

The Effects of Early Postnatal Diuretics Treatment on Kidney Development and Long-Term Kidney Function in Wistar Rats

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Key Words

Renal development · Pediatrics · Diuretics · Furosemide · Kidney

Abstract

Background: Diuretics are administered to neonates to control fluid balance. We studied whether clinical doses affected kidney development and function and whether extrauterine growth retardation (EUGR) could be a modulator. **Methods:** Wistar rats were cross-fostered in normal food or food restricted litters at postnatal day (PND) 2 and treated daily with 0.9% NaCl, 5 mg/kg furosemide or 5 mg/kg hydrochlorothiazide (HCTZ) up to PND 8. Kidneys were evaluated on proliferation, apoptosis and a set of mRNA target genes at PND 8, glomerular- and glomerular generation count at PND 35, clinical pathology parameters at 3- and 9 months, neutrophil gelatinase-associated lipocalin at PND 8, 3 and 6 months, monthly blood pressure from 3 months onward and histopathology at study end. **Results:** Treatment with furosemide or HCTZ did not have relevant effects on measured parameters. EUGR resulted in lower body weight from day 3 onwards (–29% at weaning; $p < 0.001$, –10% at necropsy; $p < 0.001$), less glomerular generations (4.4 ± 0.32 vs. 5.0 ± 0.423 ; $p = 0.025$, males only), decreased glomerular numbers ($27,861 \pm 3,468$ vs. $30,527 \pm 4,096$; $p = 0.026$), higher creatinine clearance (0.84 ± 0.1 vs. 0.77 ± 0.09 ml/min/kg; $p = 0.047$) at

3 months and lower plasma creatinine (25.7 ± 1.8 vs. 27.5 ± 2.8 $\mu\text{mol/l}$; $p = 0.043$) at 9 months. **Conclusion:** Furosemide and HCTZ did not influence kidney development or function when administered in a clinically relevant dose to rat pups at a stage of ongoing nephrogenesis. EUGR led to impaired kidney development but did not modify furosemide or HCTZ findings.

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Introduction

From a historical perspective, neonatal medicine has been mainly reliant on the translation of scientific data from the adult population. This perspective is changing because more evidence shows that neonates are different in ways of drug handling [1]. The neonate population also has another important aspect regarding the practice of medicine, which is the interaction of treatments with the still ongoing development of many organs. This is especially important if preterm birth happens. It has been hypothesized that low birth weight or early life damage may have an impact on the kidney and its functions in later life [2, 3]. It has already been shown that environmental influences during nephrogenesis, including growth restriction and medications, have a significant impact on final nephron numbers [4]. Nephrons are formed from the 5th

gestational week onwards and formation ends around the 34th–36th week of gestation in humans [5]. At that moment, a little under 1 million nephrons have been formed per kidney, but there is a very large interindividual range (i.e. 10-fold) [6]. In rodents, the timing of nephrogenesis is different and is completed approximately 7 days after birth [7].

Diuretics are the most common drugs administered to control fluid balance in neonates, especially in children with chronic lung disease after very premature birth [8]. But there is a risk of kidney toxicity. Although acute kidney toxicity is not expected or seen, higher dose levels of furosemide are associated with renal calcification in children [9]. Regarding kidney development, the described effects are more controversial. In the past, effects of furosemide on kidney development have been studied in vivo. Mallie et al. [10, 11] showed that prenatal furosemide exposure in rats tended to lower the number of differentiated glomeruli at birth and disturbed electrolyte handling. This effect was still present after a 12-day follow-up. In contrast, a recent study by our group did not identify effects on early kidney development after exposing metanephroi in vitro to more clinically relevant dose levels [12].

Our goal was to determine if a clinical neonatal dose of furosemide or hydrochlorothiazide (HCTZ) impaired kidney development and function on a short and/or long-term. HCTZ was selected as a second drug because it has not been associated with nephrotoxicity. While the nephrotoxic potential of both furosemide and HCTZ itself is low, they are often co-administered with other drugs and may thereby play a role in multi-hit toxicity. Additionally, we hypothesized that the potential effects of these drugs on kidney development may also be modulated by additional stressors such as extrauterine growth retardation (EUGR).

In this study, we investigated these hypotheses by inducing postnatal food restriction by means of litter enlargement to model EUGR in the rat. Either furosemide or HCTZ were administered for 7 days to investigate interactions on development, and vehicle and control treatments were included. Early effects on proliferation/apoptosis, a set of mRNA target genes, glomerular- and glomerular generation count on the kidney were evaluated. Furthermore, clinical pathology parameters, neutrophil gelatinase-associated lipocalin (NGAL), blood pressure and renal histopathology were evaluated in a long-term follow-up. Finally, body weight was measured to monitor overall animal health and determine the extent of EUGR.

Results

Body Weight

Body weights were evaluated in the study to follow-up on general animal health and to evaluate the effect of postnatal food restriction.

