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Neonatal Dexamethasone Treatment in the Rat Leads to Kidney Damage in Adulthood

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ABSTRACT: Recently, concern has been raised that corticosteroid treatment of preterm neonates might be associated with adverse effects later in life, including early development of hypertension. Here, we investigate the impact of neonatal dexamethasone (Dex) treatment on early renal cell proliferation and nephron number. We analyzed mitotic activity in renal cortex of rat pups neonatally treated with Dex. Nephron number was measured and possible renal damage was quantified by counting inflammatory foci, ED-1 positive cells (macrophages), and the desmin score (activated podocytes). Mitotic activity was 34 and 29% lower on d 2 and 4 in Dex-treated rats compared with saline-treated controls. The number of glomeruli was lower at 4 wk, but nephron size was unchanged after Dex treatment, as calculated from glomerular density and (lower) body- and kidney weight. At wk 50, the glomerular number was significantly lower in Dex-treated rats, whereas body and kidney weight were the same as in Sal controls. Dex rats also showed more kidney damage, manifested by a ~3.5-fold increase in inflammation foci/mm^2 and in ED-1 positive cells/mm^2 and a ~4.3-fold increased desmin score. Temporary suppression of mitotic activity during neonatal Dex treatment leads to reduction of nephron number and more kidney damage later in life. **(*Pediatr Res* 67: 72–76, 2010)**

Preterm infants suffering from severe respiratory distress syndrome have a high risk of developing chronic lung disease (CLD) due to a proinflammatory reaction occurring in immature lungs (1,2). Glucocorticoids, predominantly dexamethasone (Dex), are widely used to prevent or reduce this complication because of their anti-inflammatory properties (3,4). However, recent animal and human studies showed that neonatal Dex treatment is accompanied with several adverse effects, short term as well as long term, and concern arose with respect to the development of important organ systems and neurodevelopmental outcome (3,5–11). Furthermore, neonatal Dex treatment in rats was associated with a poor survival (12).

Recently, our group reported on the effects of neonatal Dex treatment on the development of the heart. It was shown that in rats a 3-d course of Dex significantly suppressed proliferative activity of cardiomyocytes in the first week of life. At 50 wk of age this resulted in a lower number of cardiomyocytes in the heart, cardiomyocyte hypertrophy and increased interstitial fibrosis (13–15).

However, not much is known about the effects of Dex on the kidney. In a survival study, Kamphuis *et al.*(12) found that neonatally Dex-treated rats showed more signs of kidney damage, like glomerulosclerosis and cysts, in global histopathological examination. However, in this study, no quantitative measurements of renal damage were done.

Most other studies concerning effects of glucocorticoids on the kidney focused only on the prenatal treatment of Dex. One study (16) indicated that neonatal birth weight was significantly lower in both male and female rats after prenatal Dex treatment, whereas kidney weight at 70 d was significantly lower in their Dex-treated rats. The nephron number in the Dex-treated rats was significantly lower compared with the untreated control rats (16). Ortiz *et al.*(17) found that maternal Dex treatment at d 15 and 16 of rat pregnancy leads to a 30% reduction of nephrons in the offspring compared with the untreated controls. These studies indicate that prenatal Dex may have potentially negative effects like a reduction in nephron number. However, to the best of our knowledge, studies concerning the effects on the kidney of neonatal Dex are scarce (18).

Therefore, the aim of this study was to investigate short- and long-term effects of neonatal Dex treatment on the development of the kidney and to look for possible Dex-induced renal damage. First, we aimed to investigate the proliferative activity in renal cells in rat pups, in analogy with our earlier studies that observed suppression of proliferative activity in the heart (14). Second, we investigated whether changes in proliferative activity may be associated with changes in glomerular density later in life. Finally, we investigated whether Dex treatment results in kidney damage at middle age (50-wk-old rats).

