time of ongoing postnatal nephrogenesis), found reduced glomerular size in neonates and a 25% reduction in nephron number in adulthood, suggesting that the kidney is adversely affected [14, 39]. Conversely, neonatal hyperoxia exposure (65% O₂) did not have any adverse renal effects in a mouse model [40]. Unlike these rodent models, in this more clinically relevant large animal model, nephrogenesis in the lamb was already completed, mechanical ventilation was used (and for a shorter time period of 3 days), and all animals were initially exposed to 100% O₂ then weaned to lower concentrations.

To determine the effects of ventilation independently of preterm birth, we examined glomerular capillary growth in term-born lambs that had been ventilated for three days postnatally. Importantly, a major finding from this study is that early postnatal ventilation appears to impair glomerular capillary growth, with term ventilated animals exhibiting significantly reduced glomerular capillary length and surface area compared to unventilated term controls (similar to the preterm ventilated animals). It should be noted, however, that although capillary length and surface area per renal corpuscle were significantly reduced, this did not translate into a statistically significant difference in TRFSA and thus may not have had an effect on renal functional capacity in the short term. In the long-term, however, this impaired vascular development and/or injury may predispose to nephron loss and renal disease [5, 38]. As with the preterm ventilated animals, future studies of renal function and morphology in lambs grown to adulthood would be required in order to determine the long-term impact of neonatal ventilation on renal health.

A possible explanation for the reduced glomerular capillary growth following ventilation is the relative increase in blood oxygen concentrations associated with postnatal ventilation. Indeed, previous studies have shown that in the presence of high circulating oxygen concentrations there is prolyl hydroxylase-mediated degradation of the HIF-1 α transcription factor [41] and this in turn, leads to down regulation in the expression of the angiogenic growth factor, VEGF [11, 12]. In the present study, however, there was no evidence of down-regulation of HIF-1 α or VEGF in the glomeruli of lambs that had been ventilated when compared to those that were not ventilated; thus, the reduced glomerular capillary growth following ventilation does not appear to be mediated via changes in the levels of either HIF-1 α or VEGF expression in the glomeruli. However, there does remain the possibility that the effects on HIF-1 α and VEGF expression may have occurred acutely when the lambs were initially ventilated and the levels may have normalised by 72 hours after birth (the time of necropsy).

Another possible explanation for the impaired glomerular capillary growth following ventilation may relate to changes in renal blood flow (not measured in this study); however, since none of the animals were hypotensive suggests that blood flow was adequate. In addition, given the key role of the glomeruli in blood filtration, there is also the potential for an adverse impact of the sedative and/or analgesic medications on the glomeruli and on glomerular capillary growth. Follow-up studies are required to investigate the mechanisms of normal glomerular capillary growth after birth, and why it is impaired following mechanical ventilation.

Given that in this study, we have observed negative effects of both preterm birth and postnatal ventilation on the growth of the glomerular capillaries, in future studies it is important to extend these studies to examine the effects of preterm birth on the growth of the vasa recta capillaries. The capillaries of the vasa recta, located in the renal medulla, play a key role in concentrating the urine and importantly, there is experimental evidence indicating that blood flow in the renal medulla is a key factor in maintaining a chronic elevation in blood pressure. Hence, impaired growth of the vasa recta capillaries in the preterm neonate may also be a contributing factor to the observed long-term elevation in blood pressure in subjects born preterm.

Conclusions

The findings from this study clearly demonstrate that moderate preterm birth adversely impacts TRFSA, and is therefore indicative of reduced renal functional capacity in the preterm neonate. In addition, the findings show that mechanical ventilation, independent of preterm birth, adversely impacts glomerular capillary growth. These adverse effects of preterm birth and/or mechanical ventilation on glomerular capillary growth in the neonate may contribute to the observed elevation in blood pressure in adult subjects that were born preterm. Further studies are required to determine whether the observed impairments in glomerular capillary growth persist into later life.

Acknowledgments

SOURCES OF FUNDING

This project was supported by a National Health and Medical Research Council (NHMRC) of Australia project grant, and NIH grants ROI-HL110002 (KHA) and ROI-HL062875. Author Megan R. Sutherland was the recipient of an Australian Postgraduate Award while undertaking this study.

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Figure 1. Physiological parameters in term and preterm ventilated animals

FiO_2 (A), PaO_2 (B), PaCO_2 (C), systolic blood pressure (D), diastolic blood pressure (E), heart rate (F), blood glucose (G), pH (H), and urine output (I) in preterm ventilated (grey circles) *versus* term ventilated (black squares) animals over the first 72 hours of life. Data assessed by two-way repeated measures ANOVA with the factors group, time, and their interaction (p_{GxT}). * $p < 0.05$, *** $p < 0.0001$ as determined by Bonferroni post-hoc analysis.

Figure 2. Body and kidney weights

Mean body weight (A), kidney weight (B), and kidney to body weight ratio (C) in gestational control animals at 133 days gestation, and preterm ventilated, term ventilated, and term control animals at postnatal day 3. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$ as determined by Bonferroni post-hoc analysis.

Figure 3. Nephron number and renal corpuscle volume

Mean nephron number (A), glomerular tuft volume (B), and renal corpuscle volume (C) in gestational control animals at 133 days gestation, and preterm ventilated, term ventilated, and term control animals at postnatal day 3. * $p < 0.05$ as determined by Bonferroni post-hoc analysis.

Figure 4. Glomerular morphology

Representative photomicrographs of toluidine blue-stained sections of glomeruli from the inner cortex of kidneys from gestational control animals at 133 days gestation (A), and preterm ventilated (B), term ventilated (C) and term control (D) animals at postnatal day 3. Scale bar = 50 μm.

Figure 5. Capillary length, surface area, and total renal filtration surface area

Mean capillary length (A) and surface area (B) per renal corpuscle in the inner, mid and outer regions of the renal cortex in gestational control, preterm ventilated, term ventilated and term control groups. Data assessed for every two groups by two-way ANOVA with the factors group, region of cortex, and their interaction ($p_{G \times R}$). * $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$ between groups according to two-way ANOVA; the factors region of cortex and $p_{G \times R}$ were non-significant in all cases. Linear regression analysis of average capillary length *versus* average capillary surface area per kidney in all animals (C). Total renal filtration surface area (D) in gestational control animals at 133 days gestation, and preterm ventilated, term ventilated, and term control animals at postnatal day 3. * $p < 0.05$ as determined by Bonferroni post-hoc analysis.

Figure 6. VEGF immunostaining in glomeruli

Representative VEGF immunolocalisation in glomeruli from gestational control animals at 133 days gestation (A), and preterm ventilated (B), term ventilated (C) and term control (D) animals at postnatal day 3. Scale bar = 50 µm.