Is nephrogenesis affected by preterm birth? Studies in a non-human primate model

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Title: Is nephrogenesis affected by preterm birth? Studies in a non-human primate model

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ABSTRACT

Nephrogenesis occurs predominantly in late gestation at a time when preterm infants are already delivered. The aims of this study were to assess the effect of preterm birth and the effect of antenatal glucocorticoid treatment on nephrogenesis. Preterm baboons, which were delivered at 125 days gestation and ventilated for up to 21 days postnatally, were compared to gestational controls. A cohort of preterm baboons that had been exposed to antenatal glucocorticoids were compared to unexposed preterm baboons. The number of glomerular generations was estimated using a medullary ray glomerular counting method and glomerular number estimated using unbiased stereology. CD31 and WT-1 localisation was examined using immunohistochemistry and VEGF was localised using *in situ* hybridisation. The number of glomerular generations was not affected by preterm birth and total glomerular numbers were within the normal range. Kidneys were significantly enlarged in preterm baboons with a significant decrease in glomerular density (number of glomeruli per gram of kidney) in the preterm kidney as compared to gestational controls. Neonates exposed to antenatal steroids had an increased kidney-to-body weight ratio and also more developed glomeruli compared to unexposed controls. Abnormal glomeruli, with a cystic Bowman's space and shrunken glomerular tuft, were often present in the superficial renal cortex of both the steroid exposed and unexposed preterm kidneys; steroid exposure had no significant effect on the proportion of abnormal glomeruli. The proportion of abnormal glomeruli in the preterm kidneys ranged from 0.2% - 18%. In conclusion, although nephrogenesis is on-going in the extrauterine environment our findings demonstrate that preterm birth, independent of steroid-exposure, is associated with a high proportion of abnormal glomeruli in some, but not all

neonatal kidneys. Whether final nephron endowment is affected in those kidneys exhibiting a high proportion of abnormal glomeruli is yet to be confirmed.

Keywords: Preterm birth, Baboon, Kidney, Nephrogenesis, Glomerulogenesis

INTRODUCTION

The incidence of preterm birth is currently high, with 13% of all babies born preterm in the United States (17) and 8% in Australia (25). In addition, the survival of preterm infants, including extremely preterm infants has improved substantially over recent years such that infants born as early as 26 weeks of gestation now have a 60-80% chance of survival (28, 34).

In the human kidney, nephrogenesis commences at around the 5^{th} week of gestation and is complete by 36 weeks gestation (32). After this point, no more new nephrons are formed for the life of the individual. Nephrogenesis predominantly occurs in late gestation at a time when preterm infants are already delivered (20). It is important to gain an understanding of the effects of preterm birth on nephrogenesis since there is accumulating epidemiological data linking premature birth with an increased incidence of hypertension (8, 22) and adverse renal function (23) later in life; this may be linked to a reduced nephron endowment after birth (4).

To our knowledge there has only been one previously published study which has investigated the effects of preterm birth on nephrogenesis. In the autopsy study, conducted by Rodriguez *et al.* (31), a reduced number of glomerular generations, potentially indicative of a nephron deficit, was reported in kidneys of infants that were born preterm. It is important to note, however, that the cohort of preterm infants in the human autopsy study included a number of infants that were not only preterm but also intrauterine growth restricted (IUGR). Since it is well known that IUGR adversely impacts on nephrogenesis (19, 40) it is difficult to clearly differentiate the effects of

preterm birth and IUGR on nephrogenesis in the previous study. In addition, any abnormal effects observed in the human autopsied kidneys may have been a direct result of a failure of the baby to thrive after birth rather than preterm birth *per se*. Hence in this study we have examined the effects of preterm birth, in the absence of IUGR, in a non-human primate model where the neonates were in relatively good health after birth.

The improved survival of preterm neonates, such as those born as early as 27 weeks gestation, can be largely attributed to the use of antenatal glucocorticoids. These have been shown to accelerate lung maturation, thus reducing neonatal morbidity and mortality (11). Previous experimental studies have demonstrated a link between glucocorticoid exposure early *in utero* and a reduction in nephron endowment (5, 30, 38) . There is no evidence to date, however, as to the effects of clinical doses of antenatal glucocorticoids on nephrogenesis.

Hence, the aims of the current study were to firstly assess the effects of preterm birth on nephrogenesis and secondly, to determine the effects of prenatal glucocorticoid treatment on nephrogenesis. To address these aims we have used a baboon (non-human primate) model, where the ontogeny of the kidney closely resembles that of the human (18) and the preterm neonates are cared for in a neonatal intensive care unit after birth in a similar manner as human infants (6). In our model, the baboons are delivered at 125 days of gestation (0.67 of total length of gestation), a time point at which nephrogenesis is still on-going in the baboon (18) and is approximately equivalent to 27 weeks of gestation in the human (27).

METHODS

INDUCTION OF PRETERM DELIVERY AND POSTNATAL CARE

All animal experiments were undertaken at the Southwest Foundation for Biomedical Research, San Antonio, Texas. All animal handling procedures were approved to conform to the American Association for Accreditation of Laboratory Animal Care guidelines. Fetal baboons were delivered prematurely by caesarean section at 125 days gestation (Term=185 days). After birth, all preterm neonates were intubated, administered 100 mg/kg surfactant (Survanta; donated by Ross Products, Columbus, OH, USA) and were ventilated with pressure-limited infant ventilators (InfantStar; donated by Infrasonics, San Diego, CA, U.S.A). All preterm neonates were also treated with ampicillin and gentamycin for the first 7-10 days of life. Further doses of antibiotics were only administered in cases of clinically suspected infection; two preterm animals were administered additional doses of vancomycin and a cephalosporin antibiotic, Fortaz (day 10-13 of life in one preterm neonate and day 10-17 of life in the other preterm neonate).

A detailed description of the postnatal clinical and nutritional management of the preterm baboons has been previously published (39). Briefly, during the first 24 hours of life, all animals received heparinised normal saline and 5% dextrose/water and supplemental calcium infusion. Sufficient fluids were administered in order to maintain electrolyte homeostasis, a minimal urine output of 1-2 ml/kg/h and blood pressure within the normal range. Parenteral nutrition was initiated at 24 hours of life with amino acids, electrolytes, vitamins and trace elements. If clinically stable, enteral nutrition was initiated on day 7 of life; 10 ml/kg/day of donated human breast milk was administered by intermittent

gastric infusion and once 100 ml/kg/day was tolerated, feeds were changed to Primilac (Bio-Serv, Frenchtown, NJ, U.S.A). Serum electrolytes, glucose and hematocrit were maintained within the normal range for the extremely low birth weight infant.

None of the animals had, or required, Foley catheters or any other form of urinary drainage device. There were also no animals that had identifiable urinary tract anomalies or obstructions at the time of necropsy.

