Tenofovir during pregnancy in rats: a novel pathway for programmed hypertension in the offspring

Pedro Henrique França Gois¹*, Daniele Canale¹, Weverton Machado Luchi¹, Rildo Aparecido Volpini¹, Mariana Matera Veras², Natália de Souza Xavier Costa², Maria Heloisa Massola Shimizu¹ and Antonio Carlos Seguro¹

¹Laboratory for Medical Research—LIM12, Nephrology Department, University of São Paulo School of Medicine, São Paulo, Brazil; ²Laboratory of Experimental Air Pollution—LIM05, Department of Pathology, University of São Paulo School of Medicine, São Paulo, Brazil

*Corresponding author. Faculdade de Medicina da Universidade de São Paulo, Avenida Dr Arnaldo, 455, Sala 3310, Pinheiros, CEP: 01246-930, São Paulo, SP, Brazil. Tel/Fax: +55-11-30617281; E-mail: pedrogoismd@usp.br

Received 9 September 2014; returned 20 October 2014; revised 29 October 2014; accepted 31 October 2014

Objectives: To evaluate the occurrence of systemic and renal abnormalities in the offspring of Wistar rats exposed to tenofovir disoproxil fumarate (DF) during pregnancy.

Methods: Female Wistar rats received a standard diet, with or without addition of tenofovir DF (100 mg/kg diet), 1 week before mating and during pregnancy. Offspring from the tenofovir DF group were placed with an untreated foster mother during breastfeeding and compared with offspring from rats maintained on a standard diet during mating and pregnancy (control). Control and tenofovir DF were followed up at 3 and 6 months of age. Monthly body weight and systolic blood pressure (SBP), glomerular counts, renal function, biochemical parameters, angiotensin II, renal renin angiotensin aldosterone system (RAAS) and renal sodium transporters were analysed.

Results: Tenofovir DF offspring showed lower birth weight compared with the control group. After the third month, growth among the tenofovir DF group experienced a rapid catch-up. SBP increased progressively after the second month of age in the tenofovir DF group. Nephron number did not differ between the groups; however, the tenofovir DF group showed glomerular structural changes. Plasma aldosterone was higher in the tenofovir DF group, associated with a significant increase in renal expression of RAAS. The tenofovir DF rats showed up-regulation of renal sodium transporters and consequently lower urinary sodium excretion.

Conclusions: This is the first demonstration using an experimental model that maternal exposure to tenofovir DF during gestation results in overactivation of RAAS, up-regulation of renal sodium transporters and hypertension in the offspring.

Keywords: tenofovir, pregnancy, hypertension, HIV, AIDS, hepatitis B

Introduction

HIV infection is a public health problem that affects ~35 million people worldwide, half of them women.¹ Some 8700 HIV-positive women give birth each year and >4000 children in the USA are exposed in utero to several antiretroviral (ARV) drugs.² Tenofovir disoproxil fumarate (DF) is an orally bioavailable prodrug form of tenofovir, a nucleotide analogue of adenosine monophosphate.³ Currently, tenofovir DF is considered a first-line medication in the treatment of AIDS, among several combinations in HAART, and in 2008 it was also approved for the treatment of chronic hepatitis B in adults.⁴

The latest statistics of the global HIV and AIDS epidemic reported that >900000 pregnant women living with HIV received ARVs for treatment or prophylaxis of mother-to-child transmission.¹ Data from the Antiretroviral Pregnancy Registry showed that 3276 pregnancies from January 1989 through January 2013 were exposed to tenofovir DF, of which 2313 were in the first trimester.⁵ Furthermore, despite being classified as a pregnancy category B drug, tenofovir DF has been added to recent WHO guidelines for prophylaxis of HIV vertical transmission.⁶,⁷ A number of adverse events have been associated with the use of ARVs during pregnancy, such as premature birth, spontaneous abortion and low birth weight.⁸–¹⁰ In pregnant rhesus monkeys, administration of tenofovir DF at high doses in the first trimester resulted in lower crown–rump length, lower body weight (BW) and smaller adrenal glands compared with tenofovir-unexposed control monkeys.¹¹ Additionally, high-dose
Tenofovir administration to infant macaques was associated with infant growth restriction.\textsuperscript{12} Tenofovir DF has also been associated with nephrotoxicity.\textsuperscript{13–16} However, there are no currently published data on the renal adverse effects of tenofovir DF regimens during gestation. To date, caution in the use of tenofovir DF during pregnancy has been recommended, based on potential deleterious effects on fetal growth and bone mineralization.\textsuperscript{17} The aim of this study was to evaluate the occurrence of systemic and renal abnormalities in the offspring of Wistar rats exposed to tenofovir DF during gestation.

**Materials and methods**

All experimental procedures were approved by the local research ethics committee (Comitê de Ética e Pesquisa da Faculdade de Medicina da Universidade de São Paulo, process no. 071/13) and developed in strict conformity with local institutional guidelines and with well-established international standards for the manipulation and care of laboratory animals.

Tenofovir DF (Viread\textsuperscript{\textregistered}, Gilead Sciences, Foster City, CA, USA) was kindly provided by the Reference Center for Treatment of AIDS from Brazil’s National Health System.