Body weights of male and females were different only incidentally (days 2, 3 and 35) and were therefore pooled for analysis. The mean body weight was 6.4 (SD 0.5 and 0.6) g for normal food (NF) and food restricted (FR), respectively for both NF and FR animals at cross-fostering on postnatal day (PND) 2.

From day 3 onwards (online suppl. fig. 1a; for all online suppl. material, see www.karger.com/doi/10.1159/000442674), the body weight of FR animals was lower when compared with that of NF animals: on day 3, NF mean was 6.9 (SD 0.7) g, FR mean was 6.7 (SD 0.6) g, $p < 0.001$; on the day of weaning, NF mean was 59.8 (SD 3.3) g and FR mean was 42.2 (SD 5.3) g, difference was 29%, $p < 0.001$. Relative body weight difference declined after weaning. However, an absolute body weight difference of 30–40 g was observed for the remainder of the study ($p < 0.001$; online suppl. fig. 1b). No drug treatment-related effects were detected on body weight, except for an incidental difference between furosemide and HCTZ on day 10 within NF animals ($p = 0.037$). At day 189, by chance, the heaviest and lightest animal of the sham-NF and the HCTZ-FR groups, respectively, were excluded from the routine body weight measurement due to urine sampling. This resulted in the observed abnormality in the body weight evolution graph.

Day 8 Investigations (Gene Expression, Proliferation/Apoptosis Ratio)

To determine the direct effects of treatment on kidney development, gene expression and proliferation/apoptosis and NGAL were examined on PND 8.

Gene expression analysis on kidney tissue, collected on day 8 (fig. 1), showed a 0.7-fold change of *Fgf7* in FR animals ($p = 0.009$) and 0.68-fold change in furosemide treated animals vs. sham ($p = 0.031$). *Casr* and *Gdnf* genes were downregulated in furosemide-treated animals with fold changes of 0.67 ($p = 0.04$) and 0.72 ($p = 0.02$), respectively and *Grfa1* was upregulated in FR animals compared with the NF animals (1.39-fold, $p = 0.002$). All gene expression analyses were performed on pooled samples of both genders, as no gender effect was noted. Gene expression of the loop diuretic sensitive *Nkcc2* and the thiazide sensitive *Ncc* transporters did not change.

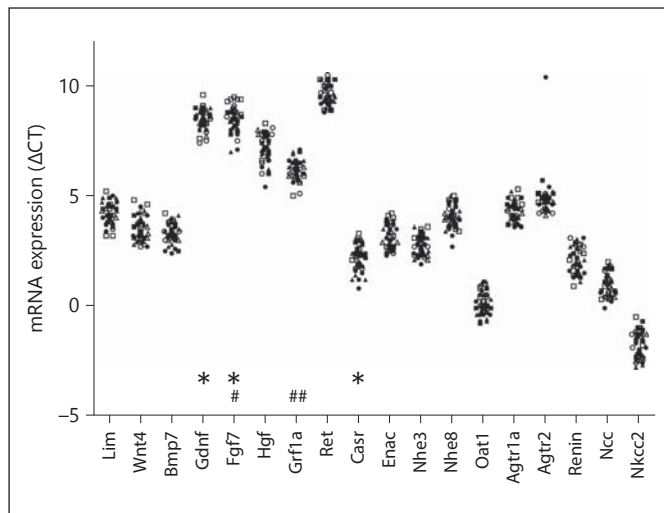


Fig. 1. Renal gene expression of relevant transporters, growth factors and receptors in kidney development measured on day 8. Δ Ct values from all individual animals are plotted for each target gene. Shown are sham (dot), furosemide (square) and HCTZ (triangle) treatment. NF and FR are distinguished by closed and open symbols ($n = 5$ – 8 per group). Statistical analysis was performed with Kruskal–Wallis 1-way analysis of variance. * $p < 0.05$ furosemide vs. sham; # $p < 0.05$, ## $p < 0.01$ FR vs. NF.

No difference was noted on the ratio between proliferating and apoptotic cells in kidney tissue on day 8 of the study (online suppl. fig. 2). The mean values for males and females were 47.1 (482 (SD 358) proliferating- and 13 (SD 8) apoptotic cells/mm²), and 29.8 (430 (SD 244) proliferating and 14 (SD 4) apoptotic cells/mm²), respectively ($f = 2.852$, $p = 0.10$). Similarly, no differences were noted between NF- and FR-treated groups (means 40.3 (516 (SD 378) proliferating and 15 (SD 6) apoptotic cells/mm²) vs. 38.0 (380 (SD 158) proliferating and 12 (SD 6) apoptotic cells/mm²), respectively; $f = 0.107$, $p = 0.75$). No influence of the drug treatment (mean ratio's: sham 44.0 (357 (SD 192) proliferating and 11 (SD 6) apoptotic cells/mm²), furosemide 45.8 (621 (SD 380) proliferating and 16 (SD 8) apoptotic cells/mm²) and HCTZ 28.5 (420 (SD 300) proliferating and 15 (SD 3) apoptotic cells/mm²); $f = 1.032$, $p = 0.37$) and no interactions between the different variables were detected.