THE EFFECT OF PRETERM BIRTH ON NEPHROGENESIS IN THE CONTEXT OF ANTENATAL STEROIDS

Fetal baboons were delivered prematurely by caesarean section at 125 days gestation (term=185). Neonates were euthanized at delivery (125 days gestation; n=4; 2 males and 2 females) or maintained in intensive care for 6 days (n=2; all males), 14 days (n=2; all males), or 21 days (Preterm + 21 days; n=4; all males) before being euthanized. Gestational-age-matched controls for the Preterm + 21 days group were delivered and euthanized at 146 days gestation (n=4; 3 males and 1 female). All animals in the Preterm + 21 days group and the 125 and 146 day gestational control groups were exposed to antenatal maternal steroids *in utero*. In those animals, pregnant baboons weighing approximately 15 kg received 6 mg betamethasone (Celestone Soluspan; Schering Pharmaceuticals, Kenilworth, NJ, USA) by intramuscular injection at 123 and 124 days of gestation (a dose of approximately 0.4 mg/kg/day) (24, 33). Those same animals were compared to non-betamethasone exposed controls in the subsequent study described below. It is to be noted that only individual data is shown for the baboons euthanized at 6 and 14 days and they were only included in the linear regression analyses; these baboons were not exposed to antenatal steroids. Kidneys from time points late in

gestation (175 and 185 days; n=5; 4 males and 1 female) were used for comparison when looking at the number of glomerular generations formed within the kidney. Previously, we have shown that nephrogenesis in the baboon is on-going at 125 days gestation and complete by 175 days gestation (18), so the kidneys from the 175 and 185 day gestational control groups were combined.

THE EFFECT OF PRENATAL MATERNAL GLUCOCORTICOIDS ON NEPHROGENESIS

The four betamethasone-exposed baboons euthanized at 125 days gestation (utilized in the study described above) were compared to non-betamethasone exposed controls (125 days gestation; n=6; 3 males and 3 females). The four betamethasone-exposed preterm neonates maintained for 21 days postnatally (utilized in the study described above) were compared to preterm controls (not exposed to antenatal betamethasone) at postnatal day 21 (Preterm + 21 days; n=4; 2 males and 2 females). At postnatal day 21, the baboons were euthanized.

TISSUE PROCESSING, EMBEDDING AND SECTIONING

Kidneys were immersion-fixed at necropsy, cut into halves, sliced into 2 mm slices and sampled using a smooth fractionator approach (29). The sampled slices (8-15 slices per kidney) were embedded in glycolmethacrylate to be used for the estimation of the number of glomeruli, the number of glomerular generations, kidney volume, mean renal corpuscle volume and the proportion of abnormal glomeruli. Complete slices containing both cortex and medulla were randomly selected from the remaining slices, embedded in paraffin and sectioned at five μm for the immunohistochemical analyses.

QUALITATIVE AND QUANTITATIVE ASSESSMENT OF NEPHROGENSIS

Morphological assessment of nephrogenesis

The presence of undifferentiated metanephric mesenchyme, the branching ureteric bud, and developing glomerular structures in the form of Comma- and S-shaped bodies in the outer cortex indicated that nephrogenesis was on-going. Developed glomeruli exhibited a well defined glomerular tuft surrounded by a distinct Bowman's space and capsule.

Measurement of nephrogenic zone thickness

The width of the nephrogenic zone was measured using image analysis software (Image Pro Plus v. 6.0 for Windows, Media Cybernetics; Silver Spring, MD, USA). This method was based on a previous method used to measure nephrogenic zone thickness to assess renal maturity in human neonatal kidneys (9, 12). From the serially sectioned glycolmethacrylate sections, one complete intact section from each sampled kidney slice (8-15 complete sections per kidney) was used to estimate the width of the nephrogenic zone. Each section was viewed at 200X magnification and the width of the nephrogenic zone was measured in 3 separate regions of each kidney section. The nephrogenic zone was defined as the area in the outer renal cortex exhibiting developing glomerular structures in the form of Comma and S-shaped bodies. An average nephrogenic zone width was determined for each kidney.

Estimation of glomerular generation number

One complete intact section from each glycolmethacrylate block (8-15 blocks per kidney) was examined. In each sampled section five clearly distinguishable medullary rays from separate regions were identified. The number of developed glomeruli (inclusive of normal and abnormal glomeruli) along one side of each medullary ray were counted, and an average number for each kidney was then determined (36).

Estimation of the number of developed glomeruli, kidney volume and mean renal corpuscle volume

Glycolmethacrylate blocks (8-15 per kidney) were serially sectioned at 20 μms with every 10^{th} and 11^{th} sections collected and stained with H&E. Kidney volume was then estimated using the Cavalieri principle (29). One pair of complete intact sections from each block was used for the estimation of glomerular number. Using an unbiased physical disector/fractionator technique, renal corpuscle volume and the number of glomeruli (and thereby nephrons) in the kidneys were stereologically estimated (3, 18). In the counting of glomeruli, only developed glomeruli (inclusive of normal and abnormal glomeruli) exhibiting a well defined Bowman's space and capsule were included; developing glomerular structures such as Comma-shaped and S-shaped bodies were not counted.

CHARACTERISATION OF ABNORMAL GLOMERULI

Quantitative assessment of abnormal glomeruli

Whilst undertaking the stereological estimation of glomerular number, in each field of view the number of normal and abnormal glomeruli (exhibiting a shrunken glomerular tuft and dilated Bowman's space) was recorded and the percentage of abnormal glomeruli within the whole kidney was determined.

Immunohistochemical analysis with the endothelial cell marker, CD31 and podocyte marker, Wilms-tumour suppressor gene-1 (WT-1)

Five μm paraffin sections were de-paraffinized, re-hydrated and rinsed in water and 10 mM Tris hydrochloride. For WT-1 staining, heat-induced antigen retrieval (3x5min in

microwave) was undertaken in Tris-EDTA buffer (10mM Tris Base, 1mM EDTA, 0.05% Tween 20; pH 9.0). Endogenous peroxidase activity was blocked by placing slides in an endogenous enzyme block solution (Dako, CA, U.S.A) for 15 minutes. Sections were then incubated with 1% goat serum for 20-30 minutes. Subsequently, sections were incubated with the primary antibody, either a mouse anti-human CD31 monoclonal antibody (1:15 dilution) (JC70A; Dako, California) or a mouse anti-human WT-1 monoclonal antibody (1:100 dilution) (M3561; Dako, CA, U.S.A) overnight. The negative control consisted of a mouse IgG antibody raised against bacterial glucose oxidase (Dako, CA, U.S.A). The sections were then incubated for 2 hours with the 'Envision' molecule (Dako, CA, U.S.A), and 3'3'-diaminobenzidine tetrachloride (DAB) was used to detect antibody binding. All sections were counterstained with hematoxylin.

In situ hybridization of vascular endothelial growth factor (VEGF) mRNA

For the synthesis of riboprobes, a cDNA fragment of human $VEGF_{121}$ (gift of Steven Stacker, Ludwig Institute, Melbourne, Australia) was cloned into BSKS plasmid (Stratagene, La Jolla, CA, U.S.A) and linearized with HindIII. An anti-sense riboprobe was generated from the template incorporating DIG-UTP (Roche Applied Science, Mannheim, Germany) into run off transcripts using T7 RNA polymerase. A sense riboprobe was also generated. *In-situ* hybridization was undertaken in 4μm paraffin sections as described by Sutherland *et al.* (36).

STATISTICAL ANALYSIS

Statistical analyses were performed using GraphPad Prism Version 4.0 for Windows (GraphPad Software, San Diego, CA, U.S.A.). A one-way analysis of variance was

utilized to compare data between the 125 day and 146 day gestational control groups, and the Preterm +21 days group. This was followed by a Tukey's post-hoc analysis.

