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Renal developmental disturbances and their long-term consequences in female pups from vitamin D-deficient mothers: involved mechanisms

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Brief Report

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Abstract

The mechanisms involved in kidney disturbances during development, induced by vitamin D_3 deficiency in female rats, that persist into adulthood were evaluated in this study. Female offspring from mothers fed normal (control group, $n = 8$) or vitamin D-deficient (Vit.D-, $n = 10$) diets were used. Three-month-old rats had their systolic blood pressure (SBP) measured and their blood and urine sampled to quantify vitamin D_3 (Vit.D_3), creatinine, Na^+, Ca^{+2} and angiotensin II (ANGII) levels. The kidneys were then removed for nitric oxide (NO) quantification and immunohistochemical studies. Vit.D- pups showed higher SBP and plasma ANGII levels in adulthood ($P < 0.05$) as well as decreased urine osmolality associated with increases in urinary volume ($P < 0.05$). Decreased expression of JG12 (renal cortex and glomeruli) and synaptopodin (glomeruli) as well as reduced renal NO was also observed ($P < 0.05$). These findings showed that renal disturbances in development in pups from Vit. D- mothers observed in adulthood may be related to the development of angiogenesis, NO and ANGII alterations.

Introduction

The association between intrauterine events and subsequent renal diseases has been recognized for the last few years.[1,2] Several reports have shown that changes in the number of nephrons and/or renal corpuscles that occur during renal development have consequences in adulthood, such as hypertension and kidney disease.[3,4]

Vitamin D (Vit.D) is mostly known for its role in the regulation of calcium homeostasis and bone metabolism.[5] However, recent studies have shown that Vit.D is also involved in the homeostasis of several other cellular processes, including inflammation, blood pressure control,[6,7] cell proliferation and differentiation[7] and renin gene regulation, which are all important events in kidney development.[6] Song *et al.*[8] demonstrated that the renin angiotensin system (RAS) contributes to increased proliferation and branched ureteric buds *in vivo* and stimulates renal vessel formation during kidney development.[9] Pups from mothers subjected to a vitamin D_3 (Vit.D_3)-deficient diet during pregnancy and lactation exhibit changes in kidney development associated with alterations in the RAS.[10,11] This study investigates the influence of Vit.D_3 deficiency on changes in kidney development in female rats, the morphological and functional consequences in adulthood and the mechanisms involved in this process.

Methods

Animals and experimental design

The experiments were performed in accordance with the ethical principles for animal experimentation adopted by the Brazilian College of Animal Experimentation, and the Animal Experimentation Committee of the University of São Paulo at Ribeirão Preto Medical School approved the study protocol (COBEA/CETEA/FMRP-USP, protocol no. 190/2016).

Female Hannover rats 6-week-old ($n = 9$) were separated into two groups that received standard rat chow (SC) (made according to the AIN93G protocol, including 1000.0 IU/kg of Vit.D_3) or a vitamin D-deficient (Vit.D-) chow (made according to the same AIN93G protocol

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but with no Vit.D_3 added) for 6 weeks. The diets were produced and marketed by PragSolucoes (Jau, SP, Brazil), and their components are shown in the Supplementary Table 1. The rats were housed four per cage according to the groups, with a temperature of 20 ± 2°C, a 12 h ultraviolet B-free light/dark cycle and *ad libitum* access to food and water. Male rats of the same age ($n = 3$) were separated and fed only the SC diet. After the 6-week period of the SC or Vit.D- diets, the rats were subjected to mating overnight. For mating, each male was housed with three females, and the first gestational day was determined based on the presence of copulatory plugs. Pregnant females were separated from males and fed with their respective experimental diets until the 21st day of lactation, when the Vit.D- diet was switched to the SC diet. This procedure guaranteed that offspring received the experimental diet only during the kidney developmental period. At birth, the litter was reduced to eight pups per mother to ensure adequate and standardized nutrition until weaning, and then the female was separated from the pups. After weaning, the mothers were killed, and the blood samples were used for the quantification of Vit.D and calcium levels. Body weight (BW) was evaluated at birth and weekly until the end of lactation (21st day) as well as at 3 months of age.