**Animals and experimental protocol**

Female Wistar rats with a mean BW of 231 ± 22 g were obtained from the animal facilities of the University of São Paulo, School of Medicine, housed in standard cages and given ad libitum access to water. Animals were allocated to be fed with either a standard diet (Nuvilab CR-1, Curitiba, PR, Brazil) or a standard diet with the addition of tenofovir DF (100 mg/kg food) 1 week before mating and during pregnancy (n = 7 for the tenofovir DF group and n = 8 for the control group). Water and food intake were measured daily during pregnancy. Females were allowed to mate with mature males (in a proportion of three females:one male) for 21 days. Soon after delivery, each Wistar dam nursed six male pups to minimize variation in nutrition of the pups during suckling. Offspring from tenofovir DF were placed with an untreated foster mother during the breastfeeding period (25 days) in order to simulate the recommendation that HIV-infected women should not breastfeed. Offspring from the tenofovir DF-fed rats and offspring from rats maintained with a standard diet during mating and pregnancy were followed up at 3 months (n = 9 for each group) and 6 months of age (n = 10 for tenofovir DF and n = 12 for control). BW was evaluated at birth and weekly until the end of the study. Naso–anal length was measured at birth and at 1 and 2 weeks of age. Unneeded animals were euthanized by intraperitoneal administration of excess of thiopental, away from other animals and in accordance with the guidelines of our animal research ethics committee. Figure 1(a) illustrates the experimental protocol.

**Figure 1.** (a) Experimental protocol. (b) Birth weight in tenofovir disoproxil fumarate (TDF) group is reduced compared with control. (c) Absolute weights from birth until 6 months of age. Rats from the TDF group showed a catch-up growth, reaching the sixth month heavier than the control group (C). Data are expressed as mean ± SEM; unpaired t-test was used to evaluate statistical significance; *P < 0.01, **P < 0.001 and ***P < 0.0001 for the comparison TDF group versus C group.
Metabolic cage studies and analysis of urine samples

On the day before inulin clearance studies, animals (offspring from tenofovir DF and control groups) were moved to metabolic cages (one rat per cage), maintained on a 12 h light/dark cycle and given free access to drinking water. Rats were acclimated to the housing conditions for 1 day before the experimental procedures, which began with collection of 24 h urine samples. The volume of each 24 h urine sample was measured gravimetrically. Urine samples were centrifuged in aliquots to remove suspended material and supernatants were analysed.

Blood pressure measurements

Systolic arterial pressure was measured monthly at 4 weeks of age in conscious rats by the indirect tail-cuff method using an electr osphygmonanometer (RTBP 2000, Kent Scientific Corporation, Torrington, CT, USA). Rats were placed in a darkened room and preheated to 39°C for 10 min. A suitably sized cuff was placed over the tail and inflated to 200 mmHg. Changes in blood flow were detected by a Doppler ultrasonic flowmeter. There were six to eight blood pressure readings, with a 1 min interval between them, and the mean was used as the blood pressure for that rat.

Morphometric measurements and stereological estimation of kidney volume, glomerular number and glomerular volume

At 3 months of age, the left kidney was flushed with 10% formalin in order to carry out histomorphological evaluation (n = 5 for tenofovir DF and n = 8 for control group). The left kidney was cleaned of connective tissue and fixed in 10% formalin. The total volume of the kidney (VTkidney) was estimated macroscopically using the Cavalieri principle and these fixed kidneys were sectioned transversally into 4 mm slices (n = 7–8 slices per kidney). A point test system [a(p) = 7.84 mm²] printed on an acetate sheet was superimposed on each slice and the number of points falling on each compartment and on the entire kidney were counted. Volume fractions (Vv) of compartments within kidneys were estimated using equation (2):

\[
\text{VTkidney} = \sum \text{ptK} \cdot a(p) \cdot T
\]  

where \(\sum \text{ptK}\) is the sum of points falling on the kidney in all sections, \(a(p)\) is the point reference area and \(T\) is the slice thickness (\(T = 4\) mm).

All slices were then processed for paraffin embedding and for each slice a block was prepared. Blocks were sectioned into 5 \(\mu\)m thickness sections, with the aid of a rotating microtome, and two random pairs of sections 400 \(\mu\)m distant from each other were collected on glass slides and stained with haematoxylin and eosin (HE). Also, from these blocks, two physical dissector pairs of sections were prepared and two random pairs of sections falling on each compartment and on the entire kidney were counted. Volume fractions (Vv) of compartments within kidneys were estimated using equation (2):

\[
\text{Est Vv} = \sum \text{Pcomp} = \sum \text{Pkidney}
\]  

where \(\sum \text{Pcomp}\) and \(\sum \text{Pkidney}\) were summed over all fields and sections from each kidney.

Subsequently, the total volume of each compartment (VTcomp) within a kidney was estimated by multiplying its volume fraction (Vv) by the corresponding kidney volume (VTkidney).

The same procedure was applied to estimate the volume and volume fractions of renal corpuscle and glomeruli on random selected fields of view (n = 60). For this analysis, random fields of view sampled on the renal cortex were observed under a 20x objective lens and a point test system with an area per point of 1000 \(\mu\)m² on the specimen scale was used.

CAST system software (Visionpharm, Denmark) was used to generate the test systems and do the counting.