Data between steroid exposed and unexposed neonates were analysed using a Student's t-test. In order to compare data between steroid exposed and unexposed neonates from different post-conceptional time points, a two-way analysis of variance was utilized. Physiological data was analysed using a repeated measures two-way analysis of variance followed by a Bonferroni's post-hoc analysis.

Linear regression analyses were performed to determine if there were significant correlations between glomerular number and post-conceptional age, birth weight, kidney weight, kidney volume and renal corpuscle volume. Included in these analyses was data from steroid exposed and unexposed animals and animals from the Preterm + 6 days and Preterm + 14 days groups. An analysis of covariance was used to determine any differences in the linear regressions between the preterm group and the gestational controls. Statistical significance was accepted as $p < 0.05$.

RESULTS

THE EFFECT OF PRETERM BIRTH ON NEPHROGENESIS IN THE CONTEXT OF ANTENATAL STEROIDS

Postnatal fetal physiology

Arterial blood gases (pH, PaCO2, PaO2), fluid intake, urine output and mean arterial blood pressure of preterm neonates from birth until postnatal day 21 were all within the accepted clinical range.

Body weights, kidney weights and kidney volumes

All fetal baboons had birth weights above the 10% reference range for premature baboons delivered at this gestational time point. There was no significant difference in birth weights between the 125 day gestational control group and the Preterm + 21 days group (Table 1). Necropsy weights of the Preterm + 21 days group were significantly less (P=0.002) compared to the 146 day gestational-age-matched controls. Although all preterm baboons lost weight after birth, relative kidney weights and volumes were significantly increased compared to the 125 and 146 day gestational controls.

Assessment of nephrogenesis

In the preterm kidneys at postnatal day 6, 14 and 21 and in the 125 and 146 day gestational controls, there was morphological evidence of on-going nephrogenesis (Figure 1A). In the preterm kidneys at postnatal day 21, nephrogenic zone thickness was significantly less compared to the 125 day gestational control group but not significantly different to the 146 day gestational control group (Figure 1B).

The number of glomerular generations increased significantly from 125 days gestation to 175/185 days gestation (Figure 1C). The number of glomerular generations in the Preterm + 21 days group was not significantly different to the number of generations

in the 146 day gestational-age-matched controls, and was significantly higher than the 125 days group.

In accordance with the glomerular generation data, the number of developed glomeruli in the Preterm + 21 days group was significantly greater compared to the 125 day gestational controls, but was not significantly different to the 146 day gestational age-matched controls (Table 1).

Statistically significant correlations were found between glomerular number and post-conceptional age (r^2=0.781, P=0.0001) and between glomerular generations and post-conceptional age (r^2=0.613, P=0.003) when all preterm animals were combined (Figure 1D-E). In the two kidneys from the Preterm + 6 days group, there were 184,234 and 202,316 developed glomeruli and in the kidneys from postnatal day 14 (n=2) there were 140,185 and 178,661 developed glomeruli.

Birth weight correlated significantly with glomerular number in both the preterm neonates (r^2=0.438, P=0.02) and the gestational controls (r^2=0.680, P=0.01). In the preterm neonates, there was no significant correlation between necropsy weight and glomerular number.

Importantly, there was a very strong correlation between kidney weight and glomerular number in both the preterm neonates (r^2=0.703, P=0.0007) and gestational controls (r^2=0.664, P=0.01); however, there was a significant difference in the slopes of the regression lines (P=0.048) such that in the preterm kidneys there were 83,840 glomeruli/gram, compared to 193,400 glomeruli/gram in the gestational controls (Figure 2).

There was no significant difference in the mean renal corpuscle volume between the Preterm + 21 days groups and the 146 day gestational-age-matched control group

(Table 1). There was no significant correlation between renal corpuscle volume and glomerular number.

THE EFFECT OF PRENATAL MATERNAL GLUCOCORTICOIDS ON NEPHROGENESIS

Postnatal fetal physiology

Exposure to antenatal maternal glucocorticoids at 123 and 124 days gestation did not significantly affect arterial blood gas levels (pH, PO_2, PCO_2) following preterm delivery at 125 days gestation (Table 2). There was no significant difference in fluid intake or urine output between the two groups (Figure 3A-B). At 72 hours of life, however, mean arterial blood pressure was significantly elevated in the steroid exposed group ($P<0.05$) (Figure 3C). There was no significant difference in mean arterial blood pressure between the two groups at postnatal day 21.

Body weights, kidney weights and kidney volumes

Antenatal exposure to steroids (123 and 124 days gestation) did not affect fetal birth weight at 125 days gestation, necropsy weight at postnatal day 21, or absolute kidney weights or kidney volumes (Table 3). Kidney weight-to-body weight ratio, however, was significantly greater in the animals exposed to antenatal steroids ($P=0.02$).

Assessment of nephrogenesis

There were no apparent morphological differences in kidney structure between the steroid exposed and unexposed preterm groups at postnatal day 21. Kidneys from both the Preterm + 21 days group and the Preterm + 21 days + steroids group exhibited a clearly visible nephrogenic zone and there was no significant difference in the width of the nephrogenic zones (94.2±6.5 μm versus 100.5±10.6 μm, respectively).

There was no difference in the number of glomerular generations formed within the kidney between the steroid exposed and unexposed groups (Table 3).

There was a significant increase in the number of developed glomeruli in the steroid exposed kidneys (Table 3). The number of developed glomeruli was 9% higher in the steroid exposed kidneys compared to unexposed controls at 125 days gestation, and 27% higher at postnatal day 21.

The response of renal corpuscle volume in relation to maternal steroid treatment was different in kidneys at 125 days gestation compared to preterm kidneys at postnatal day 21 (Table 3). At 125 days gestation there was a significant increase in renal corpuscle volume whereas on postnatal day 21 there was a significant decrease.

CHARACTERISATION OF ABNORMAL GLOMERULI

We observed in many of the preterm kidneys (both steroid exposed and unexposed), at all postnatal time points, the presence of abnormal glomeruli; these were grossly enlarged and exhibited a cystic Bowman's space and shrunken glomerular tuft. The abnormal glomeruli were only located in the outer renal cortex and exhibited an immature morphology; the clearly recognizable glomerular anlage was surrounded by a cup-shaped layer of epithelial cells.

Quantitative assessment of abnormal glomeruli

There was a wide variation in the proportion of abnormal glomeruli within the preterm kidneys (Table 4 and 5); steroid exposure did not affect the proportion of abnormal glomeruli in the kidney. The proportion of abnormal glomeruli in the preterm kidneys ranged from 0.2% to 18.3%. Of the twelve preterm kidneys analyzed (inclusive of steroid exposed and unexposed), 50% had more than 4% of their glomeruli appearing abnormal. In three preterm kidneys the proportion of abnormal

glomeruli was greater than 10%. In one of the preterm baboons the morphology of the kidney was grossly abnormal with 18% of the glomeruli abnormal. The proportion of abnormal glomeruli in the kidneys of the gestational controls was considered negligible (Table 4).

Immunohistochemical localization of the endothelial cell marker, CD31 (Figure 4A)

In kidney sections at 146 days gestation, the well developed glomeruli adjacent to the nephrogenic zone demonstrated profuse positive staining for CD31. In the preterm kidneys, the abnormal glomeruli exhibited little CD31 immunostaining compared to well developed glomeruli observed in the same section.