Ngal (online suppl. table 1) was investigated in plasma as a marker for acute kidney damage. Plasma values were on average 0.168 (SD 0.031) and 0.149 (SD 0.028) μ g/ml in NF and FR, respectively, and did not differ significantly ($f = 3.880$, $p = 0.58$). Additionally, no changes were noted between the different drug treatments and the sham control.

Day 35 Investigations (Kidney Weight, Glomerular Number and Glomerular Generation Count)

On day 35, the impact of the treatments on kidney organogenesis was assessed. To investigate whether the kidney developed normally, kidney weight, glomerular number and glomerular generation count were determined.

The mean kidney weight (table 1) in FR animals was lower compared to the weight of NF animals and a difference between the sexes was detected (males 0.64 (SD 0.07) g, females 0.57 (SD 0.08) g; $f = 12.745$, $p = 0.001$). Corrected for body weight ($\times 100$), these were 0.72 (SD 0.07) and 0.73 (SD 0.09). No differences were noted for the treatments studied (sham 0.61 (SD 0.09) g, furosemide 0.61 (SD 0.07) g, HCTZ 0.58 (SD 0.1) g; $f = 0.274$, $p = 0.762$) and interactions between the variables sex, litter size and treatment were not present. When corrected for body weight, the kidney weight in FR animals was higher compared with that of the NF animals (0.77 vs. 0.69; $f = 15.511$, $p < 0.001$) and an interaction between treatment and litter size was detected ($f = 4.812$, $p = 0.015$). The higher kidney weight in the FR group could be attributed to the slightly higher kidney weights in the HCTZ-FR group.

Glomerular generation count (table 1) was performed and it yielded on average 4.6 (SD 0.66) generations in the sham-NF dosed animals. FR animals had on average less glomerular generations (4.4 (SD 0.39)) compared with the NF animals (4.7 (SD 0.41); $f = 5.666$, $p = 0.025$). This decrease was dependent on sex ($f = 4.260$, $p = 0.049$) and was detected in males, but not females (male NF 5.0 (SD 0.23); FR 4.4 (SD 0.32), female NF 4.5 (SD 0.41); FR 4.5 (SD 0.44)). Overall analysis did not show a difference between sexes (male 4.7 (SD 0.40), female 4.5 (SD 0.42); $f = 1.861$, $p = 0.18$) or drug treatments (sham 4.5 (SD 0.61), furosemide and HCTZ 4.6 (SD 0.31 and 0.27, respectively); $f = 0.553$, $p = 0.58$).

FR animals showed a decreased amount of glomeruli compared with NF animals (means 27,861 (SD 3,468) vs. 30,527 (SD 4,096); $f = 5.492$, $p = 0.026$) on day 35 (table 1). Treatment ($f = 0.626$, $p = 0.54$) or sex ($f = 1.938$, $p = 0.17$) did not influence the glomerular number. However, an interaction was detected between treatment, sex and litter size ($f = 3.922$, $p = 0.031$). In contrast to all other groups, average glomerular number was comparable between male sham-FR (29,394) and male sham-NF (29,454) animals.

Glomerular generation count did not correlate with the total number of glomeruli (Pearson's correlation 0.046) indicating that glomerular generation counts are not a suitable predictor for glomerular numbers.

Long-Term Follow-Up (Clinical Chemistry, NGAL, Blood Pressure and Histopathology)

Long-term endpoints were included in the study to investigate any delayed effects of the food restriction and drug treatments.

Clinical biochemistry analysis at 3 months of age (online suppl. table 2a) showed a higher creatinine clearance in FR animals compared with that of the NF animals (NF 0.77 (SD 0.09) ml/min/kg, FR 0.84 (SD 0.1) ml/min/kg; $f = 4.374$, $p = 0.047$). Additionally, an interaction was detected between treatment and nest type ($f = 6.443$, $p = 0.006$), indicating plasma magnesium concentration in the HCTZ-treated groups to be lower in NF animals (0.74 (0.03) mmol/l) compared with that in FR animals (0.81 (0.04) mmol/l). No other changes were detected.

At 9 months of age (online suppl. table 2b), plasma creatinine was lower in FR animals compared with the NF animals (NF 27.5 (SD 2.8) μ mol/l, FR 25.7 (SD 1.8) μ mol/l; $f = 4.551$, $p = 0.043$), but this did not result in differences in creatinine clearance. No other differences were noted.

NGAL levels (online suppl. table 1) in urine reduced by 25% between 3 and 6 months of age in the long-term follow-up period. This effect was similar within all the different treatment groups. No differences were noted between feeding regimes and/or drug treatment.