The number of glomeruli was estimated using physical dissectors and counted in fields within the renal cortex that were selected using a systematic uniform random scheme. To count glomeruli, regions of interest in both sections (pair) were found and aligned, an unbiased counting frame was superimposed [a(f) = 2.63 mm²] on both sections and those glomeruli sampled by the frame applied on the first section that were not present in the corresponding frame in the second section were counted (Q). To double the efficiency, those glomeruli in the frame of the second section that were not present in the frame of the first section were then counted (Q−1). Using the following formulae, the number density (equation 3) and the total number of glomeruli (equation 4) were estimated. Glomeruli presenting histopathological changes were counted differentially (Qa and Qa−1):

\[
\text{Nvglom} = \frac{\sum Q + \sum Q - 1 + \sum Qa + \sum Qa - 1}{(2 \times \text{nndis} \times \text{Vdis})}
\]  

\[
\text{Nvglom} = \text{Nvglom} \times \text{Vcortex}
\]  

Determination of renal function

To determine the glomerular filtration rate (GFR), inulin clearance studies were conducted at 3 and 6 months of age. On the day of the experiment, animals were anaesthetized intraperitoneally with sodium thiopental (50 mg/kg BW). The trachea was cannulated with a PE-240 catheter and spontaneous breathing was maintained. To control mean arterial pressure (MAP) and allow blood sampling, a PE-60 catheter was inserted into the right carotid artery. For infusion of inulin and fluids, another PE-60 catheter was inserted into the left jugular vein. In order to collect urine samples, a suprapubic incision was made and the urinary bladder was cannulated with a PE-240 catheter. After surgical procedure, a loading dose of inulin (100 mg/kg BW diluted in 0.9% saline) was administered through the jugular vein. Subsequently, a constant inulin infusion (10 mg/kg BW in 0.9% saline) was started and was continued at 0.04 mL/min throughout the experiment. Blood samples and three urine samples were obtained at the beginning and at the end of the experiment. Blood and urine inulin were determined using the anthrone method. GFR data are expressed in mL/min.

Evaluation of biochemical parameters

Plasma and urinary sodium and potassium were measured by flame photometry (model FC280, CELM, São Paulo, SP, Brazil). Plasma calcium and phosphate were determined with specific electrodes (ABL800Flex, Radiometer, Brønshøj, Denmark). The enzymatic colorimetric method (Labtest, Lagoa Santa, MG, Brazil) was used to quantify levels of plasma total cholesterol. Plasma aldosterone was measured by radioimmunoassay using a commercially available kit (Immunotech, Marseille, France).
Glucosuria was assessed by colorimetric tests. Biochemical data were evaluated in offspring at 3 and 6 months of age and the maternal evaluation was performed 48 h after delivery.

**Tissue sample collection and preparation**

At the end of inulin clearance studies (at 6 months of age), the organs were perfused with PBS solution (0.15 M NaCl and 0.01 M sodium phosphate buffer, pH 7.4). The right kidney was removed and frozen in liquid nitrogen and stored at −80°C for assessment of angiotensinogen, angiotensin II receptor type 1 (AT1r), angiotensin-converting enzyme (ACE), renin, Na-K-2Cl (NKCC2) co-transporter, Na-Cl (NCC) co-transporter and epithelial sodium channel (ENaC, α, β and γ subunits) protein expression.

**Total protein isolation**

Kidney sections were homogenized in an ice-cold isolation solution (200 mmol/L mannitol, 80 mmol/L HEPES and 41 mmol/L KOH, pH 7.5) containing a protease inhibitor cocktail (Sigma, St Louis, MO, USA), using a Teflon® pestle glass homogenizer (Schmidt and Co., Frankfurt am Main, Germany). The homogenates were centrifuged at low speed (4000 rpm) for 30 min at 4°C to remove nuclei and cell debris. Pellets were suspended in isolation solution with protease inhibitors. Protein concentrations were determined using the Bradford assay method (Bio-Rad Protein Assay Kit, Bio-Rad Laboratories, Hercules, CA, USA).

**Electrophoresis and immunoblotting**

Kidney samples were run on 8% (for NKCC2, NCC and ACE) and 10% (for ENaC, angiotensinogen and AT1r) polyacrylamide gels. After transfer by electroelution onto nitrocellulose membranes (PolyScreen, PVDF Transfer, Life Science Products, Boston, MA, USA), blots were blocked with 5% skimmed milk and 0.1% Tween 20 in Tris-buffered saline for 1 h. Blots were then incubated overnight with anti-angiotensinogen (1:1000), anti-AT1r (1:500), anti-ACE (1:1000), anti-NKCC2 (1:2000), anti-NCC (1:500), anti-αENaC (1:500), anti-βENaC (1:200) and anti-γENaC (1:500) antibodies. The labelling was visualized with a horseradish peroxidase-conjugated secondary antibody (anti-rabbit IgG, diluted 1:2000 or anti-goat IgG, diluted 1:10000), using the enhanced chemiluminescence detection system ECL western blotting detection reagents (GE Healthcare, Buckinghamshire, UK). Specific polyclonal antibodies for AT1r, angiotensinogen, ACE and βENaC were obtained from Santa Cruz Biotechnology, CA, USA, specific polyclonal antibodies for NKCC2, αENaC and γENaC were obtained from Millipore, CA, USA and specific polyclonal antibodies for NCC were obtained from Stressmarq, Victoria, BC, Canada. As a loading control, blots were incubated with an α-actin antibody (Santa Cruz, CA, USA; 1:2000 with anti-goat 1:10000).

**Quantification of renal protein levels**

Images were obtained using the chemiluminescence image system Alliance 4.2 (Uvitech, Cambridge, UK). The semi-quantification of proteins was done using the software Image J for Windows® (National Institute of Standards and Technology, USA). The target protein bands were normalized through evaluation of densitometric α-actin protein abundance. Results were expressed as percentages of control values.