Immunohistochemical localisation of the podoctye marker, WT-1 (Figure 4B)

Profuse WT-1 staining was observed in the glomerular tuft of well developed, normal glomeruli from preterm kidneys. In the abnormal glomeruli from the preterm kidneys, however, WT-1 positive immunostaining was localised to the layer of epithelial cells surrounding the spherical mass of cells of the glomerular tuft. Positive immunostaining was also localised to the epithelial cells of the Bowman's capsule in the abnormal glomeruli.

In situ localisation of vascular endothelial growth factor, VEGF mRNA (Figure 4C)

VEGF mRNA was localised to the glomerular podocytes in both the preterm kidneys and gestational controls including the abnormal glomeruli.

DISCUSSION

The findings of this study clearly demonstrate that nephrogenesis continues after preterm birth in the steroid exposed and unexposed primate kidney. There was an increase in the number of glomerular generations and total glomeruli in the extrauterine environment, with no differences found between the preterm kidneys and their gestational age matched controls, in the context of antenatal steroids. Interestingly, exposure to antenatal glucocorticoids prior to preterm birth led to renal hypertrophy and an increase in the number of developed glomeruli in the kidney compared to unexposed kidneys. Many of the glomeruli located in the outer renal cortex of the preterm kidney, in both the steroid exposed and unexposed baboon neonates, often appeared abnormal. Immunohistochemical analyses of these abnormal glomeruli showed that they were in a relatively immature state of development, poorly vascularized and are therefore likely to be non-functional.

Although all preterm baboons lost weight after birth, there appeared to be substantial postnatal kidney growth, with the relative kidney weights and kidney volumes significantly higher in the preterm animals compared to the gestational controls. Similar findings have been previously reported in preterm babies (21). The renal hypertrophy observed in the preterm kidneys did not appear to be attributed to differences in the thickness of the nephrogenic zone or in the size of glomeruli thus implying tubular hypertrophy.

Our results demonstrate that nephrogenesis unequivocally occurs postnatally in both steroid exposed and unexposed preterm neonates; morphologically there was evidence of a nephrogenic zone and when assessed quantitatively the number of glomerular

generations and the total number of developed glomeruli increased with postnatal age. The average number of developed glomeruli in the preterm kidneys (inclusive of steroid exposed and unexposed preterm kidneys) was approximately 245,673, ranging from 138,078 to 304,186, which appears to be within the normal range for term baboon kidneys, albeit at the lower end (18). Total glomerular number is also expected to increase further since nephrogenesis, although nearing completion was not finished by postnatal day 21.

In the context of antenatal steroids, there was no significant difference in the number of glomerular generations formed in the kidney between the Preterm + 21 day kidneys and their 146 day gestational-age-matched controls. These findings are not consistent with those of Rodriguez *et al*. (31) where they reported fewer glomerular generations within the kidneys of autopsied preterm infants. However, the discrepancy in findings is likely explained by a number of the preterm human neonates being intrauterine growth restricted in the study of Rodriguez *et al.* (31), which is known to influence nephron endowment (19, 40).

We have previously reported a very strong correlation between renal size and glomerular number (18) and this association appears to be maintained after premature delivery since kidney weight significantly correlated with glomerular number in the preterm baboons. However, in the present study our linear regression analyses indicate that glomerular density (the number of glomeruli per gram of kidney) is substantially less in the preterm kidneys (83,840 glomeruli/gram) compared to gestational controls (193,400 glomeruli/gram); this is likely to be due to the relative increase in kidney size after preterm birth and not a change in the absolute number of

glomeruli formed. Further studies would be necessary in order to investigate whether this difference in glomerular density reflects a change in renal tubular mass.

Our findings have demonstrated that antenatal exposure to glucocorticoids prior to preterm birth increases the number of developed glomeruli within the preterm baboon kidney. Certainly, this indicates that glucocorticoid administration has accelerated glomerular maturation, which is in accordance with previous studies demonstrating that glucocorticoids induce organ maturation (13). Our findings also support the improvement in renal function demonstrated to occur in preterm infants exposed to steroids (1). Exposure to steroids also resulted in a greater increase in kidney weight-to-body weight ratio, suggesting that glucocorticoid treatment may be leading to renal hypertrophy. Previous studies in the preterm lamb, baboon and human neonate have shown that glucocorticoid treatment increases mean arterial pressure, renal blood flow and glomerular filtration rate, implicative of renal functional maturation, (1, 10, 35) which may be contributing to the renal hypertrophy observed in the current study. Indeed, mean arterial blood pressure was observed to be significantly elevated at 72 hours of life in the preterm baboons exposed to antenatal steroids. Similar findings have also been reported in human infants, in which the effects of prenatal glucocorticoids appear to be limited to the early postnatal period (1, 37).

In accordance with previous studies (31, 36) abnormal glomeruli exhibiting a dilated Bowman's space surrounding an underdeveloped glomerular tuft were observed in the outer renal cortex of both steroid exposed and unexposed preterm kidneys, suggesting glomeruli formed in the extra-uterine environment are 'at risk'; developed glomeruli deep in the cortex were not affected. The glomerular abnormalities appear to be a

direct consequence of premature birth and/or treatments in the postnatal care of the preterm neonate, since the number of abnormal glomeruli in the gestational control kidneys was negligible. Immunostaining with the endothelial cell marker, CD31 showed that the abnormal glomeruli in the preterm kidneys were poorly vascularised, even though VEGF was expressed. The abnormal glomeruli appeared to be in a relatively immature state of development with a layer of WT-1 positive epithelial cells (indicative of podocytes) surrounding a spherical mass of relatively undifferentiated cells. Positive WT-1 immunostaining was also localised to the epithelial cells of the Bowman's capsule (parietal podocytes), which has been demonstrated previously in the human kidney (2). Interestingly, Bariety *et al.* (2) noted that capsules without a glomerular tuft, or a retracted tuft, contained a greater number of parietal podocytes compared to normal glomeruli, lining the entirety of the Bowman's capsule. Furthermore, Gibson *et al*. (14), have also shown that in atubular cystic glomeruli in human kidneys, the Bowman's capsule is always lined by parietal podocytes. It is therefore conceivable that the cystic abnormal glomeruli observed in the preterm kidneys may be atubular, and as such would never be functional.

Not all kidneys from the premature baboons contained the same proportion of abnormal glomeruli, ranging from 0.2% to as high as 18%. Hence, preterm birth may not always adversely impact on kidney development, or alternatively there may be a difference in the rates of resorption of dysfunctional glomeruli (26). Another likely explanation is that factors in the postnatal care of the neonate (which varies between neonates) adversely impact on nephrogenesis. In particular, pharmacological agents administered to the neonate in the postnatal period, such as aminoglycoside antibiotics, are known to be nephrotoxic (7, 15, 16). In the present study, since all

preterm neonates were exposed to antibiotics after birth, it is possible that the glomerular abnormalities in the kidneys have been caused by exposure to nephrotoxic antibiotics. However, if this is the case, it is difficult to explain why there was such a variation in the proportion of abnormal glomeruli within the preterm kidneys given that all neonates received the same regime of antibiotics, except for two animals that were administered additional doses; these animals were not the neonates with the high proportion of abnormal glomeruli. Further studies are required to elucidate whether exposure to antibiotics is nephrotoxic to the preterm infant and/or whether other medications, or factors in the postnatal care of the preterm infants lead to the adverse renal effects that we observe. If definitive associations are found, this may lead to potential interventions to improve the renal health of preterm babies.

