Antibiotics and renal branching morphogenesis: comparison of toxicities

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BACKGROUND: Many premature born neonates receive antibiotic drugs to treat infections, which are applied during active nephrogenesis. We studied the impact of clinical concentrations of gentamicin and alternatives, ceftazidime and meropenem, on ureteric branching.

METHODS: Mice metanephroi were dissected at embryonic day 13 and cultured in media with or without various concentrations of gentamicin, ceftazidime, or meropenem. Zero and 24 h kidney size were assessed by surface area measurements, and the ureteric tree was visualized by whole mount staining and confocal microscopy. Branching was evaluated by counting and gene expression levels of *Wt1*, *Sox9*, *Bmp7*, *Fgf8*, and *Gdnf* were investigated.

RESULTS: A concentration of 2,000 µmol/l ceftazidime impaired ureteric development. In addition, a 4.5-fold and a 2.5-fold downregulation was noted in *Fgf8* and *Gdnf*, respectively. No adverse effects were noted after gentamicin or meropenem treatment. No relationship was noted between surface area expansion and ureteric bud formation, but surface area at explantation related to bud count after 24 h of culture.

CONCLUSION: Ceftazidime, but not gentamicin or meropenem reduced ureteric branching in mice and suggest a role for *Fgf8* and *Gdnf* in its mechanism. Metanephros surface area measurements can be used to reduce intra- and inter-litter variation.

Kidney development, leading to the formation of nephrons, starts around the 5th wk of gestation and terminates before term birth, around the 34th–36th wk of gestation. Many factors have been described to disturb this developmental process, leading to long-term problems such as hypertension and chronic kidney disease (1).

One such disturbing factor may be the use of (nephrotoxic) drugs during kidney development, such as in pregnant women or neonates born before termination of nephrogenesis. Gentamicin, as well as other aminoglycosides, is widely used as part of the first line treatment of (suspected) bacterial infection in neonates to combat Gram-negative infections. Based on data from the Netherlands Perinatal Registry, 62% of neonates born before 32 wk, who can be considered the most vulnerable group, are treated with aminoglycosides (2). However, due to the fact that gentamicin is classified as a nephrotoxic drug, controversy remains on its safety as aminoglycosides have been shown to disturb kidney development and lead to a reduced nephron number in some experimental animals (3–6) and organ culture studies (7).

The aim of our research was to study the impact of antibiotic treatments on nephrogenesis in a model of early nephrogenesis. Gentamicin was studied as well as clinically relevant, alternative drug treatments to compare the toxic potentials of these drugs in a clinical dose range. As alternative treatments, we chose a third generation cephalosporin, ceftazidime, and the carbapenem meropenem. These two drugs both have properties to deal with Gram-negative bacteria and have different mechanisms of action compared to gentamicin. Although beta-lactams have their own potencies to be nephrotoxic, ceftazidime can be given at a fairly high dose before proximal tubule damage is noticeable (8). As for meropenem, this drug is regarded as one of the safer carbapenems (9) due to its stability to renal dehydropeptidase-I (10), which was confirmed in a large clinical trial (11). Although these alternatives to gentamicin appear quite safe, information on the potential toxic effects on renal branching morphogenesis is lacking. We hypothesized that gentamicin would hamper early kidney development, and ceftazidime and meropenem would prove to be safe.

RESULTS

Metanephric Growth

First, we studied the usefulness of surface area growth as a good marker for renal development. Based on previous studies (7,12,13), a relationship between metanephric size expansion and ureteric tip count, a more direct measurement of renal development, was expected. However, as can be noted in **Figure 1a**, surface area expansion did not correlate with ureteric tip formation ($R^2 = 0.005$, $P = 0.77$) and was therefore not used as a marker in our studies.