**Immunohistochemistry studies**

Sections of kidney (4 μm) were incubated overnight at room temperature (1:2000) with a polyclonal primary antibody against angiotensin II (Peninsula Laboratories, USA). The reaction product was detected with an avidin-biotin-peroxidase complex (Vector Laboratories, Burlingame, CA, USA). The colour reaction was developed with 3,3-diaminobenzidine (DAB) and the material was counterstained with Harris haematoxylin, dehydrated and mounted.

To evaluate the immunostaining for angiotensin II, the proportions of positive areas in tissue sections, determined by the limit colour, were obtained by image analysis with Image-Pro Plus software (Media Cybernetics, Silver Spring, MD, USA) on a computer connected to a digital camera and coupled to a microscope (Axioskop 40, Carl Zeiss, Göttingen, Germany). The volume proportion is expressed as the area of the tissue section testing positive for angiotensin II divided by the total area of the section. Results were expressed as percentages.

**Maternal evaluation**

We also conducted series of experiments [inulin clearance, measurement of arterial pressure, renal blood flow (RBF) and renal vascular resistance (RVR), metabolic cage studies, evaluation of biochemical parameters and urinary thiobarbituric acid reactive substances (TBARS)] with mothers fed with standard diet (control mothers, n=8) or standard diet with tenofovir DF (tenofovir DF mothers, n=8) 48 h after delivery.

**Haemodynamic studies**

To evaluate the MAP and allow blood sampling, a PE-60 catheter was inserted into the right carotid artery during the inulin clearance studies. MAP was assessed using an MP100 system (Biopac Systems, Santa Barbara, CA, USA).

After the inulin clearance experiment, a midline incision was made to measure the RBF. We carefully dissected the left renal pedicle and isolated the renal artery, taking precautions to avoid disturbing the renal nerves. An ultrasonic flow probe was placed around the exposed renal artery. RBF was measured using an ultrasonic flowmeter (T402, Transonic Systems, Bethesda, MD, USA) and is expressed as mL/min. RVR was calculated by dividing the blood pressure by RBF and is expressed as mmHg/mL/min.

**Assessment of reactive oxygen metabolites**

Urinary levels of TBARS, which are markers of lipid peroxidation, were determined using the thiobarbituric acid assay. Briefly, a 0.2 mL plasma or urinary sample was diluted in 0.8 mL of distilled water. Immediately thereafter, 1 mL of 17.5% trichloroacetic acid was added. Following the addition of 1 mL of 0.6% thiobarbituric acid (pH 2), the sample was placed in a boiling water bath for 15 min, after which it was allowed to cool. Subsequently, 1 mL of 70% trichloroacetic acid was added and the mixture was incubated for 20 min. The sample was then centrifuged for 15 min at 2000 rpm. The OD of the supernatant was read at 534 nm against a reagent blank using a spectrophotometer. The quantity of TBARS was calculated using a molar extinction coefficient of 1.56×10^5 M⁻¹ cm⁻¹.

**Statistical analysis**

All quantitative data are expressed as mean ± SEM. The unpaired t-test was employed to compare the means of two groups of data. Values of P<0.05 were considered statistically significant. Data were analysed using GraphPad Prism software 5.0.

**Results**

Tenofovir DF offspring showed a significant reduction in BW (6.18 ± 0.48 versus control 6.55 ± 0.58 g, P=0.0006) (Figure 1b). This reduction in BW was maintained up to the third month of age, after which animals in the tenofovir DF group experienced rapid catch-up growth, reaching the sixth month heavier than
the control group (Figure 1c). Naso–anal length was also reduced in the tenofovir DF group compared with controls at birth (4.78 ± 0.02 versus control 4.93 ± 0.03 cm, P = 0.009), 1 week of age (7.22 ± 0.05 versus control 7.78 ± 0.07 cm, P = 0.001) and 2 weeks of age (8.95 ± 0.23 versus control 10.13 ± 0.08 cm, P < 0.0001). No differences were found in litter size at birth between the groups. In order to rule out the influence of the adoption process in growth restriction of the tenofovir DF offspring, soon after delivery we carried out an exchange of offspring from two control mothers during the breastfeeding period. Growth parameters did not differ between the control group and offspring from the control group with foster mother. However, both control groups maintained the differences with the tenofovir DF group.

**Blood pressure data**

As seen in Figure 2, the systolic arterial pressure increased after 30 days of age in the tenofovir DF group (138 ± 3 versus control 111 ± 2 mmHg, P < 0.0001) and rose progressively until 6 months of follow-up (155 ± 2 versus control 119 ± 2 mmHg, P < 0.0001).

**Volume and volume densities of kidney compartments and number of glomeruli**

In the kidneys obtained from animals at 3 months of age, total volume of the kidney (tenofovir DF 1625 ± 165 versus control 1528 ± 88 mm³, P = 0.5), total volume of the renal cortex (tenofovir DF 1181 ± 144 versus control 1067 ± 72 mm³, P = 0.4), volume of the renal medulla (tenofovir DF 316 ± 25 versus control 304 ± 24 mm³, P = 0.7) and total number of renal corpuscles (tenofovir DF 43261 ± 2982 versus control 38141 ± 3175 U, P = 0.2) did not differ between the groups. We found that tenofovir DF offspring showed glomerular structural changes in ~12% of the total nephron number (4846 ± 1455 altered glomerular units). Glomerular shrinkage was the most common abnormality encountered (Figure 3a–e). Thickening of the parietal layer of

![Figure 2](image-url)  
*Figure 2.* Systolic blood pressure increased progressively after 30 days of age in the tenofovir disoproxil fumarate (TDF) group compared with controls (C). Unpaired t-test was applied to evaluate statistical significance of the comparison of TDF and C groups. ***P < 0.0001.