Blood pressure was measured monthly from 3 months of age onwards (fig. 2) and analyzed over the course of the study. Diastolic blood pressure (DBP) and mean blood pressure (MBP) declined over the course of the study within NF ($f = 3.728$, $p = 0.018$) and FR groups ($f = 3.161$, $p = 0.027$). No effects were noted on systolic blood pressure (SBP) or pulse pressure (PP).

No differences were detected between the treatment groups on the individual time points except for the measurement at the age of 9 months. DBP, SBP and MBP were all increased in NF animals and PP was slightly reduced compared with FR animals (DBP (NF 88.6 (SD 23.0) mm Hg, FR 69.1 (SD 11.4) mm Hg; $f = 7.688$, $p = 0.016$); SBP (NF 128.6 (SD 18.3) mm Hg, FR 114.9 (SD 10.7) mm Hg; $f = 4.647$, $p = 0.050$); MBP (NF 101.4 (SD 21.1) mm Hg, FR 84.8 (SD 11.4) mm Hg; $f = 5.770$, $p = 0.032$); PP (NF 40.3 (SD 6.2) mm Hg, FR 45.8 (SD 5.8) mm Hg; $f = 5.334$, $p = 0.038$)).

Kidney weight at the end of the experiment (table 1) was significantly lower in FR animals compared with NF animals (mean 2.80 (SD 0.27) g vs. 3.08 (SD 0.12) g; $f = 11.440$, $p = 0.002$). After correction for body weight, no difference could be noted. Neither drug-related effects nor abnormal findings were observed on tubular and glomerular morphology in the kidney after microscopic evaluation.

Table 1. Renal parameters

Treatment	Sham NF	Furosemide NF	Hydrochlorothiazide NF	Sham FR	Furosemide FR	Hydrochlorothiazide FR	Statistical significance
<i>Day 35</i>							
Body weight, g	94.0 (4.47)	93.5 (9.76)	96.7 (8.78)	72.9 (4.97)	78.3 (9.12)	67.5 (9.55)	NF vs. FR ($p < 0.001$) M vs. F ($p = 0.005$)
Right kidney weight, g	0.67 (0.05)	0.65 (0.05)	0.63 (0.12)	0.54 (0.06)	0.57 (0.08)	0.55 (0.07)	FR vs. NF ($p < 0.001$) M vs. F ($p = 0.001$)
Right kidney/body weight (ratio $\times 100$)	0.71 (0.04)	0.70 (0.05)	0.65 (0.08)	0.74 (0.06)	0.73 (0.09)	0.81 (0.05)	FR vs. NF ($p < 0.001$) Attributed to HCTZ ($p = 0.015$)
Glomerular generations (count)	4.6 (0.66)	4.8 (0.20)	4.7 (0.28)	4.3 (0.59)	4.4 (0.23)	4.6 (0.26)	FR vs. NF ($p = 0.025$) only in males
Glomerular number	29,454 (4,022)	30,640 (4,272)	31,488 (4,366)	29,648 (4,446)	26,394 (3,433)	27,540 (1,961)	FR vs. NF ($p = 0.026$)
<i>End of experiment</i>							
Body weight, g	483 (27.4)	496 (8.7)	498 (23.9)	435 (12.7)	455 (21.7)	456 (20.4)	FR vs. NF ($p < 0.001$)
Right kidney weight, g	2.98 (0.13)	3.13 (0.07)	3.14 (0.12)	2.75 (0.10)	2.96 (0.12)	2.7 (0.43)	FR vs. NF ($p = 0.002$)
Right kidney/body weight (ratio $\times 100$)	0.62 (0.02)	0.63 (0.01)	0.63 (0.02)	0.63 (0.03)	0.65 (0.02)	0.59 (0.10)	
Data are presented as mean (SD). PND 35: n = 5–8/group; end of experiment n = 4–6/group.							