In addition, we noted a variation in metanephric size within a litter (up to 11%) and between our litters ($\pm 20\%$), based on differences in surface area measured directly after explantation. The impact of this variation on the ureteric bud tip count after 24 h was studied. As can be seen in **Figure 1b**, a significant

a

b
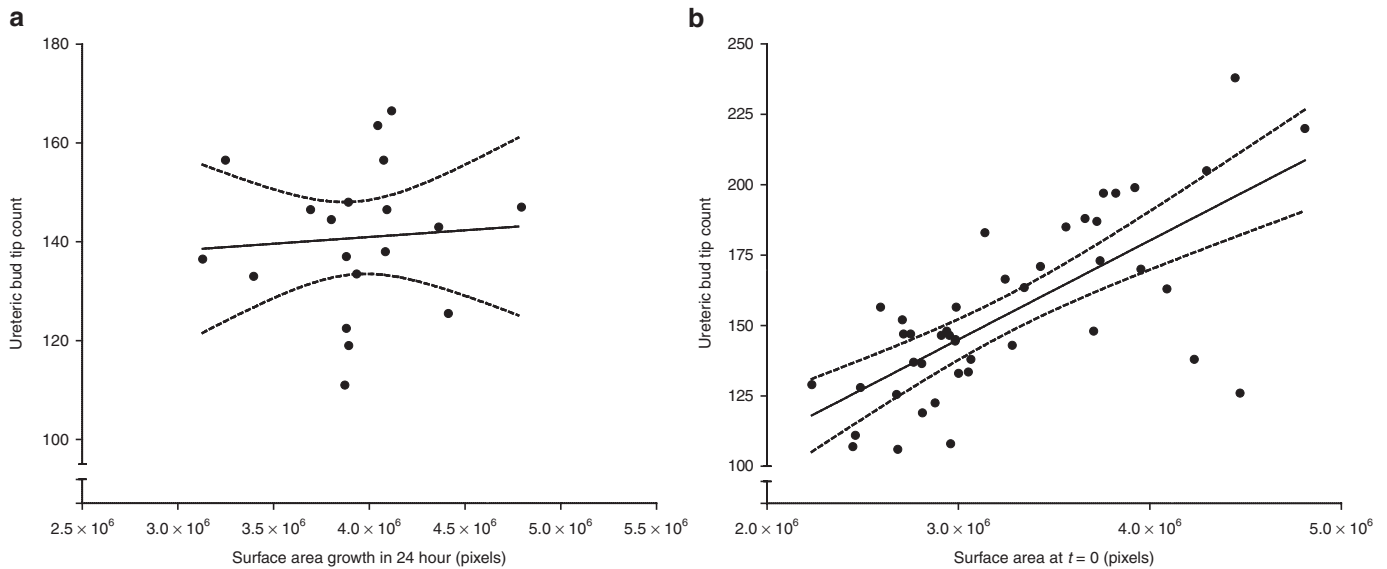
Figure 1. Correlation (with 95% confidence interval) between surface area measurements and ureteric bud formation (**a**) after 24 h of organ culture and (**b**) at explantation. Pearson's correlation coefficients were $R^2 = 0.005228$ and $R^2 = 0.5082$ for **a** and **b**, respectively.

correlation was noted between metanephric size and ureteric bud development ($R^2 = 0.5$, $P < 0.01$). Based on this finding, all ureteric tip counts were corrected for metanephric surface area at explantation.

Ureteric Tip Imaging
Ureteric tip development was studied in the three different antibiotic classes. A range of three doses was tested to evaluate the broad drug response and investigate a possible dose response relationship (**Figure 2**).

As can be noted in **Figure 2b**, only the 2,000 µmol/l ceftazidime concentration resulted in a statistically significant lower ureteric tip count after 24 h of exposure (28.37 vs. 48.16, $P < 0.01$). This effect was also clearly detectable visually (**Figure 3**). No effects on development compared to control were noted at the lower and the middle dose. In our experiments with gentamicin (**Figure 2a**) a trend was noticeable towards a lower ureteric tip count. Meropenem treatment (**Figure 2c**) did not show an effect on ureteric tip development compared to control at any of the concentrations studied.