![Figure 3](image-url)  
*Figure 3.* Histopathology of renal cortex. Low (a) and high (b) magnification photomicrographs of control kidneys showing normal renal corpuscles and normal-sized glomeruli. Tenofovir disoproxil fumarate rat kidneys showing: (c, asterisk) glomerular shrinkage (atrophy); (d, arrow) enlargement of the capsular space; and (e, arrow) reduced vascularization of the glomerulus. This figure appears in colour in the online version of JAC and in black and white in the print version of JAC.
Bowman’s capsule and decreased vascularization were also seen in a few glomeruli.

**Renal function data**

Tenofovir DF animals showed no statistically significant difference in inulin clearance at 3 and 6 months of age: third month, tenofovir DF 3.3 ± 0.2 versus control 3.8 ± 0.2 mL/min, \( P = 0.1 \); and sixth month, tenofovir DF 3.2 ± 0.2 versus control 3.0 ± 0.2 mL/min, \( P = 0.4 \).

**Biochemical parameters**

Animals from the tenofovir DF group showed higher plasma sodium at 6 months of age and lower urinary sodium excretion than the control group at 3 and 6 months of age. Plasma aldosterone was higher in the tenofovir DF group. Animals of the tenofovir DF group exhibited higher total cholesterol levels at 6 months of age. Proteinuria was not observed in animals of either group (tenofovir DF 11.1 ± 0.8 versus control 11.8 ± 0.5 mg/24 h, \( P = 0.6 \)). Glucosuria was not detectable in all animals from both groups. Plasma phosphate was not different between the groups at 3 and 6 months of age. Table 1 shows the biochemical parameters evaluated at 3 and 6 months of age.

**Western blot analysis**

It is well known that apical NKCC2 and NCC are the major transporters for sodium reabsorption, respectively, in the thick ascending limb of the loop of Henle and in the distal convoluted tubule. Rats from the tenofovir DF group showed higher NKCC2 (148% ± 6% for tenofovir DF versus 100% ± 7% for control, \( P = 0.0007 \)) and NCC (120% ± 5% for tenofovir DF versus 99% ± 7% for control, \( P = 0.03 \)) protein abundance in the kidneys compared with the control group (Figure 4a and b). Another key renal sodium transporter is ENaC. It consists of three different subunits (\( \alpha, \beta \) and \( \gamma \)) involved in the regulation of sodium transport in

---

### Table 1. Biochemical measurements in animals from the tenofovir disoprophil fumarate (TDF) and control groups at 3 and 6 months of age

<table>
<thead>
<tr>
<th>Evaluated data</th>
<th>Third month</th>
<th></th>
<th></th>
<th></th>
<th>Sixth month</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>control (n=9)</td>
<td>TDF (n=9)</td>
<td>( P ) value</td>
<td>control (n=12)</td>
<td>TDF (n=10)</td>
<td>( P ) value</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Plasma</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Na(^+) (mEq/L)</td>
<td>139 ± 2</td>
<td>140 ± 1</td>
<td>0.6</td>
<td>142 ± 3</td>
<td>152 ± 3</td>
<td>0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>K(^+) (mEq/L)</td>
<td>5.0 ± 0.2</td>
<td>5.0 ± 0.2</td>
<td>0.8</td>
<td>4.4 ± 0.2</td>
<td>4.5 ± 0.2</td>
<td>0.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PO(_4)(^{3-}) (mg/dL)</td>
<td>6.3 ± 0.3</td>
<td>6.0 ± 0.3</td>
<td>0.3</td>
<td>4.3 ± 0.3</td>
<td>4.3 ± 0.4</td>
<td>0.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>total cholesterol (mg/dL)</td>
<td>52 ± 3</td>
<td>54 ± 5</td>
<td>0.7</td>
<td>52 ± 4</td>
<td>72 ± 3</td>
<td>0.002</td>
<td></td>
<td></td>
</tr>
<tr>
<td>aldosterone (pg/mL)</td>
<td>112 ± 23</td>
<td>256 ± 45</td>
<td>0.01</td>
<td>116 ± 26</td>
<td>247 ± 28</td>
<td>0.002</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Urine</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UNaV (mg/24 h)</td>
<td>0.86 ± 0.07</td>
<td>0.61 ± 0.07</td>
<td>0.02</td>
<td>0.55 ± 0.07</td>
<td>0.33 ± 0.02</td>
<td>0.02</td>
<td></td>
<td></td>
</tr>
<tr>
<td>UKV (mg/24 h)</td>
<td>2.56 ± 0.11</td>
<td>2.30 ± 0.11</td>
<td>0.1</td>
<td>1.67 ± 0.18</td>
<td>1.30 ± 0.05</td>
<td>0.1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

aldo, aldosterone; UNaV, urinary sodium excretion; UKV, urinary potassium excretion.

Data are reported as mean ± SEM; unpaired \( t \)-test was employed to compare the means of data; \( P \) value is for the comparison of TDF versus control offspring at 3 and 6 months of age.