In conclusion, using a non-human primate model the current study has clearly demonstrated on-going nephrogenesis after preterm birth. The rate of glomerular formation remains similar following preterm birth; however, glomerular density (number of glomeruli per gram of kidney) was significantly reduced in the preterm kidney suggesting that the non-glomerular compartments are growing at a faster rate. Of concern, many preterm neonates exhibited abnormal glomeruli in the outer renal cortex suggesting that extra-uterine nephrogenesis leads to an increased risk of abnormal glomerular development. Whether this will impact on final nephron endowment is yet to be determined, since nephrogenesis was still on-going.

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Figure Legends

Figure 1: (A) Representative photomicrographs of kidney sections from the 125 (top panel) and 146 (middle panel) day gestational control groups and the Preterm + 21 days group (bottom panel) all demonstrating evidence of on-going nephrogenesis in the outer renal cortex (NZ=nephrogenic zone, C=Comma-shaped body, UB=ureteric bud). (B) Nephrogenic zone thickness of gestational controls (125 days gestation and 146 days gestation) compared to the Preterm + 21 days group. Data was analysed using a one-way analysis of variance followed by a Tukey's Post-Hoc Analysis. Nephrogenic zone thickness in the 125 day gestational controls were significantly greater (*$P<0.05$) compared to the 146 day gestational controls and the Preterm + 21 days group (C) The number of glomerular generations in the gestational controls (125 days gestation, 146 days gestation, 175/185 days gestation) compared to the Preterm + 21 days group. Data was analysed using a one-way analysis of variance followed by a Tukey's Post-Hoc Test. The number of glomerular generations was significantly greater in the 125 day gestational controls compared to the 146 day and 175/185 day gestational controls and the Preterm + 21 days group (*$P<0.0001$) (D) Linear regression analysis of glomerular number versus post-conceptional age in preterm kidneys showing a significant linear relationship between post-conceptional age and glomerular number. (E) Linear regression analysis of glomerular generations versus post-conceptional age in preterm kidneys showing a significant linear relationship between post-conceptional age and the number of glomerular generations.

Figure 2: Linear regression analyses of glomerular number versus kidney weight in preterm neonates (circles) and gestational controls (triangles). There was a significant linear relationship between kidney weight and glomerular number in both

678 the preterm neonates and gestational controls (P<0.05). An analysis of covariance
679 demonstrated a significant difference in the slopes of the two regression lines
680 (P=0.048).
681
682 **Figure 3:** (A) Fluid intake, (B) urine output and (C) mean arterial blood pressure of
683 the Preterm + 21 days group; n=4 compared to the Preterm + 21 days + steroids
684 group; n=4. Steroid exposed neonates are represented in squares and unexposed
685 controls are in circles. Data was analysed using a repeated measures two-way
686 analysis of variance followed by a Bonferroni's Post-Hoc analysis. At 72 hours of
687 life, mean arterial blood pressure was significantly higher in the steroid exposed
688 animals compared to unexposed controls (*P<0.05).
689
690 **Figure 4:** Representative photomicrographs of kidney sections from the 146 day
691 gestational control group and the Preterm + 21 days group immunostained with (A)
692 an endothelial cell marker(CD31), (B) the Wilm's tumour suppressor gene-1 (WT-1),
693 and (C) *in situ* hybridization for vascular endothelial growth factor (VEGF). The
694 glomeruli from the 146 day gestational control group show profuse positive brown
695 staining for CD31 (A). The abnormal glomeruli in the Preterm + 21 days group are
696 poorly vascularized as shown by the scant positive brown staining for CD31 (A).
697 WT-1 positive staining is localized to podocytes within the glomerular tuft of
698 developed, normal glomeruli (B). In abnormal glomeruli, WT-1 staining is localized
699 to podocytes surrounding the immature glomerular anlage (B). WT-1 positive
700 immunostaining can also be observed in the parietal epithelial cells of the Bowman's
701 capsule (arrows). VEGF positive immunostaining (dark purple staining) was
702 localized to the podocytes in the glomerular tuft (arrows) (C). In an abnormal

glomerulus from a preterm kidney, VEGF positive podocytes stained dark purple were also observed (arrows) (C).

Table 1: Birth weights, necropsy weights, kidney weights, kidney volumes, kidney weight to body weight ratios, kidney volume to kidney weight ratios, glomerular number and mean renal corpuscle volume of gestational controls at 125 days gestation and 146 days gestation and preterm neonates at postnatal day 21 (analyzed using a one-way analysis of variance followed by a Tukey's Post-Hoc analysis).

	Gestational Controls		Preterm
	125 days (n=4)	**146 days (n=4)**	**Preterm + 21 days (n=4)**
Birth weight (g)	353 ± 12$^{\#}$ (329 – 375)	597 ± 25* (532 – 654)	419 ± 22$^{\#}$ (354 – 460)
Necropsy weight (g)	353 ± 12$^{\#}$ (329 – 375)	597 ± 25* (532 – 654)	400 ± 30$^{\#}$ (320 - 466)
Kidney weight (g)	1.37 ± 0.16 (1.01 – 1.77)	1.82 ± 0.18 (1.31 – 2.16)	2.73 ± 0.41* (1.90 – 3.87)
Kidney volume (mm^3)	903 ± 113 (636 – 1170)	1348 ± 196 (819 – 1768)	1719 ± 270 (1391 – 2523)
Kidney weight-to-body weight ratio (g/kg)	3.9 ± 0.5 (2.7 – 5.4)	3.1 ± 0.4 (2.0 – 3.7)	6.7 ± 0.5$^{*\#}$ (5.9 – 8.3)
Kidney volume-to-body weight ratio (mm^3/g)	2.6 ± 04 (1.7 – 3.6)	2.3 ± 0.4 (1.2 – 3.0)	4.3 ± 0.4$^{*\#}$ (3.5 – 5.4)
Glomerular number	117,235 ± 8,766 (101,439 – 137,765)	270,486 ± 33,631* (202,266 – 352,621)	283,535 ± 12,358* (249,772 – 304186)
Average renal corpuscle volume x 10^{-4} (mm^3)	5.87 ± 0.52 (5.03 – 7.38)	3.97 ± 0.35 (2.37 – 4.29)	3.65 ± 0.43 (3.14 – 4.72)

All animals were exposed to antenatal glucocorticoids
Data presented as mean ± SEM with data range in parentheses.
(*P<0.05 versus 125 days gestation, #P<0.05 versus 146 days gestation)

724 **Table 2:** Arterial blood gases (pH, $PaCO_2$, PaO_2) of the Preterm + 21 days group compared to the
725 Preterm + 21 days + steroids group, at postnatal day 21. Data was analysed using a student's t-test.