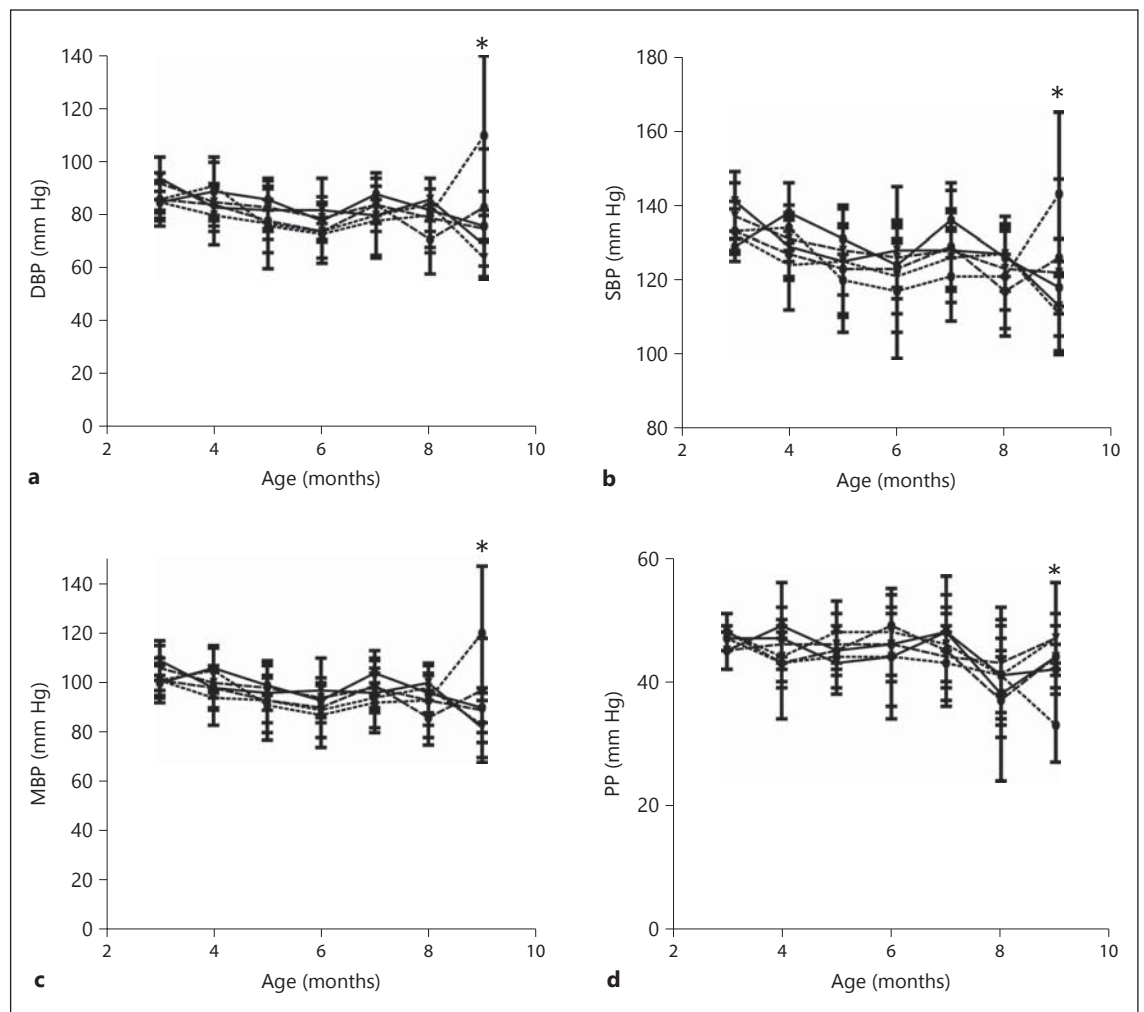


Fig. 2. Blood pressure measurements in rats over the study period showing DBP (**a**), SBP (**b**), MBP (**c**) and PP (**d**). Means \pm SD of NF animals are plotted with dots and FR with triangles. Sham-dosed groups are indicated with a solid line, furosemide groups with a striped line and HCTZ groups with a dotted line. $n = 4\text{--}6/\text{group}$, with exception of 9-month time point where $n = 2\text{--}4/\text{group}$. Uni-

variate analysis with Tukey post-hoc test was performed for all individual time points. * NF vs. FR ($p \leq 0.05$). A multivariate analysis for repeated measurements was performed to investigate the effect of study time and showed a decrease of diastolic and MBP within nest type over course of the study period ($p = 0.018$).

Discussion

The goal of this study was to determine whether the clinical neonatal dose of furosemide and HCTZ affected kidney development and function on a short- and/or long-term basis.

Overall, we concluded that these drugs do not impact kidney development. Furosemide was able to induce slight changes in *Fgf7*, *Casr* and *Gdnf* gene expression levels. However, these changes were less than twofold and therefore, the biological relevance of these changes with respect to a disturbed organ development is questionable.

Indeed, no alterations in any of the structural renal parameters were noted (table 1). In addition, no abnormalities in any of the renal functions tested were observed. A renal concentration effect, as was noted after prenatal dosing of furosemide [10], could not be found within our experimental conditions. It may therefore be concluded that the renal phenotype after prenatal and postnatal furosemide exposure is different. However, more plausibly we did not find this effect because we administered more clinically relevant dose levels that are significantly lower compared with the dose levels used in that study (i.e. 75 mg/kg).

Similar to furosemide, treatment with HCTZ did not change any parameters of kidney development. No alteration in the *Ncc* expression level was noted, which was contrary to our expectations because HCTZ blocks this thiazide-sensitive Na^+Cl^- cotransporter and it has been shown to affect gene expression [13]. The lack of effect could be due to either the slightly lower dose levels used in this study and the different route of administration or the time between the last dose administration and sampling. Similarly, we did not see any response on *Nkcc2* after furosemide treatment.