Gene Expression Analysis
We studied a selection of targets in known nephrogenesis and apoptosis pathways by mRNA expression analysis (**Figure 4**). Robust CT levels indicated that the targets were present in our metanephric organ cultures.

Treatment with ceftazidime in a concentration of 2,000 µmol/l resulted in a 2.5-fold downregulation of *Gdnf* mRNA, a well known factor in the ureteric branching pathway (14). In addition, a 4.5-fold downregulation of the *Fgf8* mRNA was noted (**Figure 4c**). No changes in expression patterns were noted after treatment with 200 µmol/l of ceftazidime. Gentamicin or meropenem treatment did not have an effect on the mRNA levels of our chosen targets. Furthermore, mRNA levels of Caspase 3 and Caspase 9 remained similar

between controls and high dose treatments of gentamicin and ceftazidime.

DISCUSSION
We have shown that an upper clinical range concentration of ceftazidime reduces ureteric branching morphogenesis and suggest a role for *Fgf8* and *Gdnf* pathways in its toxicity. No adverse effects of gentamicin and meropenem within their postulated clinical dose range were noted on ureteric branching or basic nephrogenesis pathways.

Previous reports have used surface area expansion as a measurement for renal development (7,12,13). Our study shows no correlation between the surface expansion and a more direct kidney developmental marker such as ureteric bud count. This lack of correlation may be explained by flattening of the metanephros in organ culture and its partial loss of three dimensional structure as has been reported previously (15). However, the impact on actual measurements had not been studied. We show that surface area measurements in the first 24 h after explantation are not a proper marker for renal development.