---

**Figure 4.** Immunoblotting of kidney fractions from tenofovir disoprophil fumarate (TDF) and control (C) animals at 6 months of age. TDF rats exhibited enhanced renal expression of Na-K-2Cl co-transporter (NKCC2) — 160 kDa (a) and Na-Cl (NCC) co-transporter — 160 kDa (b). Data are expressed as percentages of the control. Data are presented as mean ± SEM; \(^* P = 0.03\) and \(^{***} P = 0.0007\) (unpaired \( t \)-test) for the comparison TDF versus C.
the collecting duct. As shown in Figure 5(a–c), protein expression of ENaC in tenofovir DF-treated rats was significantly higher than in the control rats (α-ENaC: tenofovir DF 147% ± 2% versus control 102% ± 2%, P < 0.0001; β-ENaC: tenofovir DF 138% ± 11% versus control 99% ± 8%, P = 0.02; and γ-ENaC: tenofovir DF 151% ± 12% versus control 99% ± 1%, P = 0.007). Interestingly, renal protein expression of angiotensinogen (tenofovir DF 129% ± 9% versus control 101% ± 1%, P = 0.04), AT1r (tenofovir DF 173% ± 23% versus control 100% ± 11%, P = 0.03) and ACE (tenofovir DF 214% ± 27% versus control 100% ± 6%, P = 0.01) were also up-regulated in the tenofovir DF group (Figure 6a–c), supporting the activation of the renal renin angiotensin aldosterone system (RAAS).

Immunohistochemistry studies

Animals from the tenofovir DF group exhibited increased expression of angiotensin II in the renal cortex at 6 months of age (tenofovir DF 8.3% ± 1.6% versus control 1.0% ± 0.2%, P = 0.0009). Intense cortical staining for angiotensin II was observed predominantly in the tubular cells. Figure 7 shows the quantification and immunolocalization of the cells stained for angiotensin II in the renal cortex.

Maternal evaluation

There were no differences among the groups in terms of food and water intake during pregnancy. Daily food intake varied from 20 to

![Figure 5. Immunoblotting of kidney fractions from tenofovir disoproxil fumarate (TDF) and control (C) animals at 6 months of age. TDF rats exhibited enhanced renal expression of epithelial sodium channel (ENaC) subunits (a) α (97 kDa), (b) β (95 kDa) and (c) γ (85 kDa). Data are expressed as percentages of the control values. Data are presented as mean ± SEM; *P = 0.02, **P = 0.007 and ***P < 0.0001 (unpaired t-test) for the comparison TDF versus C.](image1)

![Figure 6. Immunoblotting of kidney fractions from tenofovir disoproxil fumarate (TDF) and control (C) animals at 6 months of age. TDF animals showed higher renal protein abundance of angiotensinogen (Ang)—60 kDa (a), angiotensin II receptor type 1 (AT1r)—43 kDa (b) and angiotensin-converting enzyme (ACE)—195 kDa (c). Data are expressed as percentages of the control values. Data are presented as mean ± SEM; *P < 0.05 (unpaired t-test) for the comparison TDF versus C.](image2)
25 g/animal/day. There were only small and not statistically differences in blood pressure, RBF and RVR between the groups, which were not accompanied by a significant change in GFR. No differences were observed in biochemical parameters between the mothers from the control and the tenofovir DF groups. Plasma aldosterone levels in the mothers from both the tenofovir DF and the control groups were higher than in the offspring, which may represent the physiological increase in RAAS activity during pregnancy.

Table 2 provides an overview of the biochemical and functional parameters in mothers from the control and the tenofovir DF groups 48 h after delivery.

**Discussion**

Developmental programming is the term used to describe the concept that many adult conditions or diseases occur in response to environmental stimuli experienced during critical periods of fetal and early postnatal growth. \(^2^2\) Many studies support the finding that small modifications in fetal growth may have profound consequences in later life. \(^2^3\) - \(^2^8\) The present study reports several important findings regarding the deleterious effects in offspring exposed to tenofovir DF during gestation. Our data show that tenofovir DF might be related to intrauterine growth retardation (IUGR) with consequences in adult life.

Compared with the study by Tarantal et al., \(^1^1\) in which high doses of tenofovir DF were administrated to pregnant rhesus monkeys, we found similar results regarding the lower anthropometrical parameters of the offspring exposed in utero to tenofovir DF. On the other hand, we further followed up the offspring from the tenofovir DF-exposed mothers until adulthood and reported systemic and renal adverse effects. Another important difference of our study concerns the tenofovir DF dose. We used the principle

### Table 2. Biochemical and functional parameters in mothers from the tenofovir disoproxil fumarate (TDF) and control groups 48 h after delivery

<table>
<thead>
<tr>
<th>Evaluated data</th>
<th>Control (n=8)</th>
<th>TDF (n=6)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Plasma</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Na(^+) (mEq/L)</td>
<td>143 ± 2</td>
<td>141 ± 3</td>
<td>0.5</td>
</tr>
<tr>
<td>K(^+) (mEq/L)</td>
<td>4.6 ± 0.6</td>
<td>3.8 ± 0.2</td>
<td>0.1</td>
</tr>
<tr>
<td>Ca(^{2+}) (mg/dL)</td>
<td>3.7 ± 0.5</td>
<td>3.1 ± 0.5</td>
<td>0.2</td>
</tr>
<tr>
<td>PO(_4)(^{3-}) (mg/dL)</td>
<td>5.1 ± 0.6</td>
<td>5.5 ± 0.5</td>
<td>0.6</td>
</tr>
<tr>
<td>Aldo (pg/mL)</td>
<td>219 ± 31</td>
<td>324 ± 68</td>
<td>0.1</td>
</tr>
<tr>
<td><strong>Urine</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GFR (mL/min)</td>
<td>2.93 ± 0.13</td>
<td>2.78 ± 0.17</td>
<td>0.4</td>
</tr>
<tr>
<td>TBARS (nmol/24 h)</td>
<td>151 ± 20</td>
<td>158 ± 20</td>
<td>0.8</td>
</tr>
<tr>
<td><strong>Haemodynamic studies</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MAP (mmHg)</td>
<td>128 ± 4</td>
<td>136 ± 5</td>
<td>0.2</td>
</tr>
<tr>
<td>RBF (mL/min)</td>
<td>6.0 ± 0.4</td>
<td>5.5 ± 0.1</td>
<td>0.2</td>
</tr>
<tr>
<td>RVR (mmHg/mL/min)</td>
<td>21 ± 1</td>
<td>24 ± 1</td>
<td>0.1</td>
</tr>
</tbody>
</table>