MATERIALS AND METHODS

Animals. The Animal Research Committee of the University of Leiden approved the study protocol. The investigation conforms to the Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publication No.85–23, revised 1996). Pregnant Wistar rats (270–300 × g) were housed in groups and kept under conventional housing conditions and had free access to food and water. An artificial 12-h light/12-h dark cycle was

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Abbreviations: CLD, chronic lung disease; **Dex,** dexamethasone; **Sal,** saline

Table 1. *Anatomical parameters of 2-, 4-, and 7-d-old rats*

Age	Day 2		Day 4		Day 7	
Treatment (*n*)	Sal (8)	Dex (8)	Sal (9)	Dex (8)	Sal (7)	Dex (6)
Body weight (g)*	7.60 ± 0.37	6.95 ± 0.49 ($p = 0.012$)	9.73 ± 0.38	6.43 ± 0.71 ($p = 0.002$)	13.78 ± 1.08	10.66 ± 1.58 ($p = 0.016$)
Kidney weight (mg)	53.75 ± 6.94	52.50 ± 3.78 (ns)	65.56 ± 6.82	55.63 ± 6.78 ($p = 0.021$)	94.29 ± 9.32	80.00 ± 10.49 ($p = 0.022$)

Data are presented as mean ± SD (p Dex *vs* Sal).
* Published by de Vries *et al*., (14).
ns, not significant.

Table 2. *Anatomical parameters of 4-wk-old and 50-wk-old rats*

	Week 4		Week 50	
Treatment (*n*)	Sal (8)	Dex (8)	Sal (7)	Dex (6)
Body weight (g)	85.87 ± 8.48	72.22 ± 3.21 (−16%, $p = 0.0006$)	521.29 ± 37.90	522.67 ± 31.44 (ns)
Kidney weight (g)	0.46 ± 0.04	0.35 ± 0.03 (−24%, $p = 0.0002$)	1.68 ± 0.25	1.61 ± 0.29 (−4%, ns)
Number of glomeruli/kidney section	177 ± 16.37	145.88 ± 10.41 (−18%, $p = 0.0003$)	205.71 ± 19.41	165 ± 23.19 (−20%, $p = 0.022$)
Cortex area (mm^2)	19.71 ± 2.36	16.35 ± 1.77 (−17%, $p = 0.0104$)	52.52 ± 4.40	47.65 ± 7.78 (−9%, ns)
Number of glomeruli/mm^2	9.08 ± 1.24	9.01 ± 1.10 (−1%, ns.)	3.92 ± 0.31	3.45 ± 0.35 (−12%, $p = 0.022$)

Data are presented as mean ± SD (p Dex *vs* Sal).
ns, not significant.

used. The pups were born on d 21–22 of gestation. On the day of birth, male pups from the same litters were randomly assigned to the treatment group and control group.

Experimental design. Rat pups were injected i.p. with Dex (Dexamethasone Sodium Phosphate; BUFA, Uitgeest, The Netherlands) on d 1 (0.5 mg/kg body weight), d 2 (0.3 mg/kg body weight), and d 3 (0.1 mg/kg body weight) after birth. This treatment was based on a 21-d tapering treatment given to preterm infants to prevent or reduce CLD (19). Rat pups treated with equal volumes (10 μL/g body weight) of sterile pyrogen-free saline (Sal) at d 1, 2, and 3 served as control group. The rats (total $n = 75$) were killed at d 2 [$n =$ (Sal *versus* Dex) 8 *versus* 8], 4 ($n = 9$ *versus* 8), and 7 ($n = 7$ *versus* 6) after birth, and at 4 ($n = 8$ *versus* 8) and 50 ($n = 7$ *versus* 6) weeks of age. Rats were killed by decapitation. Body weight was measured before sacrifice. The kidneys were harvested and weighted and immediately immersion fixed in buffered formalin 4%. The kidneys were further dissected after 24 h of fixation.

Histopathological assessment of renal damage. After formalin fixation, the kidneys were cut transversally in two equal parts and embedded in paraffin. The paraffin-embedded kidneys were then cut into 2 μm sections for Periodic Acid Schiff (PAS) and immunohistochemical staining.

Kidney sections of 50-wk-old rats were used for renal damage measurements using ED-1 staining (macrophages) and desmin staining (activated podocytes).