Although flattening of the organ rudiment during organ culture renders surface analysis unhelpful for quantification of kidney development, surface area at explantation can partially predict the amount of tips that will develop in metanephroi. We found a significant correlation between the surface area of explant size directly after dissection and the amount of ureter tips counted after 24 h. One could expect such a correlation because kidney development is more pronounced *in utero* than in organ culture (16). Therefore, a more evolved (and therefore branched and larger) metanephros at time of dissection can be expected to have more end tips after 24 h of growth. These data allow for correction of variation in gestational age. By using our correlation to correct all ureteric tip counts for the measured surface area after explantation, variability of developmental stage will be more limited, and more precise
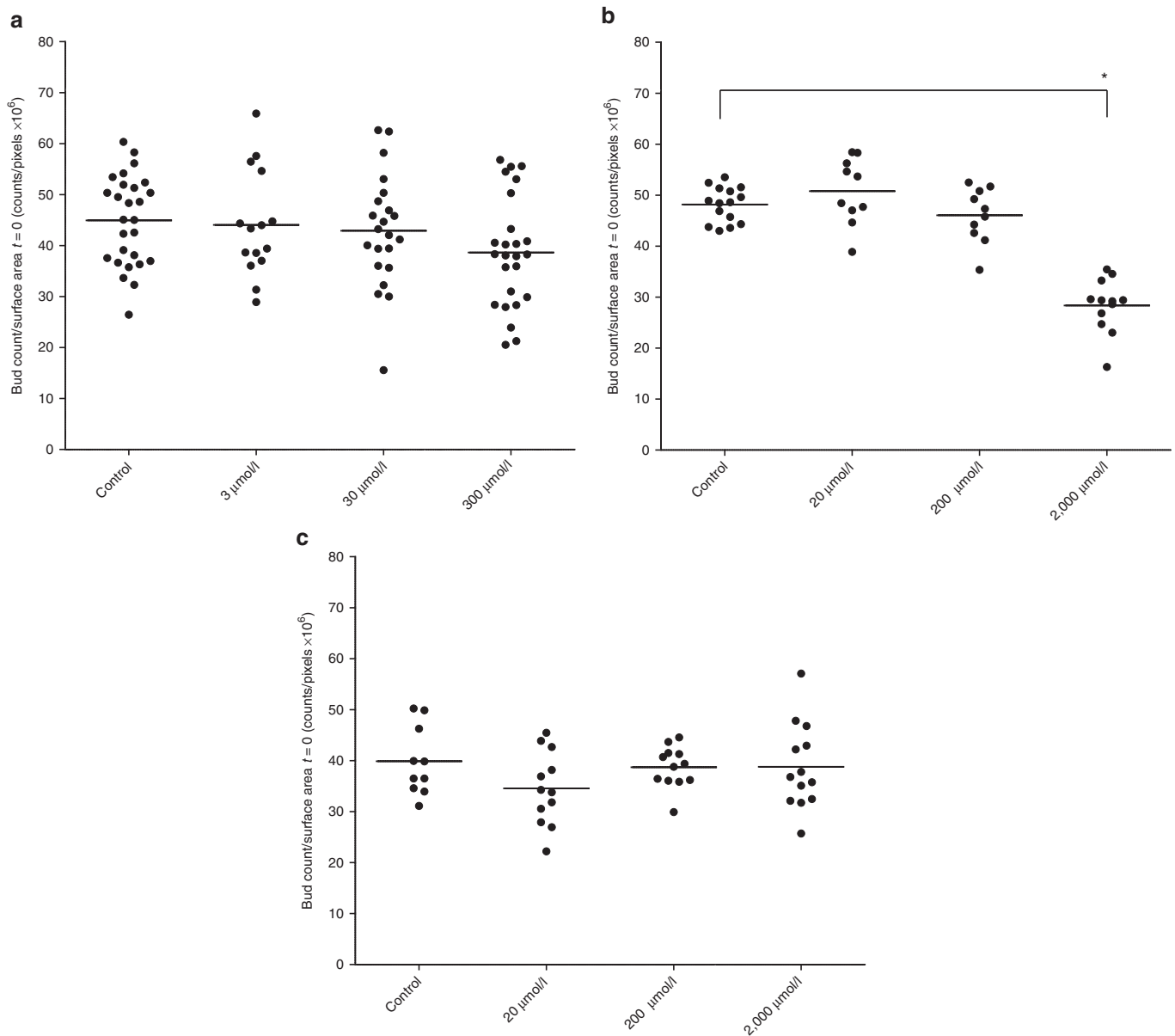
Figure 2. Quantitative analysis of ureteric buds in metanephroi cultured for 24 h in media with three antibiotic drugs, i.e., (**a**) gentamicin, (**b**) ceftazidime, and (**c**) meropenem. Individual data points as well as means per treatment group are presented. A low, clinical, and high concentration were tested. Sample sizes of treatment groups were between 15 and 27; 10 and 15; and 10 and 13 for gentamicin, ceftazidime, and meropenem, respectively. *$P < 0.01$.

comparisons between dose groups, but also between litters can be obtained.

Our initial research question was whether clinical relevant levels of gentamicin could influence nephrogenesis and whether other classes of antibiotics might be safer.

The potential of gentamicine to influence nephrogenesis has already been established in rat. Studies were mostly performed by either maternal dosing (3–5) or postnatal dosing (3,17). In these studies, no effect of gentamicin was noted on renal development after early postnatal dosing during active nephrogenesis. However, after maternal dosing gentamicin accumulation was confirmed in the fetal kidney. In addition, exposure to gentamicine *in utero* resulted in a reduction of glomerular numbers up to 20% and alterations in the tubular structure.

However, the dose administered to the pregnant females in these studies was 75 mg/kg and was almost 20-fold higher than the 4 mg/kg clinical dose we extrapolated the dose levels from in this study.