Our second hypothesis stated that a multi-hit on the kidney may modulate developmental pathways and that EUGR would modify drug toxicity. Based on our results, such a modulating effect of the combination of growth restriction with diuretic treatment could not be demonstrated. The interactions noted between treatment and nest type were limited to HCTZ and FR leading to a slightly higher kidney weight at day 35. No interactions were detected between furosemide and EUGR.

Our model was successful in obtaining growth retardation evidenced by the changes in body weight observed throughout the study. Additionally, we confirmed that EUGR has an impact on glomerular numbers [12], which were decreased by 11% after food restriction. Of note was the absence of any difference between the male sham-FR and sham-NF animals. This may indicate that the decrease noted in the FR group was more drug-driven, but this was not confirmed in our analysis. In other studies, the effect between NF and FR was more pronounced, thereby substantiating the presence of a higher average total count of glomerular numbers, which was relatively low in this study [14] (Bueters et al. unpublished). Therefore, we believe that the observation of low glomerular numbers in the sham-NF group was probably related to chance. However, a more drug-driven effect could be explained as well. Although the changes that were observed were small and we question the biological relevance, furosemide did downregulate the gene expression of *Gdnf* and *Fgf7*, both of which are considered important for ureteric bud development and nephron formation [15–17]. This observation, in combination with the added stressor of food restriction, could lead to a state where nephrogenesis is disturbed. Such a link is less clear for HCTZ. The main effect on the kidney described for HCTZ is activation of the renin angiotensin system (RAS), but no changes in our mRNA targets of the RAS after either food restriction or HCTZ treatment were seen. Woods et al. [18] showed that maternal protein restriction in rats resulted in decreased glomerular num-

bers but also in a suppression of the RAS. Other studies have been performed, investigating the relations between reduced nephron endowment and the RAS in sheep, but what is not clear at this stage is whether there is a real role for the RAS system or not [19, 20]. At the moment, the relation between the RAS and nephron endowment is not clear.

The changes in glomerular number did not correlate with the number of glomerular generations, which were slightly reduced in male FR vs. NF animals. Again changes in mRNA expression of 2 targets, *Fgf7* and *Grfa1* with a role in kidney development were detected [15, 16, 21]. The observed changes were very small and the impact on organogenesis was questionable. However, it may be that the effects of small reductions in several pathways cumulate into a significant reduction in the final common pathway, i.e. nephron formation.

Brenner et al. [22] described in the hyperfiltration hypothesis that glomerular damage leads to hyperfiltration, and that the first sign of kidney damage is mostly noted by an increase in creatinine clearance caused by hyperfiltration instead of the classical creatinine clearance decrease, which is increasingly evident in more serious kidney damage [23]. In this study, this was evidenced by a minimally higher creatinine clearance in FR at 3 months and the lower creatinine plasma levels at 9 months. Although these findings are in line with our hypothesis, the observed changes are very small and without any confirmed histopathological kidney abnormalities. At the end of the experiment, it is difficult to determine whether these were related to treatment.

In the same hypothesis, Brenner et al. [22] described that hyperfiltration would lead eventually to a systemic increase in blood pressure. Indeed, it has been described that intrauterine growth retardation leads to a reduced glomerular number [24] and an increase in blood pressure [25]. However, there are also studies performed in which this phenomenon was not observed [26]. We could not detect any differences in blood pressure variables between our groups. This might be because the effects noted in the study of Schreuder et al. [25] were quite small in absolute values and a larger variation was noted with our experimental set-up. Although we detected an increase in the MBP of the NF groups at the 9-month time point, we believe this may be caused by a bias in the measurements because we were not able to record signals of approximately half of the animals on that time point. This was probably related to the age of the animals, which resulted in a suboptimal tail blood flow.

There are some limitations to the experimental study design. Although this experiment tried to simulate a multi-hit of both drug use and EUGR, these can actually only approach the real situation in the neonatal care units. For example, there is no full control on the nutrients that every rat FR pup received. Although the mothers are well fed with a balanced diet, it is unknown whether the reduced food intake of the pups might have led to an imbalance of nutrient intake and whether this is similar to the clinical practice. Additionally, our rat pups were healthy pups in contrary to the patients, which are likely to be suffering from chronic lung disease and are therefore fluid restricted. This physiological condition could potentially modify the adverse effects on nephrogenesis as well and should be investigated separately in fluid restriction models. Because of the multi-factorial situation in the clinic, our results are most likely an underestimation of the true effect.

Another limitation was the use of a noninvasive blood pressure technique (tail cuff) instead of a golden standard technique such as telemetry. Another option to measure blood pressure is the transducer method, which is considered a terminal experiment. For a long-term follow-up study, this would have resulted in adding many animals to the study. All methods have their advantages and disadvantages. Although telemetry has a very sensitive read-out, it does require the rats to undergo surgery and requires an expensive set-up (Kurtz et al. [27]). Due to extensive drop-out in a previous telemetry study, we decided to use the tail cuff.