After deparaffinization and blocking of endogen peroxidase activity, sections were heated in citrate buffer in an autoclave sterilizer for antigen retrieval (10 mMol and pH 6.0). Incubation with ED-1 antibody for 1 h (monoclonal α-mouse antibody, kindly provided by Ed Döpp, Department of Cell Biology, Free University, Amsterdam, the Netherlands, diluted 1:2000) was followed by incubation with Rabbit Anti Mouse Peroxidase (polyclonal RAMPO, DAKO, Denmark, 1:100) in 10% normal rat serum (IMGEN USA) for 30 min, and then with Swine Anti Rabbit Peroxidase (polyclonal SWARPO, DAKO, Denmark, 1:100) in 10% normal rat serum (IMGEN, USA) for 30 min. Nova Red (Vector NovaRED, Vector Laboratories, CA) was used for color development and Mayer's Hematoxilin solution (Merck, Germany) was used for counter staining of nuclei.

After deparaffinization and blocking of endogen peroxidase activity, sections were heated in citrate buffer (10 mMol and pH 6.0) and boiled for 20 min for antigen retrieval. Anti-desmin antibody (monoclonal α-mouse antibody, Biogenex, San Raman, CA; 1:100) was applied for 1 h and was followed by incubation with RAMPO 100) in 10% normal rat serum (IMGEN USA) for 30 min, and then with polymeric horseradish peroxidase-linker antibody (PowerVision, Immunologic, Klinipath, The Netherlands, ready to use) for 30 min Nova Red (Vector NovaRED, Vector Laboratories, CA) was used for color development and Mayer's Heamatoxilin solution was used for counterstaining (Merck, Germany).

Measurements. All the measurements mentioned later were performed in a blinded fashion as to treatment group.

Proliferative activity was measured in kidney sections of rats of 2, 4, and 7 d of age by the method described by De Vries *et al.*(14). Briefly, the number of mitotic spindles per mm^2 ("mitotic index") was counted in 15 consecutive high-power fields of the subcapsular area (nephrogenic zone) of the kidney at 400× magnification (total area = 0.23 mm^2).

Glomerular density was determined using a modification of the method described by Tsuboi *et al.*(20) to approach nephron number. Glomerular density (number of glomeruli/mm^2 cortex area) was determined in PAS stained kidney sections of 4- and 50-wk-old rats and determined by counting the number of glomeruli in one central transverse cross-section of the kidney at low-power magnification (20×). The number of glomeruli was divided by the total cortex area of the same section as measured using image analysis software (ImageJ, Cell Imaging Facility Toronto, Toronto).

Renal damage in 50-wk-old rats was assessed by ED-1 staining to determine the number of inflammatory foci and by Desmin staining to determine podocyte stress.

An inflammatory focus was defined as an aggregate of inflammatory cells containing at least 10 lymphocytes and at least one ED-1 positive cell. Inflammatory foci were counted in 20 high-power fields (200× magnification, total area of 1.84 mm^2) of the cortex in a single cross-section of the kidney and expressed as number of inflammatory foci per mm^2 cortex.

The number of ED-1 positive macrophages is associated with the severity of kidney damage (21). In brief, ED-1 positive cells were counted in 20 high power fields (200×, total area of 1.84 mm^2) of renal cortex in one cross-section and expressed as number of ED-1 positive cells per mm^2 cortex area.

In the rat, renal damage can also manifest as desmin expression in stressed podocytes (21). In brief, 50 glomeruli per kidney were analyzed at a magnification of 400×. Per glomerulus, a desmin score of 0 reflects staining of 0–5% of the glomerular cross sectional surface area; one reflects 5–25% staining; two reflects 25–50% staining; three reflects 50–75% staining; and four reflects >75% staining. The podocyte stress score was defined as the mean desmin score of 50 glomeruli.

Statistical analysis. All data are expressed as mean ± SD. Differences between the groups are determined with the Mann-Whitney *U* test using SPSS *versus* 15.0.1. A $p < 0.05$ was considered statistically significant.

RESULTS

Anatomical parameters. At d 2, bodyweight was lower in the Dex-treated rat pups but kidney weight was not different between the Dex and Sal group. At d 4, the body weight and kidney weight were 34 and 29% lower in Dex compared with Sal-treated rat pups. At d 7, this difference was 22 and 11%, respectively. Table 1 summarizes these results.

At 4 wk of age, body weight and kidney weight was 16 and 24% lower in Dex compared with Sal rats ($p < 0.001$ and $p < 0.001$, respectively). At 50 wk of age, the body and kidney weight in the two groups were not different anymore (Table 2).