A vaginal scrub was performed on all female rats, and only those in dioestrus were selected on the final day experiment to avoid hormonal differences that might influence the results. This period of the hormonal cycle primarily consists of a leukocyte predominance in the vaginal scrub.[12]

Systolic blood pressure measurement

After acclimation and preconditioning 4–5 days before the procedure, SBP was measured in conscious 90-day-old pups using the tail-cuff method (CODA System, Kent Scientific, Torrington, CT, USA) as previously described.[13]

Renal function studies

At 89 days of age, the pups were moved to metabolic cages for 24 h, and a urine sample was collected for an electroimmunoassay albumin measurement using a specific antibody against rat albumin (data expressed as urinary albumin per 24 h).[14] The determination of urine osmolality was performed using an osmometer (Fiske OS Osmometer, Advanced Instruments, Norwood, MA, USA). Then, the female rats were anaesthetized at 90 days of age (sodium thiopental, 40 mg/kg, i.p.), and the aorta was cannulated for blood sample collection. Creatinine was measured using a commercial kit (Labtest Diagnostica S.A., Lagoa Santa, Brazil) to evaluate the creatinine and sodium levels using a 9180 series electrolyte analyser (Roche, Vienna, Austria) for the plasma and urine samples. The kidneys were then removed for tissue nitric oxide (NO) quantification (right kidneys) and immunohistochemical studies (left kidneys).

Determination of plasmatic angiotensin II

Plasma concentrations of angiotensin II (ANGII) were measured by specific radioimmunoassay as previously described.[15] ANGII was extracted from 1 ml of plasma using SepPak C-18 cartridges (Waters Corporation, Milford, MA, USA). The assay sensitivity as well as intra- and inter-assay coefficients of variation were 0.39 pg/ml, 4.17 and 10.3% for ANGII, respectively.

Serum Vit.D levels

The serum was stored at −70°C for further determination of 25-hydroxy-Vit.D, which was performed in the clinical analysis laboratories at the School of Medicine of Ribeirão Preto Hospital and Clinics using a chemiluminescence analyser (DiaSorin, Liaizon® XL).

Immunohistochemical studies

The kidneys were embedded in paraffin using routine histological techniques and cut transversally (5 µm thick). After deparaffinization in xylol, nonspecific antigen binding was blocked by incubation for 20 min with normal goat serum. The sections were incubated with anti-JG12 (1:800; eBioScience, Thermo Fisher Scientific, CA, USA), anti-cubilin (1:1500; Santa Biotechnology, Santa Cruz, CA, USA) or anti-synaptopodin antibodies (1:400; Santa Biotechnology) for 60 min at room temperature. The avidin–biotin–peroxidase complex (Vector Laboratories, Burlingame, CA, USA) was used to detect the reaction products. The sections were then counterstained with methyl green and dehydrated, and then the slides were mounted with Permount mounting medium (Fischer Scientific, NJ, USA).

To quantify JG12, synaptopodin and cubilin staining, each glomerulus or cortical field (measuring 0.100 mm^2 each) was semi-quantitatively graded, and the mean score per section was calculated.[16] The scores mainly reflected changes in the extent rather than in the intensity of staining and were dependent on the percentage of the glomeruli or grid field, showing positive staining. The scores were determined as follows: 0 = absent or < 5% staining; 1 = 5–25%; 2 = 25–50%; 3 = 50–75% and 4 > 75% staining.[15] The number of JG12-positive cells in each cortical interstitial 30-grid field was determined in the renal cortex, and the mean counts were calculated for each section.[16] It has been demonstrated that the semi-quantitative scoring system is not only reproducible among different observers but that data are also highly correlated with those obtained through computerized morphometry by several authors.[17]

Statistical analysis

Comparisons between the Vit.D- and control group (CG) groups were performed using unpaired Student's *t*-tests using GraphPad Prism software version 6 (GraphPad, CA, USA). The data that were not normally distributed were statistically analysed using the nonparametric Kruskal–Wallis test followed by the Dunn post-test. Those data were expressed as medians and interquartile ranges (25–75%). For the normally distributed data, we used analysis of variance with the Newman–Keuls multiple comparisons test. Those data were expressed as the mean ± S.E.M. In all comparisons, the level of significance was set at $P < 0.05$.