aldo, aldosterone; GFR, glomerular filtration rate assessed by inulin clearance; TBARS, thiobarbituric acid reactive substances; MAP, mean arterial pressure; RBF, renal blood flow; RVR, renal vascular resistance. Data are reported as mean ± SEM; unpaired t-test was employed to compare the means of data; P value is for the comparison of TDF versus control mothers.
of scaling to extrapolate the dose from humans to rats. Scaling, in the context of biology, is a term that refers to the adaptation of a biological system to function at a different metabolism. Allometry is the study of size and its consequences. Thus, allometric scaling is used to consider the metabolic rate of an animal and its size to create an exponent to scale doses between species (dose-by-factor approach).29 According to the US FDA dose-by-factor approach, the following formula is used to scale doses between animals and humans: \[ \text{HED} = \left( \frac{\text{animal wt}}{\text{human wt}} \right)^{0.33} \times \text{animal mg/kg-dose} \times \text{animal BW} \times \text{Dose}, \] where \( \text{HED} \) is the human equivalent dose.30 Nevertheless, the FDA approach does not consider the pharmacokinetic properties of a drug. It is a dose extrapolation based on the body surface area and metabolic rate. If the FDA-recommended approach had been used in this study, the dose of tenofovir DF applied for the rat would be \( \approx 28 \) mg/kg/day (almost six times higher than the human dose, considering 300 mg/day in an adult weighting 60 kg). However, since we were performing a study of drug safety during pregnancy, we decided to use a lower dose of tenofovir DF (\( \approx 8 \) mg/kg/day, assuming a daily food intake of 20 g/day and a mean BW of 230 g).

In contrast, data from the PHACS cohort showed that tenofovir DF use during pregnancy was not associated with increased risk of lower birth weight or small for gestational age. However, lower mean length for age and head circumference for age (Z-score) were observed at age 1 year in the tenofovir DF-exposed infants.29 A major shortcoming of the PHACS study was that the median duration of tenofovir DF exposure during pregnancy varied between the participants. Furthermore, tenofovir DF deleterious effects might be related not only to the exposure duration but also to the period of gestation in which tenofovir DF was administered. In this regard, there are a few advantages of using a rat model for our study. We were able to administer tenofovir DF during the entire gestational period. Furthermore, the rat is an excellent model for kidney disease and can reflect the pathological process seen in human kidneys. The rat model may also be suitable for mechanistic studies of fetal programming, mainly because humans and rats present common outcomes of fetal growth restriction, such as hypertension, dyslipidaemia and over-activation of RAAS.30

It has been shown previously that tenofovir DF exhibits significant placental transport in rhesus monkeys, reaching peak fetal levels 1–3 h after maternal administration.31 Tenofovir DF rapidly crosses the placenta in pregnant women on HAART, achieving a maternal–cord blood tenofovir ratio of \( \approx 0.65–1.00.32 \) Neumanova et al. performed a study evaluating the transport of tenofovir using an isolated perfused Wistar rat placenta. Tenofovir exhibited a passive transport across the placenta in both maternal-to-fetal and fetal-to-maternal directions. In addition, Nirogi et al.33 studied the pharmacokinetic profile of a single dose of efavirenz/emtricitabine/tenofovir in pregnant rats. They showed that \( \approx 76% \) of circulating tenofovir DF appeared in the placenta, which was consistent with the results found in humans and monkeys.33 Thus, we assume that tenofovir DF passes over the placenta and disturbs the in utero environment and may induce a permanent response in the fetus and the newborn, leading to enhanced susceptibility to later diseases such as hypertension and dyslipidaemia. Moreover, we could not associate the restriction in fetal growth with any biochemical abnormalities, haemodynamic changes, increase in oxidative stress or overactivation of RAAS in the mothers treated with tenofovir DF during pregnancy, supporting the hypothesis of a direct effect of the drug on the fetus during organogenesis.

Other data supporting impaired fetal growth in tenofovir DF offspring are that animals from this group were born smaller than controls and changed their growth pattern after the third month of age, reaching the sixth month heavier than the control group. The increased growth rate has been previously described following IUGR and is usually called catch-up growth. Many studies have associated the rapid increases in weight after birth with higher blood pressure and hypercholesterolaemia, as shown in our experimental model.34–37 Nevertheless, birth weight should not be considered the triggering feature itself, but rather a simple measure of a more global process, leading to a marked change of organ structure, organ function or both and to later-life diseases.38

Erhuma et al.39 demonstrated that a low protein diet during pregnancy was associated with programmed dyslipidaemia of the offspring 9 months of age. Zhang et al.40 found similar results in rats subjected to dietary iron restriction during gestation. In retrospective and prospective clinical studies, lower birth weight and lower abdominal circumference at birth have been linked to dyslipidaemia in childhood as well as in adult life.41,42 In the liver, cholesterol is converted into biliary acids by 3-hydroxy-3-methylglutaryl-CoA reductase and cholesterol 7a-hydroxylase (CYP7A1). Both enzymes undergo maturation in fetal and neonatal life.43 Previous studies have revealed that the activity of these enzymes may be programmed in the context of IUGR.39,44 Consequently, we assume that tenofovir DF exposure during pregnancy might be a new pathway to programming dyslipidaemia.