Proliferative activity. Proliferative activity was determined by counting the number of mitotic spindles per mm^2 subcapsular cortical area. Figure 1 shows representative microscopic pictures of the subcortical area of kidneys from 2-, 4-, and 7-d-old rats. There were less mitotic figures in the Dex-treated compared with Sal-treated pups: 9.1 ± 2.4 *versus* 16.4 ± 4.0 ($p < 0.001$) on d 2 and 14.8 ± 4.3 *versus* 20.9 ± 8.5 ($p < 0.05$) on d 4 (mean ± SD. Dex *versus* Sal). On d 7, a similar trend was observed, but the difference was no longer significant ($p = 0.7$) (Fig. 2).

Kidney size, glomerular density, and estimated nephron number in 4-wk-old and 50-wk-old rats. In 4-wk-old rats, there were less glomeruli per transversal kidney section in the Dex compared with the Sal group (18% decrease, $p < 0.001$). The cortical surface area was smaller in 4-wk-old Dex rats (18% decrease, $p < 0.01$), but the number of glomeruli per mm^2 cut surface cortex area was not significantly different. Fifty-week-old Dex rats, had 20% less glomeruli per transversal kidney section ($p < 0.05$) than Sal rats, but differences in cortical surface area (−9%) or kidney weight (−4%) were not significant. Glomerular number per cortical surface area was 12% lower in Dex rats compared with Sal rats ($p < 0.05$). Table 2 summarizes these results.

Renal damage in 4-wk-old and 50-wk-old rats. Renal damage parameters were evaluated in 50-wk-old rats (illustrated in Figs. 3 and 4). At this age, the Dex-treated rats showed evidence of extensive kidney damage with foci of inflammatory cells, associated with sclerotic glomeruli, together with dilated tubuli with protein casts. In Sal rats, such changes were seen only sporadically. Dex rats had about 3.5 times more focal inflammatory infiltrates ($p < 0.005$), 3.3 times more ED-1 positive macrophages ($p < 0.05$), and a 4.3-fold higher glomerular podocyte stress score ($p < 0.05$).

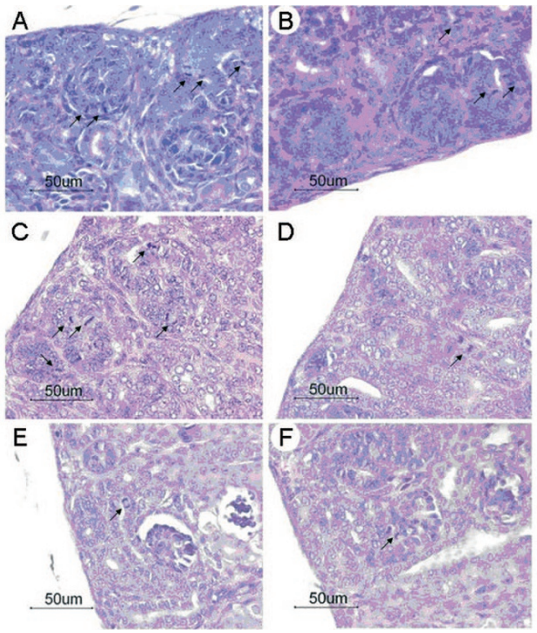

Figure 1. Histology of 2-, 4-, and 7-d-old rats neonatally treated with Sal and Dex. PAS staining of the subcapsular nephrogenic zone. *A*, 2-d-old Sal (control) rats; *B*, 2-d-old Dex rats; *C*, 4-d-old Sal rats; *D*, 4-d-old Dex rats; *E*, 7-d-old Sal rats; and *F*, 7-d-old Dex rats. Arrows indicate mitotic figures.