Our results indicate that 24 h exposure of gentamicin did not result into impaired ureteric branching or altered expression of key pathways. Even though we did notice a trend towards reduced ureteric branching this difference was not statistically significant. This contradicts earlier findings of Gilbert *et al.* who found reduced ureteric branching in rat organ cultures at a gentamicin concentration of 100 μmol/l (7). The main difference between our approaches can be found in animal species (rat vs. mice in the current study) and in developmental stage. We believe the species difference is probably of secondary importance to the
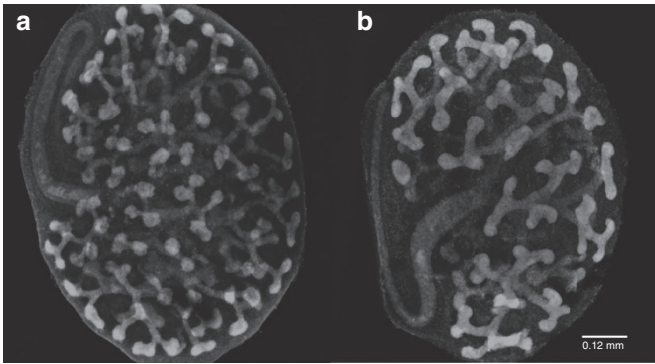
Figure 3. Representative immunohistochemic staining of ureteric bud development in metanephroi cultured for 24 h in media with (**b**) 2,000 µmol/l ceftazidime or (**a**) vehicle control.

developmental stage as effects of *in utero* gentamicin exposure on renal development were reported in rats, mice and guinea pigs alike (3–7,18). Although some differences in susceptibility may be present. Comparing Theiler and Witschi developmental stages (19), our E13 mice are approximately 12–24 h more mature compared with the E14 rat embryos. It is well known that timing of insults during organ development is of great importance for the outcome and that dosing at a day later may completely abolish any effect (20). Taken these factors into account, gentamicin has the potential to disturb nephrogenesis, but the clinical dose range seemed to be safe when administered in the time frame and species that we studied.

From the data of our selected potential alternatives, we conclude that ceftazidime does not appear to be a good substitute for gentamicin. In the high dose group, an impaired kidney
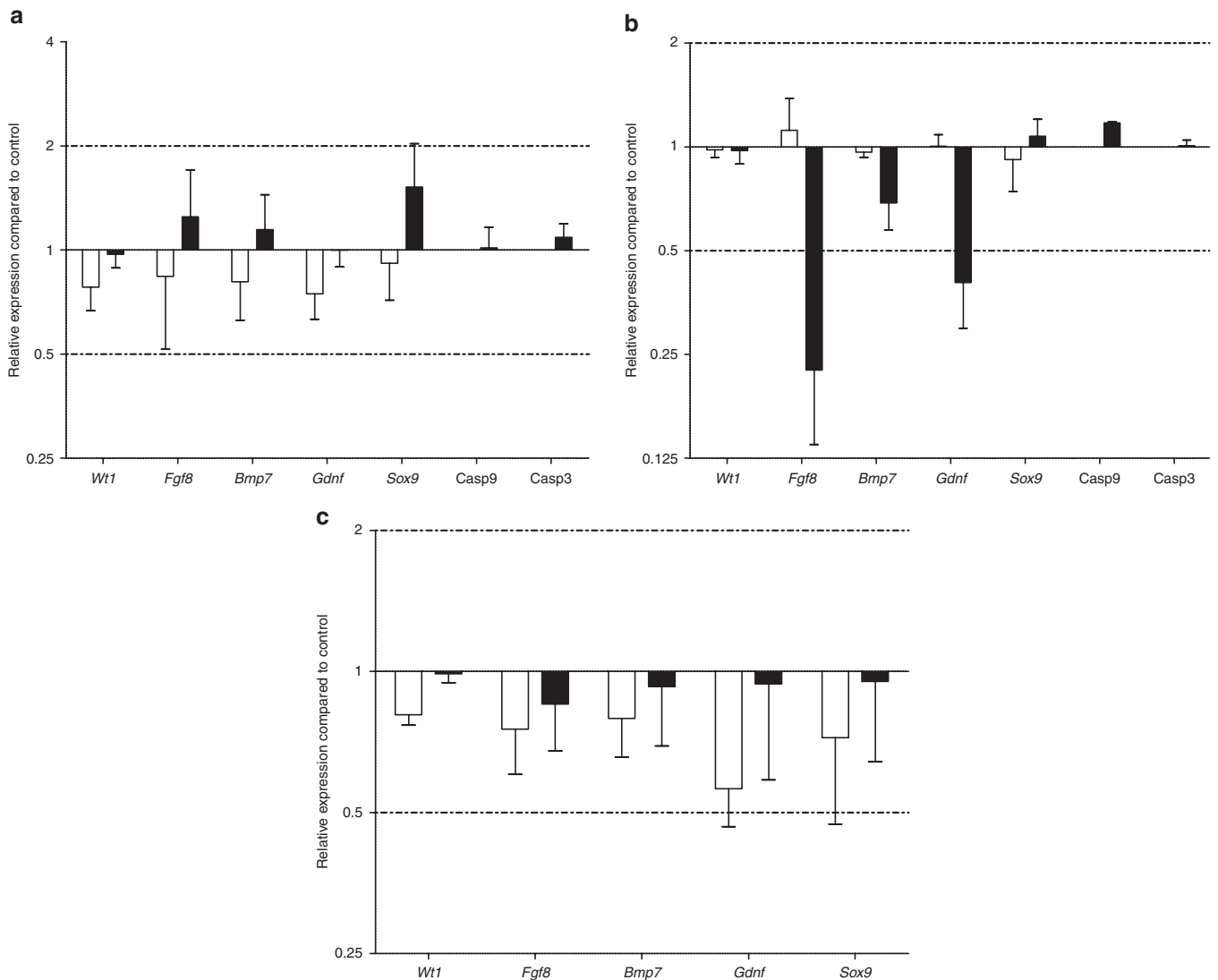
Figure 4. Gene expression of important nephrogenesis pathway targets in metanephroi cultured for 24 h in media with three antibiotic drugs, i.e., (**a**) gentamicin, (**b**) ceftazidime, and (**c**) meropenem. Means with SEM are presented for (**a**) two or (**b,c**) three experiments, with exception of the caspase targets, which were done in two experiments. Concentrations tested were 30 (white bars) and 300 µmol/l (black bars) for gentamicin, 200 (white bars) and 2,000 µmol/l (black bars) for both ceftazidime and meropenem. Six to nine metanephroi were pooled per experiment.