The RAAS plays a key role in regulating blood pressure and fluid balance, especially through the systemic effects of angiotensin II and aldosterone. However, the inappropriate activation of this system has been associated with systemic deleterious implications.45 In this regard, there are several studies addressing the involvement of RAAS in the pathogenesis of hypertension. In the Framingham Offspring Study, the investigators found a 16% increase in the risk of an elevation in blood pressure and a 17% increase in the risk of hypertension per quartile increment in the serum aldosterone level.46 Reynolds et al.47 showed higher aldosterone levels in men and women with low BW and high blood pressure. In addition, Sahajpal et al.48 demonstrated that renal expression of AT1r was increased in the offspring of dams fed with low protein diet and may have caused hypertension. Accordingly, we found renal up-regulation of several components of the RAAS, associated with higher levels of plasma aldosterone, in the animals exposed to tenofovir DF during gestation. Therefore, we showed an important involvement of the renal and systemic RAAS in the pathogenesis of hypertension in our experimental model.

Brenner et al.49 and Brenner and Chertow50 proposed that a congenital or programmed reduction in nephron number and consequently in whole kidney glomerular surface area may be a main factor in the generation of hypertension. However, the paucity of nephrons is not sufficient to explain hypertension in all models of prenatal programming. Other elements would also participate together with or independently of the number of glomeruli in the pathogenesis of hypertension.51 In humans, nephrogenesis starts at week 6 of gestation and new nephrons are formed until week 36, after which kidney development is based on further growth of existing structures.52 In the Wistar rat, glomerulogenesis continues until the second week after birth and renal tubular maturity may take even longer.53–55 Therefore, we decided to
perform the stereological analysis at 3 months of age, to assure that all nephrons had already been formed and to detect potential renal abnormalities. The final number of nephrons in Wistar rats varies by strain, but ~40000 glomeruli is considered normal.56 We did not find a reduction in nephron numbers in either group studied. However, ~12% of the total nephron number in the tenofovir DF offspring did not display normal morphology. Glomerular shrinkage, the most common alteration encountered, has been described as the initial glomerular lesion of hypertensive nephrosclerosis.57 We performed histomorphometrical analysis at the third month of age, when the animals had already been hypertensive for ~60 days; therefore, the glomerular alterations found in our study may be compatible with the glomerular structural changes of hypertensive nephrosclerosis in the initial stage.

Another important mechanism possibly involved in the pathogenesis of programmed hypertension is the up-regulation of renal transporters of sodium. The thick ascending limb of the loop of Henle, together with the convoluted tubule and the collecting duct, reabsorb >30% of sodium filtered through glomeruli.58 Enhanced sodium reabsorption in these segments of the nephron leads to many forms of hypertension.51,59,60 Thus, our study provides evidence that up-regulation of renal sodium transporters may be involved in the generation and maintenance of hypertension in offspring exposed to tenofovir DF during gestation. We did not find proteinuria or a significant difference in inulin clearance at the third and the sixth month of age between the tenofovir DF and the control groups, indicating that renal dysfunction was not associated with increased blood pressure in this model.

Prolonged tenofovir DF treatment has been associated with proximal renal tubular dysfunction (PRTD; partial or complete dysfunction), usually manifested with glucosuria and/or hypophosphatemia.53 We did not find evidence of PRTD in the offspring exposed to tenofovir DF during pregnancy, since glucosuria was negative and plasma phosphate was normal. However, the offspring in our study were not directly or for the long term exposed to tenofovir DF, which may explain the absence of PRTD in this animal sample.

In conclusion, the present study is the first demonstration using an experimental model that maternal exposure to tenofovir DF during pregnancy results in hypertension and dyslipidaemia of offspring. Our findings also suggest that overactivation of the RAAS and up-regulation of renal sodium transporters may contribute to the generation and maintenance of hypertension. Tenofovir DF exposure has increased in HIV-infected women, especially among pregnant women. Moreover, given the consistency of our results, we hope that this study motivates further clinical studies to verify the reproducibility of these data in humans, which might have a profound impact on the choice of the ARV during gestation. Tenofovir DF demands a wider assessment of the safety profile during pregnancy, particularly regarding the potential renal and cardiovascular deleterious effects in the offspring reaching adult life. It is compelling to follow up children and adolescents born to mothers who have received tenofovir DF during pregnancy.

Acknowledgements

Preliminary results of this work were presented at the Cell/The Lancet Translational Medicine Conference (‘What Will it Take to Achieve an AIDS-free World?’), San Francisco, CA, USA, 2013 (abstract no. CHIV2013_0072).

We are sincerely grateful to Dr Adriana Girardi for kindly providing us with the anti-ACE antibody. We also thank Professor Dr Joel Claudio Heimann and Dr Luzia Furukawa for allowing us to use their electro-sphygmomanometer.

Funding

This work was supported by Brazilian National Funding Agencies: ‘Fundaçao de Amparo à Pesquisa do Estado de São Paulo’—FAPESP (2012/50227-9) and ‘Conselho Nacional de Desenvolvimento Científico e Tecnológico’— CNPq (306148/2013-7).

Transparency declarations

None to declare.

References