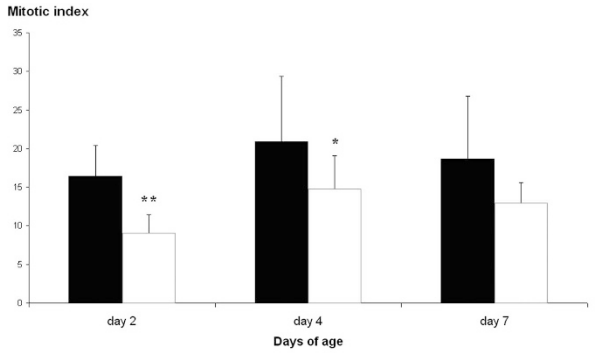

Figure 2. Proliferation of renal cells (mitotic index) of Sal- and Dex-treated neonatal rats. The mitotic index shows a significant decrease in proliferative activity of renal cells on d 2 and 4 in Dex-treated rats (*white bars*) compared with the Sal (control) group (*black bars*). On d 7, no difference in mitotic index is found between the two groups. Data are presented as mean ± SD $**p < 0.001$; $*p < 0.05$ Dex *vs* Sal. Mitotic index = number of mitotic spindles in a subcordial area per mm^2 (see Materials and Methods).

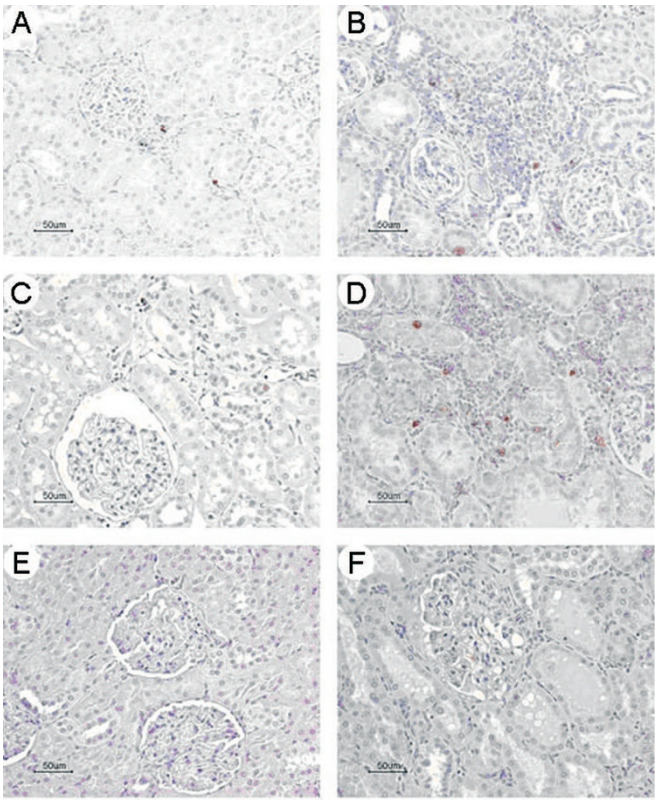

Figure 3. Histology of 50-wk-old rats neonatally treated with Sal or Dex. First row, in the ED-1 staining, the number of inflammatory foci was determined. In Sal (control) (*A*) one small inflammatory foci is seen. In Dex-treated rats (*B*), more and much larger foci are seen with more ED-1 positive cells. Second row, dex-treated rats (*D*) show much more ED-1 positive cells compared with the Sal-treated rats (*C*). Third row, in Sal- treated rats (*E*), no desmin positive podocytes are seen (score 0). In Dex-treated rats (*F*), some desmin positive podocytes can be found (score 3).

DISCUSSION

In this study, we aimed to investigate the short-term and long-term effects on the kidney of neonatal Dex treatment in rats, as a model for neonatal Dex treatment in preterm infants for prevention or reduction of CLD. The major findings were