726

727

	Preterm + 21 days (n=4)	**Preterm + 21 days + steroids (n=4)**
pH	7.32 ± 0.03	7.29 ± 0.03
$PaCO_2$ (mmHg)	47.7 ± 4.5	53.2 ± 1.9
PaO_2 (mmHg)	79.7 ± 0.3	67.0 ± 6.4

728
729 Data presented as mean ± SEM

730 **Table 3:** Birth weights, necropsy weights, kidney weights, kidney volumes, kidney weight to body weight ratios, glomerular generation
731 number, glomerular number and mean renal corpuscle volume of preterm neonates at 125 days gestation and at postnatal day 21. Neonates at
732 each time-point were exposed to maternal betamethasone treatment at 123 and 124 days gestation (+ steroids; data as shown in Table 2) or were
733 unexposed (- steroids). Data was analysed using a two-way analysis of variance.

					P-values		
	125 days gestation - steroids (n=6)	**125 days gestation + steroids (n=4)**	**Preterm + 21 days - steroids (n=4)**	**Preterm + 21 days + steroids (n=4)**	**Post-conceptional age**	**Steroid treatment**	**Post-conceptional age x Steroid treatment**
Birth weight (g)	375 ± 25 (299 - 448)	353 ± 12 (329 – 375)	414 ± 28 (373 – 496)	419 ± 22 (354 – 460)	P = 0.05	P = 0.733	P = 0.589
Necropsy weight (g)	375 ± 25 (299 - 448)	353 ± 12 (329 – 375)	395 ± 15.4 (363 – 436)	400 ± 30 (320 - 466)	P > 0.05	P > 0.05	P > 0.05
Kidney weight (g)	0.917 ± 0.10 (0.559 - 1.21)	1.37 ± 0.16 (1.01 – 1.77)	2.31 ± 0.17 (1.89 – 2.64)	2.73 ± 0.41 (1.90 – 3.87)	**P < 0.0001**	P > 0.05	P > 0.05
Kidney volume (mm^3)	548 ± 48 (391 – 660)	903 ± 113 (636 – 1170)	1690 ± 200 (1352 - 2164)	1718 ± 270 (1391 – 2523)	**P < 0.0001**	P > 0.05	P > 0.05
Kidney weight-to-body weight ratio (g/kg)	2.5 ± 0.3 (1.5 – 3.6)	3.9 ± 0.5 (2.7 – 5.4)	5.9 ± 0.3 (5.2 – 6.5)	6.7 ± 0.5 (5.9 – 8.3)	**P < 0.0001**	**P = 0.02**	P > 0.05
Glomerular Generations	7 ± 0.2 (6 – 7)	7 ± 0.1 (7 – 8)	10 ± 0.2 (10 – 11)	10 ± 0.6 (8 -11)	**P < 0.0001**	P = 0.6	P > 0.05
Glomerular number	105,632 ± 10173 (63,385 - 126,697)	117,235 ± 8,766 (101,439 – 137,765)	207,810 ± 35,384 (138,078 - 272,085)	283,535 ± 12,358 (249,772 – 304186)	**P < 0.0001**	**P = 0.03**	P = 0.1
Average Renal Corpuscle Volume x 10^{-4} (mm^3)	3.79 ± 0.45 (2.49 – 5.59)	5.87 ± 0.52 (5.03 – 7.38)	5.32 ± 0.43 (4.24 – 6.22)	3.65 ± 0.43 (3.14 – 4.72)	P = 0.7	P = 0.5	**P = 0.001**

734 Data presented as mean ± SEM with data range in parentheses.

735

Table 4. The proportion of abnormal glomeruli in kidneys of gestational controls and preterm baboon neonates.

	Gestational Controls			**Preterm**		
	125 days	146 days	175/185 days	Preterm + 6 days	Preterm + 14 days	Pre-term + 21 days
Proportion of abnormal glomeruli (%)	1.3	0.2	0.0	4.3	10.7	18.3
	1.2	0.0	0.0	6.5	6.1	1.4
	0.3	0.0	0.0			2.0
	2.4	0.0	0.0			2.6
			0.0			
Average	**1.3 ± 0.4%**	**0.05 ± 0.05%**	**0.0 ± 0.0%**	**5.4 ± 1.1%**	**8.4 ± 2.3%**	**6.1 ± 4.1%**

Animals from the 125 days gestation, 146 days gestation and Preterm + 21 days groups were all exposed to antenatal glucocorticoids.

Data presented as mean ± SEM

752 **Table 5.** The proportion of abnormal glomeruli in steroid exposed baboons (data as shown in Table 5) compared to unexposed controls.

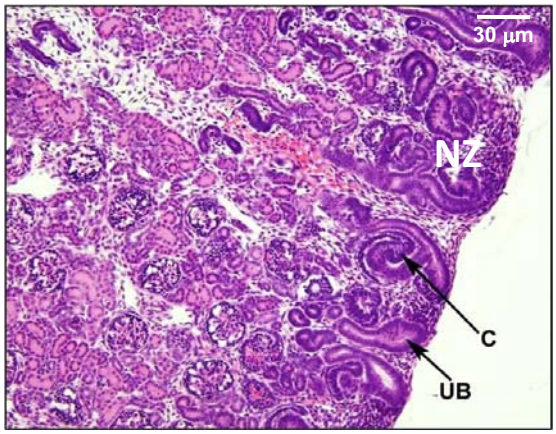
	125 days gestation -steroids	**125 days gestation + steroids**	**Preterm + 21 days - steroids**	**Preterm + 21 days + steroids**
Proportion of abnormal glomeruli (%)	0.2	1.3	0.2	18.3
	2.9	1.2	12.8	1.4
	0.6	0.3	0.2	2.0
	0.2	2.4	4.7	2.6
	0.5			
	0.0			
Average	**0.7 ± 0.4 %**	**1.3 ± 0.4%**	**4.5 ± 3.0%**	**6.1 ± 4.1%**

753

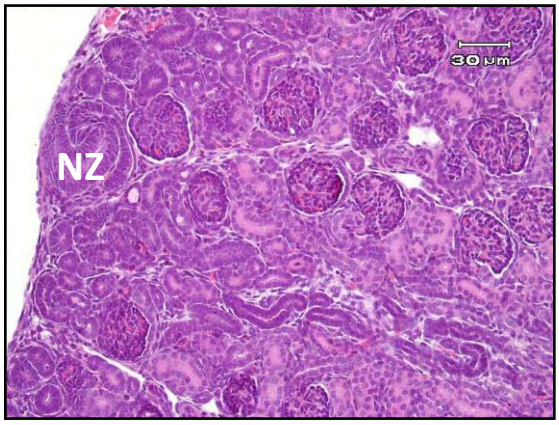
754 Data presented as mean ± SEM

A

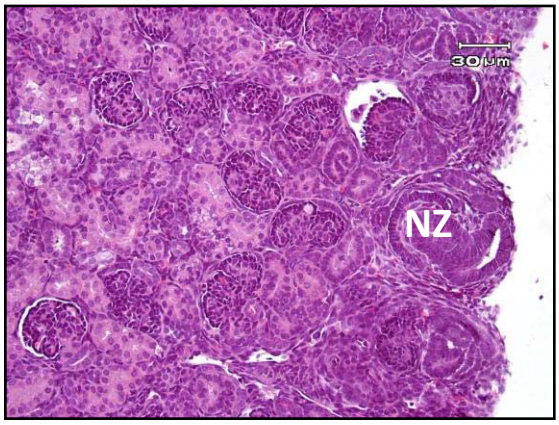
125 days gestation



146 days gestation



Pre-term + 21 days



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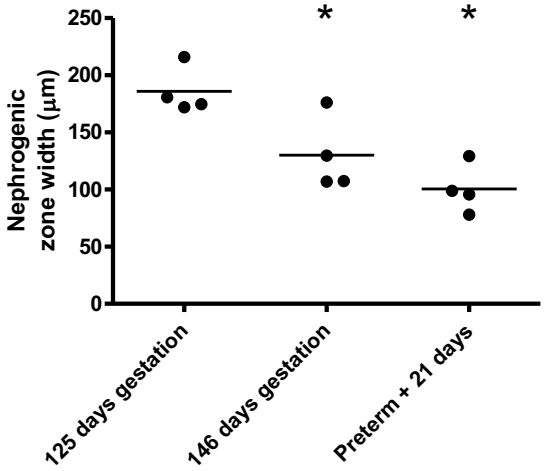