In conclusion, furosemide and HCTZ did not influence the development or function of the kidneys when administered in a clinically relevant dose to young rat pups at a stage of ongoing nephrogenesis. Growth retardation based on the increase of litter size led to a reduction in glomerular numbers but had no modifying effect on furosemide or HCTZ treatment. In the future, experiments should be performed in which the neonatal environment is even more precisely simulated, preferably in a disease model to investigate other potential synergies. In regard of neonatal safety, care should be taken to avoid growth retardation as best as possible.

Material and Methods

Animal Experiments

All experiments were approved by the Animal Ethics Committee at the Radboud University Nijmegen.

Timed-pregnant Wistar WU rats (Charles River laboratories, Sulzfeld, Germany) were delivered at the central animal labora-

tory Nijmegen approximately 1 week before delivery. Dams were housed individually and checked for litters at least twice daily. The day a full litter was found was labeled as PND 1. Pups were cross-fostered on PND 2 to generate control (NF, 12 pups per litter) and FR (20 pups per litter) animals. Additional NF and FR litters were formed in order to replace potential losses in the study litters. Rats had ad libitum access to water and food (Ssniff R/M-H diet, Soest, Germany) with a sodium, potassium and chloride content of 0.24, 0.91 and 1.00%, respectively. Animals received a daily intraperitoneal dose of 0.9% NaCl (sham), 5 mg/kg furosemide (Lasix, Aventis Pharma BV, Gouda, Netherlands) or 5 mg/kg HCTZ (H2910) (Sigma-Aldrich, The Netherlands) for 7 days starting on PND 2. In total, 6 treatment groups per gender were formed (sham-NF and FR, furosemide-NF and FR, HCTZ-NF and FR).

Body weight was measured daily up to weaning (day 28) and weekly thereafter till 9 months of age. After day 35, only males were kept in the study as they are generally more prone to develop hypertension [28]. Some mortality was noted in the first weeks of the study (5 animals: 2 male NF (sham and furosemide), 2 female FR (sham and furosemide), 1 non-treated NF animal). This was considered to be related to the nature of the study (neonatal manipulations) but unrelated to the study interventions.

At day 8, (at least) 3 animals/sex/group were sacrificed and kidneys were collected. The left kidney was frozen in liquid nitrogen for mRNA analysis and the right kidney was fixed in 10% formalin.

At day 35, (at least) 3 animals/sex/group were sacrificed by exsanguinations followed by perfusion with 10% formalin. Kidneys were stored in 10% formalin and the right kidneys were processed for stereology.

The remaining (male) animals were followed until 9 months of age and were sampled for electrolyte concentrations and creatinine clearance at the age of 3 and 9 months. Blood pressure was recorded monthly from 3 months of age onwards.

mRNA Analysis

On day 8, left kidneys were pulverized by a microdismembrator (Sartorius-Stedim, The Netherlands) in a deep frozen state. Isolation of RNA was performed by combining the TRIzol extraction method (Invitrogen, Carlsbad, Calif., USA) with the Nucleospin RNA II isolation kit (Machery-Nagel, Düren, Germany). The manufacturer's protocol for TRIzol extraction was followed up to the isopropanol separation step. Subsequently, the aqueous phase was added 1:1 to 70% ethanol and loaded on the nucleospin column. Further purification was performed according to the nucleospin manufacturer's protocol. RNA concentration and quality were assessed with the nanodrop 2,000× spectrophotometer (Thermo Fisher Scientific, Waltham, Mass., USA).

One microgram of RNA was used in a subsequent reverse transcriptase reaction using random primers (Promega, Madison, Wisc., USA), oligo dT (Promega) and M-MLV reverse transcriptase (Invitrogen). mRNA levels were measured by quantitative PCR on a Bio-Rad CFX96 using gene expression mix and hydrolysis probes (online suppl. table 3) as ordered from Applied Biosystems (Pleasanton, Calif., USA) or Biolegio (Nijmegen, Netherlands), with Actb and Hmbs as internal standards. Delta Ct (Δ Ct) values, which were calculated by correcting the threshold cycle (Ct) of the gene of interest to the Ct of the housekeeping genes, were reported. Fold changes were calculated in case Δ Ct values were statistically significant.

Immunohistochemistry

On day 8, right kidneys were fixed in formalin and subsequently processed for immunohistochemistry. Citric acid was used for antigen retrieval, and endogenous peroxidase activity was blocked. Additionally, slides were preincubated with goat serum and primary antibodies for Ki67 (1:500) (Abcam, Cambridge, UK) and Caspase-3 (1:500) (BD Pharmingen, Breda, The Netherlands) were used as markers of cellular proliferation and apoptosis, respectively. As secondary antibody, 1:200 goat anti-rabbit biotinylated antibody (Vector laboratories, Amsterdam, The Netherlands) was used and signal was amplified with avidin-HRP. PowerDAB (Immunologic, Calif., USA) was used for detection.