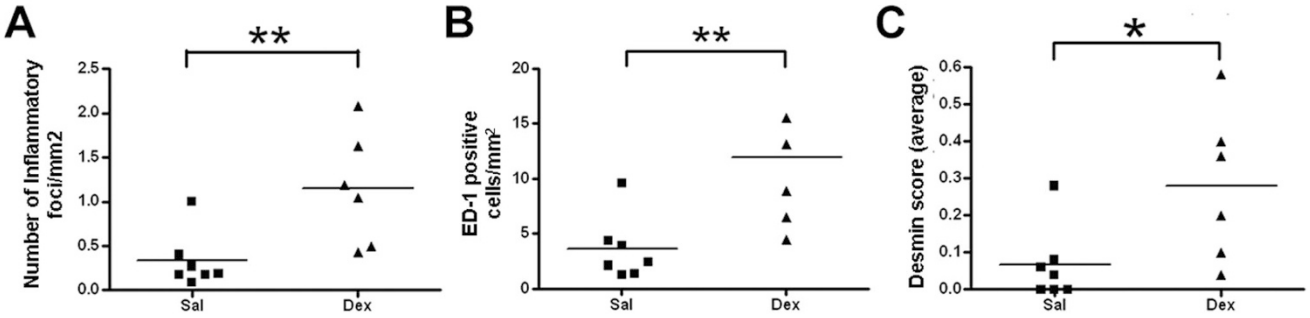

Figure 4. Renal damage scores of 50-wk-old rats treated with Sal or Dex. Assessment of renal damage in 50-wk-old rats shows a significant difference seen between Sal- and Dex-treated rats in the number of (*A*) inflammatory foci/mm^2 ($p = 0.0047$), (*B*) ED-1 positive cells/mm^2 ($p = 0.0082$), and in (*C*) desmin score ($p = 0.0221$). Data are presented as mean (*p* Dex *vs* Sal).

that neonatal Dex treatment on d 1, 2, and 3 in rats causes a temporary suppression of proliferative activity of renal cells, which was associated with lower kidney weight and nephron number and extensive renal damage in adult life and during aging. These findings are in agreement with previous observations in the heart. Also in the myocardium, a suppression of proliferative activity was found on d 2 and 4, and there was no catch-up growth at later days (14). In addition, total body weight was lower in Dex-treated rats, which suggests that neonatal Dex treatment had a negative effect on organ growth and development. This was similar to findings in earlier studies (13–15). The differences in weight may be partially due to nutritional problems in the days after the neonatal treatment. Although we suggest that the effects on the kidney were induced by a direct effect of Dex treatment, this study cannot definitely exclude the possibility that the Dex-induced growth restriction in itself, during this very active phase of nephrogenesis, may have indirectly influenced kidney weight and nephron formation (22,23).

In the human kidney, glomerulogenesis is completed around wk 34–36 of gestation, well before a term delivery (wk 37–40), and postnatal development is limited to maturation and growth (24). In rats, however, glomerulogenesis continues beyond the first week after birth. Therefore, newborn rats may constitute a model for early postnatal life of preterm infants, which implies that our findings are indicative of potential negative side effects of current Dex treatment. The mechanism behind suppression of renal cell proliferation by Dex is incompletely understood but probably involves repression of NF-kB and AP-1 (25). At 4 wk of age, Dex-treated rats still had lower body weight than controls, and the number of glomeruli and kidney weight were reduced proportionally. Thus, there was no compensatory nephron hypertrophy. However, in older rats (50 wk), bodyweight of Dex rats and controls was similar, as was kidney weight, whereas Dex rats had a lower cortical glomerular density, consistent with nephron hypertrophy, and showed a trend toward lower total cortical surface area. At this age, also renal damage parameters were significantly higher in Dex rats, consistent with observations in a previous report by Liu *et al.*(18).

There are several possible causes of renal damage. The reduced number of nephrons might have suffered from hyperfiltration and increased sodium reabsorption. Also, previous studies have demonstrated that antenatal glucocorticoid exposure induces dilatation of both afferent and efferent arterioles, with concomitant increase of GFR and proximal sodium reabsorption, in association with increased activity and expression of Na+K+ATPase (24,26). Stubbe *et al.*(27) reported that also postnatal Dex administration can lead to increased sodium reabsorption, and a permanent increase of Na+K + 2Cl cotransporter, and of Na+K+ATPase α1. Heart failure has a major negative impact on the kidney, and renal failure is a major progression factor of cardiovascular disease. This mechanism of mutual aggravation of pathologies might be involved also in the severity of organ damage observed in adult rats in this and previous studies of perinatal Dex treatment. However, the early preclinical changes that we have observed in both the heart and the kidney indicate a primary treatment-related pathologic effect of neonatal Dex treatment in both organs.

In conclusion, Dex treatment of neonatal rats leads to lower nephron numbers and extensive renal damage in aging rats. This is a warning that current practice of Dex treatment in prematurely born infants might increase the risk of developing renal damage in later life, as has been suggested previously for the impact of this therapy on the developing heart.

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