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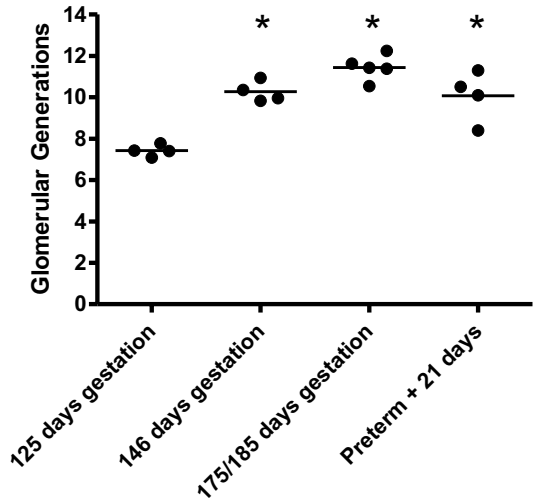

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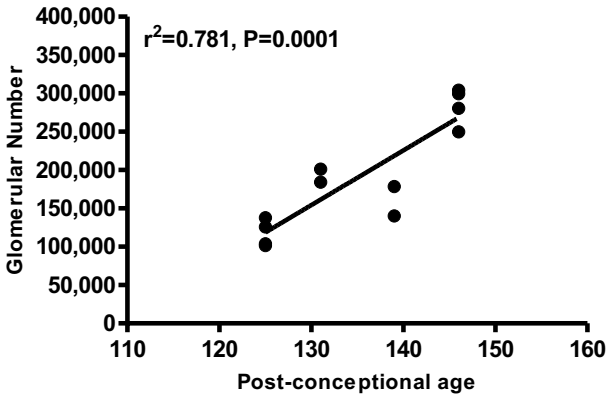

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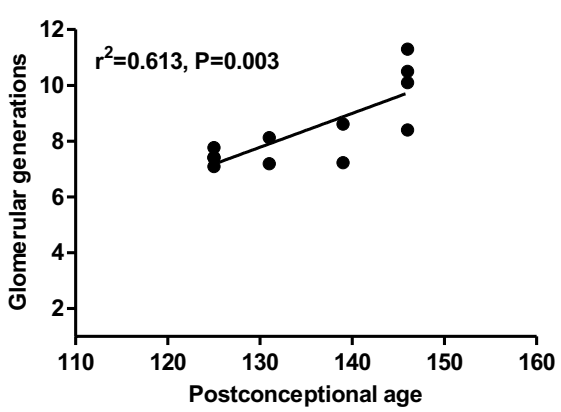
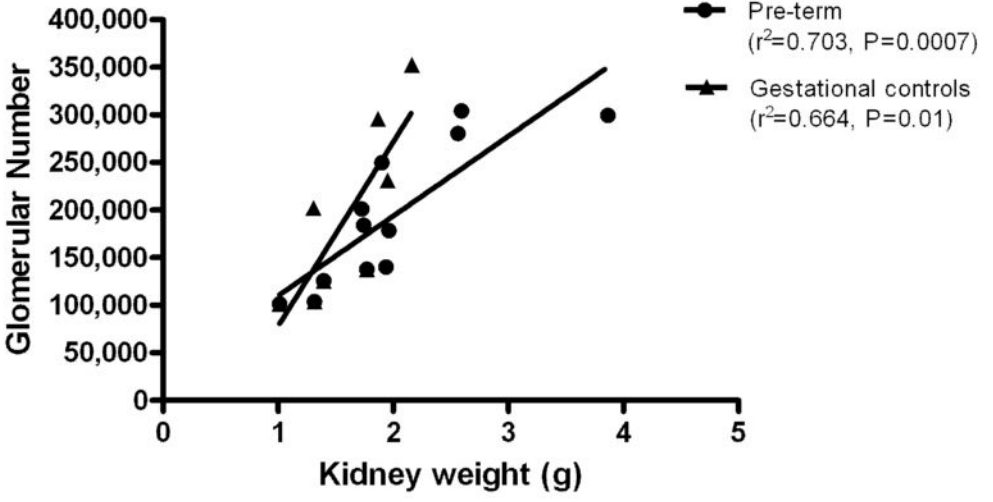
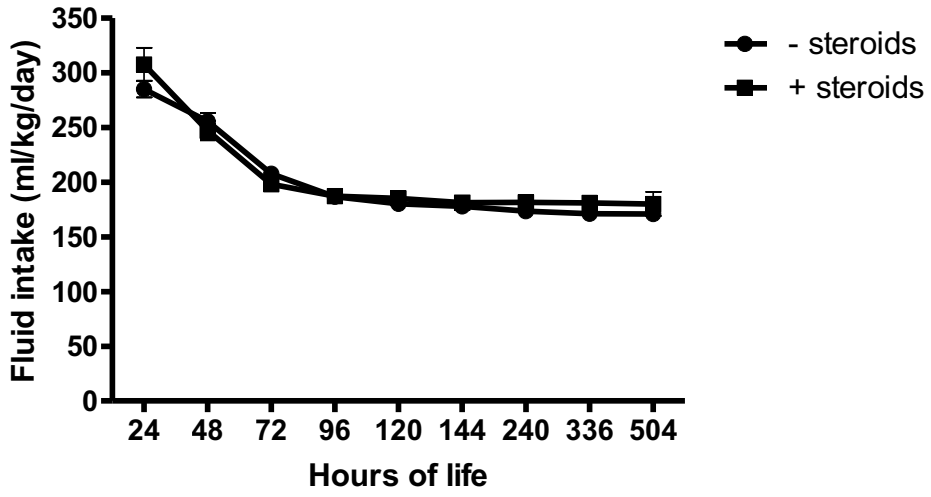
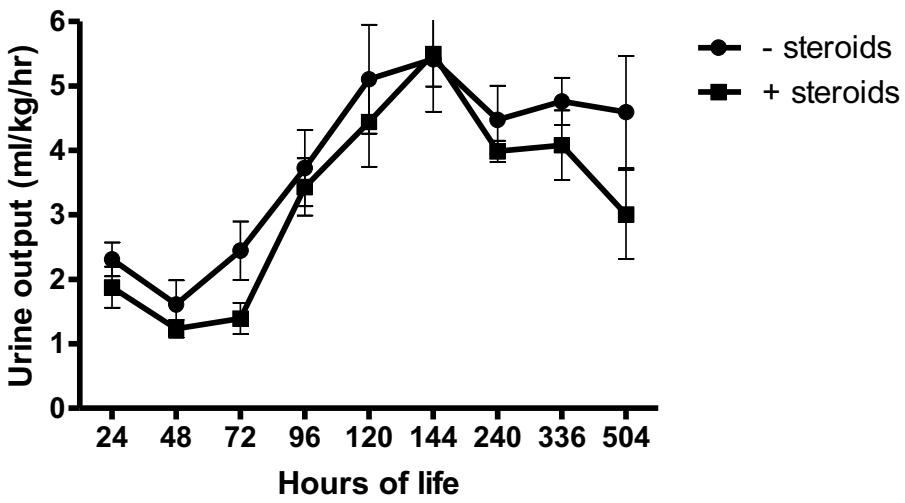