development and downregulation in corresponding pathways was identified.

We hypothesize that ceftazidime affects the renal progenitor cell population causing downregulation of *Fgf8* and subsequent downregulation of *Gdnf* resulting in ureteric bud impairment. *Fgf8* is important in cell survival of renal progenitor cells, which are an important source of Gdnf production (21,22). In contrast, no expression changes were noted in *Bmp7*, which is also localized to the progenitor cell population (23), suggesting no general progenitor cell loss with possibly cell loss in a specific sub population. This finding is supported with the normal expression levels of Caspase 3 and Caspase 9, markers of apoptotic insults and also important in the normal development of the renal tubular network (24). Alternatively, ceftazidime may directly affect both the mesenchyme and the ureteric bud. Although this high dose is tenfold higher than our theoretical calculated clinical dose, the actual intracellular concentration in human cells is unknown. Based on our results, the safety of ceftazidime for treatment of pregnant women should be reconsidered, as it may be harmful for early renal development of the fetus. In addition, ceftriaxone was earlier reported to have a negative effect on nephrogenesis in rats (25), which underlines the general potential harmful effects of maternal cephalosporine use on fetal nephrogenesis.

In contrast, meropenem did not show any negative effect on ureteric bud branching or mRNA levels of important development pathways, and may therefore be a potential useful substitution for gentamicin with regards to nephrogenesis.