Results

BW, fluid intake and food consumption

There was no difference in BW, fluid intake and food consumption between the CG and Vit.D- mothers during gestation and lactation (data not shown). The Vit.D- pups had decreased BW ($P < 0.05$) at the end of lactation (51.7 ± 2.9 *v.* 60.8 ± 1.8) and increased BW in adulthood ($P < 0.05$) compared with that of the CG group (289 ± 17 *v.* 238 ± 21). Higher water intake was also observed in the Vit.D- pups (Table 1).

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Vit.D and calcium quantification

Serum 25-hydroxy-Vit.D levels were markedly lower in mothers fed Vit.D- chow ($P < 0.05$) when compared with that of the CG (8.23 ± 1.8 *v.* 48.56 ± 3.49 nmol/l, respectively), while serum Ca^{2+} levels did not differ between groups (Vit.D- $= 2.24 \pm 0.14$ *v.* CG $= 2.35 \pm 0.09$ group). The findings also found that 90-day-old offspring also had low Vit.D levels ($P < 0.05$) compared with that of the controls (23.23 ± 2.3 *v.* 39.56 ± 3.49 nmol/l, respectively) without changes in the Ca^{2+} levels (Vit.D- $= 2.25 \pm 0.04$ *v.* CG $= 2.19 \pm 0.06$).

Table 1. Renal function and water intake in 3-month-old adult offspring in the CG and Vit.D- groups

	3 months	
	CG	Vit.D-
	$n = 8$; $m = 3$	$n = 10$; $m = 4$
Water intake (ml 100/g)	8.3 ± 1.2	11.3 ± 1.8 *
V (ml 100/g)	6.4 ± 1.2	9.2 ± 1.5*
U (mOsm kg per H_2O)	1.578 ± 135	1.170 ± 105*
FE_{Na+} (%)	0.31	0.24*
	(0.24; 0.33)	(0.19; 0.24)
Pcreat	0.59 ± (0.53; 0.62)	0.72 ± (0.50; 0.83)
GFR (ml/min/100 g)	0.81 ± 0.07	0.76 ± 0.09
ALB (mg per 24 h)	0.5 (0.3; 0.6)	0.6 (0.3; 0.7)

24-hour urine volume (*V*), urinary osmolality (*U*), plasma creatinine mg% (Pcreat), fractional excretions of sodium (FE_{Na+}), GFR, ALB in 3-month-old pups from the CG and Vit.D- groups. Data are expressed as the median and interquartile range (25–75th) (ALB, FE, Pcreat) or mean ± S.E.M. (*V*, *U*, GFR). *n* = number of animals, *m* = number of litters. *$P < 0.05$ compared with controls of the same age.
GFR, glomerular filtration rate; ALB, albuminuria

Renal function

The animals subjected to the Vit.D- diet during kidney development presented with higher blood pressure at 3 months of age ($P < 0.05$) compared with that of the CG (128 ± 1.7 mmHg *v.* 117 ± 1.4 mmHg, respectively) as well as an increase ($P < 0.05$) in plasma ANGII levels (Vit.D- $= 48 \pm 5.6$ pg/ml *v.* CG $= 25 \pm 34$ pg/ml). These changes were associated with a significant decrease in urine osmolality and sodium fractional excretion as well as an increase in water intake and urinary volume ($P < 0.05$). No difference was observed in the creatinine plasma levels (Table 1).

Immunohistochemical studies

The Vit.D- animals had decreased expression of cubilin (a differentiation marker of the renal proximal tubule brush border) in the renal cortex compared with that of the CG group ($P < 0.05$) (Fig. 1a, 1b and 1i). A decrease in the expression of JG12 (an endothelial cell marker) was also observed in the capillaries of the renal cortex and glomeruli (Fig. 1c, 1d, 1e, 1f, 1j and 1k) and of synaptopodin (a component of the glomerular capillary wall) in glomeruli in the Vit.D- group compared with the CG group (Fig. 1g, 1h and 1l). These alterations were associated with a decrease ($P < 0.05$) in renal tissue NO levels (Vit.D- $= 0.03 \pm 0.02$ μM/μg protein *v.* CG $= 0.06 \pm 0.02$ μM/μg protein).