Stereology

Perfusion-fixed, 35-days-old right kidneys were embedded in paraffin and sectioned exhaustively at 20 μm . Every 10th–11th slide, starting at a random position (determined by a random number table), were taken and stained for haematoxylin-eosin. Slides were scanned on an Olympus Olivia slide scanner and evaluation was performed with the Newcastle module of the Visiopharm integrator system software package (version 3.6.5.0, Horzholm, Denmark).

A sample and its adjacent section were aligned manually and a counting frame was superimposed. Subsequently, a region of interest was drawn manually and a randomly oriented counting grid was placed on the region with x and y step lengths of 2,500 μm . All section pairs were then scored for the presence of glomeruli.

The total number of glomeruli per kidney ($N(\text{glom})$) was estimated by the physical fractionator/dissector principle. On average, 141 glomeruli were counted per kidney and the total number of glomeruli was estimated according to the following formula: $N(\text{glom}) = 1/\text{SSF} * 1/\text{ASF} * \text{Ps}/\text{Pf} * \Sigma Q^-/2$.

In this formula, SSF is the section sample fraction and ASF is the area sample fraction (calculated as counting frame area divided by (x-step length * y-step length)). The factor Ps/Pf was introduced to correct for slides with artificial edges; Ps is the number of counting frame corners that hit the kidney tissue, Pf is the number of counting frame corners that hit the evaluated kidney tissue. Finally, ΣQ^- is the number of counted glomeruli and the factor 1/2 was introduced for counting both ways between the slide pairs.

Glomerular Generation Counting

Glomerular generations were determined with 'the direct method' as described in the paper of Hinchliffe et al. [29]. Five radials were counted per slide.

Histopathology

Animals (male) were sacrificed after 9 months and the right kidney was immersion-fixed in 10% formalin. Samples were processed and stained for haematoxylin-eosin or periodic acid-Schiff (2 slides/animal). These slides were evaluated for glomerular and tubular abnormalities by an experienced renal pathologist (S.F.) who was blinded to treatment groups, with exception of the sham-NF group in order to establish a reference.

Blood Pressure Measurements

Blood pressure was measured noninvasively in all animals (male) from 3 months onwards by volume pressure recording on the CODA apparatus (Kent Scientific Corporation, Torrington,

Conn., USA) with software version 1.0. Prior to the measurements, the room was heated to be above the ambient temperature of 22°C. Additionally, animals were kept warm by the heating plate of the apparatus during the experiment and the tails were covered by a cloth to further stimulate the thermoregulation of the animals. Before recording, animals were acclimatized for 10 min in the restrainers and subsequently twenty blood pressure measurements were collected. The first 10 measurements were used to train the animal and were discarded. The last 10 measurements were evaluated by the software and if in acceptable boundaries regarding blood flow, they were averaged per animal. PP was calculated manually from the output data of the apparatus. Blood pressure was measured on 3 subsequent days in all animals in each month and an average of these 3 days was reported. This resulted in a robust average for each animal for each time point except for the 9-month time point. At 9 months, representative results could only be obtained in 2/3 of the animals and these were reported.

Glomerular and Tubular Function

Animals were housed in urine collection cages for 24 h at 3 and 9 months of age. Urine was collected for a period of 24 h and on exiting the urine collection cage, a blood sample was collected from the tail vein. Blood was centrifuged at 3,000 rpm and plasma was directly taken to the clinical laboratory for analysis of sodium, potassium, magnesium, phosphate, urea and creatinine. Urine samples were measured for volume, urea, creatinine and osmolality or stored for possible future analysis. Creatinine and urea clearances were calculated and normalized for body weight.

NGAL Measurement

Urine samples collected at 3 and 6 months, and plasma collected at day 8 were analyzed for the presence of NGAL protein levels as a marker of tubular damage using the rat lipocalin 2 ELISA kit (R&D Systems, Abingdon, UK) with a detection limit of 78.1 pg/ml.

Statistical Analysis

Mean (SD) values are presented for all findings unless otherwise indicated. Parameters of interest were analyzed by univariate analysis using sex, litter size and treatment as fixed factors. Post-hoc analysis was performed by Tukey. The time effect on blood pressure was analyzed between and within subjects with a multivariate analysis for repeated measures, using the Greenhouse-Geisser correction. Gene expression data were analyzed by the Kruskal–Wallis test for non-parametric data. Interaction between glomerular generation and glomerular numbers was analyzed with linear regression. All analyses were performed by the use of SPSS version 20 (IBM, Armonk, N.Y., USA) at the alpha 0.05 level.

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