Our studies have some limitations. First, due to the stage of development and the method of antibody staining, we are currently unable to evaluate ureteric bud staining beyond 24 h of exposure. Therefore, it might be that prolonged exposure to gentamicin or meropenem will eventually result in renal damage. Using genetically modified mice with intrinsic fluorescent protein expression in the ureteric bud may solve this problem and allow for such longer-term studies (16). Second, we have no data on drug uptake within the cells. Gentamicin has been shown to be detectable within embryonic kidney cells after 8 h of exposure (7). Our *ex vivo* model is slightly more mature and of a different species which could have influence uptake. However, we noted a dose related trend in our experiments and are therefore confident that we had uptake of gentamicin in our model. For ceftazidime and meropenem data on uptake in organ culture are not yet available, which may play a role in the absence of effects in the meropenem treated metanephroi. However, the reduced formation of ureteric bud tips in the high dose ceftazidime group suggests uptake of ceftazidime by the metanephros within hours. Furthermore, we investigated a selection of targets in the nephrogenesis pathways and studied gene expression rather than protein expression. An immunohistochemical approach was attempted, but has not provided additional insight so far. Besides the technical difficulties of studying protein expression levels in small tissues as metanephroi, we feel that the significant morphological change in these cultured metanephroi show the relevance of the altered gene expression levels. In order to study apoptosis, we decided to study mRNA levels of 2 different caspases. Alternatively, TUNEL could be used to the same extent and should be performed in future studies. Finally, at present we do not have data on different time points during development at which the kidney may be more vulnerable to specific drugs. This would be a very important future step in relating study findings to the human embryonic kidney development.

Our objective was to determine whether these antibiotic compounds could interfere with a basal developmental process as ureteric bud differentiation when administered in a clinical dose range. Based on our data, we can conclude that short-term gentamicin or meropenem treatment at this stage of embryonic kidney development does not have an adverse impact on nephrogenesis. However, ceftazidime was shown to inhibit ureteric branching and the expression of *Fgf8* and *Gdnf*, two important players in kidney development. Furthermore, metanephric surface area growth should not be used as a renal developmental predictor, but surface area at explantation can be used to correct for intra- and inter-litter variation in developmental stages.

METHODS

Drugs
Gentamicin (G1272) and ceftazidime hydrate (C3809) were obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands). Meropenem was obtained from Fresenius Kabi (Teramo, Italy).

Organ Culture
Experiments were approved by the Animal Ethics Committee at the Radboud University Nijmegen. Embryonic day 13 time pregnant HSD:ICR female mice (Harlan, Horst, The Netherlands) were euthanized after arrival by cervical dislocation and kidneys were dissected from the embryos by means of two small needles. Intact isolated metanephroi were held on ice in pre-cooled Leibovitz medium (Gibco, Paisley, UK) until transfer to the organ culture system. Metanephroi were cultured on 0.4 µm pore size Millicell cell culture inserts (Millipore, Carrigtwohill, Ireland) placed in a six-well plate containing DMEM/F-12 (1:1) medium (Gibco), supplemented with 10 mg/l insulin, 5,5 mg/l transferrin and 5 µg/l sodium selenite (Sigma-Aldrich, St Louis, MO). Depending on treatment, drugs were added to the medium and metanephroi were incubated at 37 °C and 5% CO_2 for 24 h. For gentamicin, drug concentrations of 3, 30, and 300 µmol/l were chosen. Ceftazidime and meropenem were both tested at 20, 200, and 2,000 µmol/l. Concentration levels were calculated as follows: The clinical pediatric dosing was used and corrected for plasma binding and volume of distribution as detailed in **Table 1** (26–32). In addition, concentrations at a factor of 10 above and below were studied to take calculation uncertainties into account and to investigate a potential dose response relationship.

All metanephroi from one litter were randomly assigned to one of the treatment groups and a separate no-treatment group that consisted of metanephroi from the same litter was included for each drug. Per litter, one drug was investigated to rule out differences on basis of variability between litters. Multiple litters were studied per drug.

Table 1. Dose selection parameters

	Gentamicin	Ceftazidime	Meropenem
(Neonatal) dose (mg/kg)	4	50	20
Molecular weight	463.6	546.58	383.46
Plasma binding (%)	0	27	2
Volume of distribution (l/kg)	0.31	0.36	0.3

Data in this table were derived from multiple sources (26–32).