Glomerular Number
400,000
350,000
300,000
250,000
200,000
150,000
100,000
50,000
0
0
1
2
3
4
5
Kidney weight (g)
Pre-term
(r^2=0.703, P=0.0007)
Gestational controls
(r^2=0.664, P=0.01)

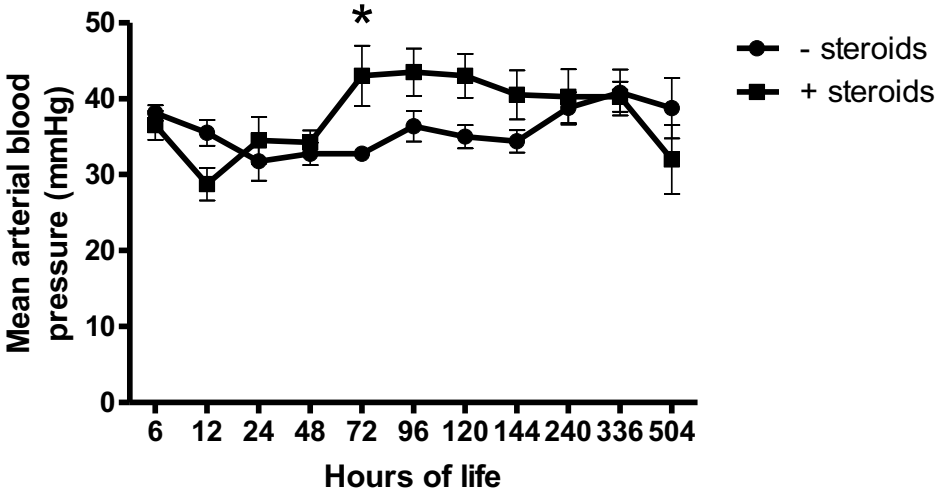
A


Fluid intake (ml/kg/day)
350
300
250
200
150
100
50
0
24
48
72
96
120
144
240
336
504
Hours of life
- steroids
+ steroids

B


Urine output (ml/kg/hr)
6
5
4
3
2
1
0
24
48
72
96
120
144
240
336
504
Hours of life
- steroids
+ steroids

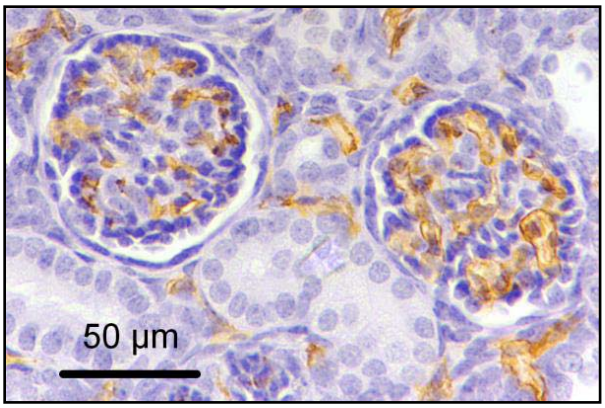
C

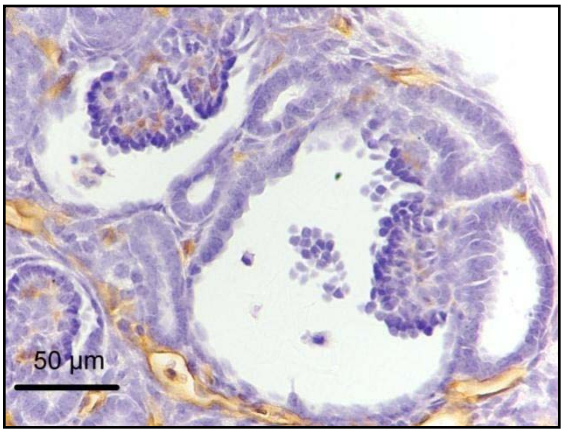

Mean arterial blood pressure (mmHg)
50
40
30
20
10
0
*
6
12
24
48
72
96
120
144
240
336
504
Hours of life
- steroids
+ steroids

146 DAY GESTATIONAL CONTROL

PRETERM + 21 DAYS

A





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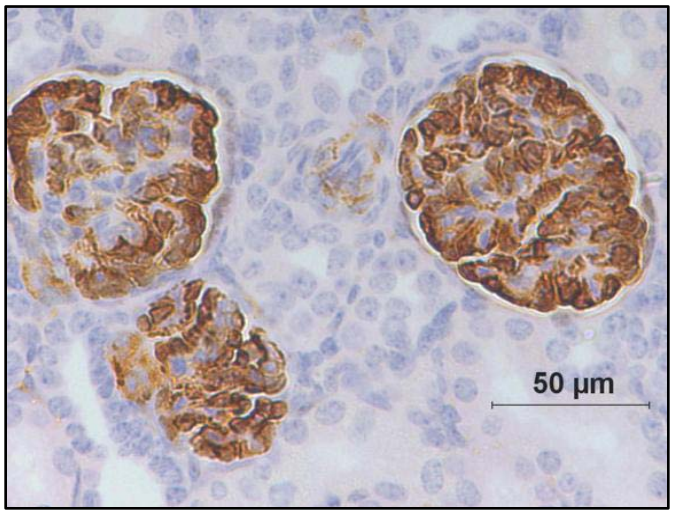

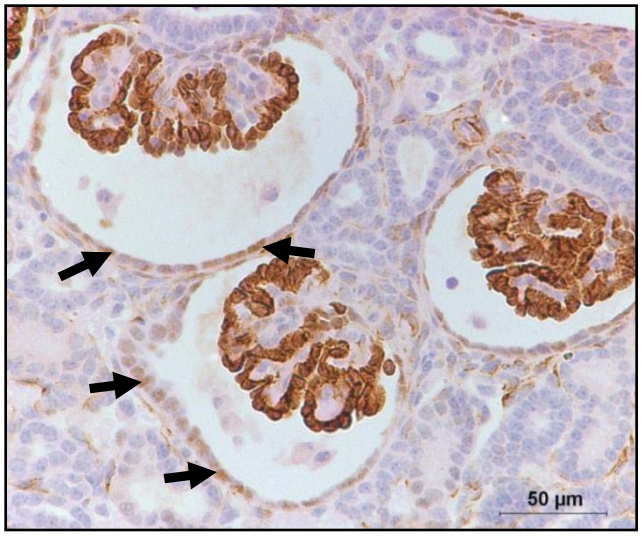

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