Metanephric Growth

Growth of the explanted metanephroi was determined by measuring surface areas expansion in 24 h. Photographs of the metanephroi were obtained with a Canon EOS 1000D camera (Canon, 's Hertogenbosch, The Netherlands) attached to a Zeiss Axiovert 25 microscope (Carl Zeiss, Sliedrecht, The Netherlands) at a total magnification of 12.5×. Background light was set on maximal intensity and a shutter speed of 40 ms was used. The photographs were taken with a resolution of 10.1 megapixels and surface area size was analyzed with FIJI/ImageJA version 1.45i (33).

Ureteric Tip Imaging

Whole mount immunostaining of the metanephroi was performed to visualize the ureteric tree after 24 h of culture. The metanephroi were fixed in ice-cold methanol for 10 min and washed with phosphate-buffered saline (PBS) for 15 min. Subsequently, the metanephroi were incubated in PBS containing 2% bovine serum albumin for 12 h to block non-specific binding. After washing with PBS containing 1% Triton X-100 (PBS-T), metanephroi were incubated with an antibody against calbindin-D28k (Sigma Aldrich), diluted 1:100 in PBS-T, for 24 h. Again after washing with PBS-T, incubation with an Alexa 488 IgG antibody (Invitrogen, Eugene, OR) was performed at a dilution of 1:300 in PBS containing 2% bovine serum albumin for 24 h. All incubation and washing steps were performed at 4 °C. After a final washing step in PBS-T of 15 min, the metanephroi were mounted on a slide in mounting medium (Dako, Carpinteria, CA) and sealed using paraffin.

For each metanephros, ureteric branching was visualized by confocal laser scanning microscopy using a Leica TCS SP2 microscope. Optical sectioning was performed at a 4 μm interval at a magnification of 10×. Per metanephros, between 18 and 30 images were acquired with a resolution of $1,024 \times 1,024$ pixels. Subsequently, the amount of ureteric tips was counted with the multipoint tool in FIJI. A tip was defined as an end point of the whole branching structure that did not show any signs of branching.

Gene Expression Analysis

RNA was isolated from the metanephroi by combining the Trizol extraction method with the NucleoSpin RNA II isolation kit (Machery-Nagel, Düren, Germany). The metanephroi were suspended in Trizol (Invitrogen, Carlsbad, CA) and incubated for approximately 30 min with occasional vortexing. After addition of chloroform (Merck), the samples were incubated on ice for 5 min and centrifuged at 14,000g, 4 °C for 15 min. The aqueous phase was added 1:1 to 70% ethanol to adjust binding conditions and loaded on the Nucleospin column. Further purification was performed according to the manufacturers protocol. RNA concentration and quality was assessed with the Nanodrop 2000c spectrophotometer (Thermo Fisher Scientific, Waltham, MA).

Complementary DNA was generated on a Biozym MJ Research PTC-200 Peltier thermal cycler using random primers (Promega, Madison, WI), oligo dT (Promega) and M-MLV reverse transcriptase (Invitrogen). mRNA levels of *Wt1*, *Sox9*, *Bmp7*, *Fgf8*, *Gdnf*, *Casp3*, and *Casp9* were measured by quantitative PCR (qPCR) with *Actb* and *Hmbs* as internal standards.

qPCR was performed on a Biorad CFX96 using the gene expression mix and hydrolysis probes (**Table 2**) as ordered from Applied Biosystems (Pleasanton, CA). Delta-delta CT values were investigated and an up or downregulation of factor 2 was considered biologically relevant.

Statistics

Metanephric surface expansion at 24 h and metanephric surface area at explantation were both investigated for correlation with ureteric bud tip development and tested with a Pearson's correlation coefficient.

Comparison of ureteric bud tip development for each treatment was investigated by one-way analysis of variance followed with Dunnett as *post hoc*. For both analysis, statistical significance was investigated at the $\alpha = 0.05$ level.

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