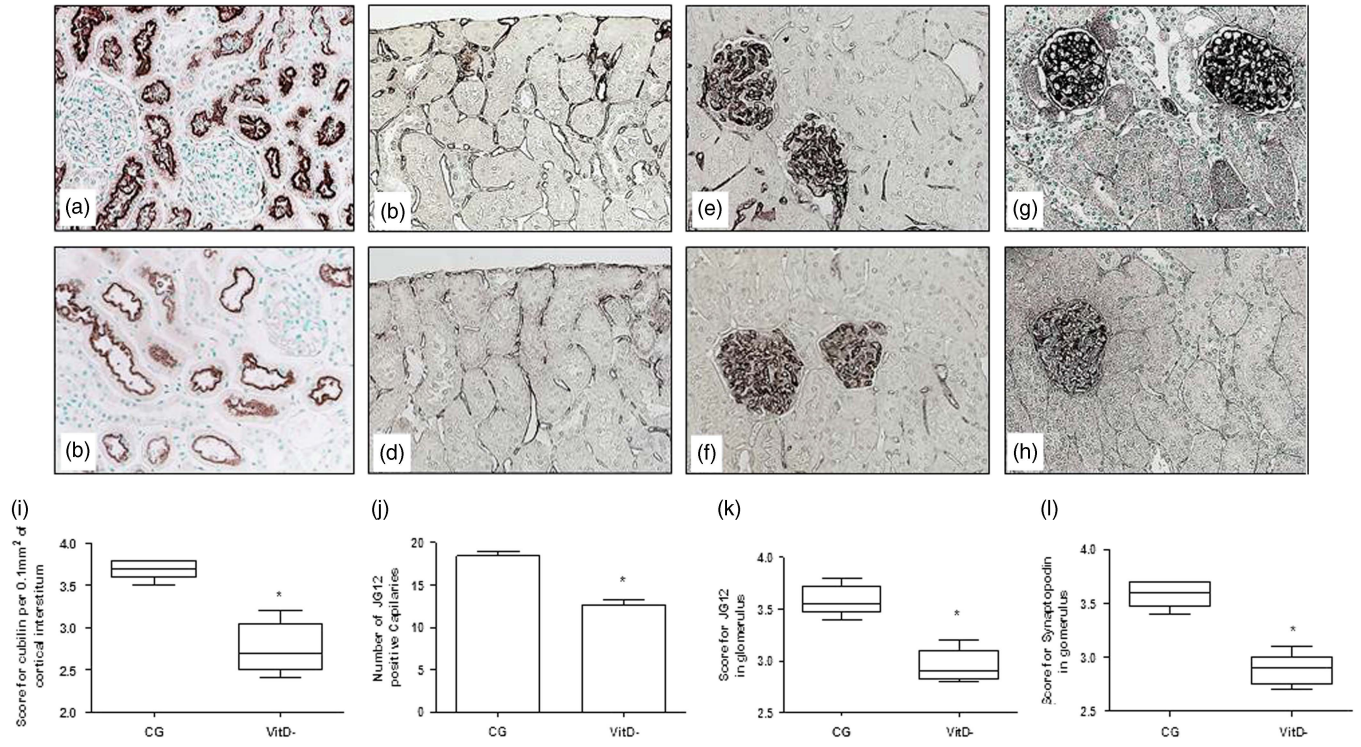

Fig. 1. Immunolocalization of cubilin (*a*,*b*), JG12 (*c*–*f*) and synaptopodin (*g*,*h*) in the renal cortex and outer renal medulla from the CG (*a*,*c*,*e*,*g*) and Vit.D- (*b*,*d*,*f*,*h*) groups. Scores for cubilin and JG12 labelling in the tubulointerstitial area of the renal cortex (*i*,*j*) and for JG12 and synaptopodin labelling in the glomeruli (*k*,*l*). Scale bar = 50 μm, ($n = 7$–12 for each group). Immunohistochemical data are expressed as the median and interquartile range (25–75th) (*i*,*k*,*l*) or mean ± S.E.M. (*j*). *$P < 0.05$ *v.* CG. Note the increase in JG12 and decrease in cubilin and synaptopodin expressions in the Vit.D- groups, which show lack of cell differentiation.

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Discussion

The present study shows that Vit.D contributes to kidney development by modulating renal cell proliferation and differentiation; this is probably due to the influence of Vit.D on RAS activity, which plays a critical role in kidney development. Our data show that rats exposed to a Vit.D- diet show alterations in renal development that lead to long-term disturbances in renal function and structure. Vit.D- pups also had lower BWs, confirming the results of previous studies.[11] However, these rats presented with a higher BW compared with the CG group in adulthood. Wen *et al.* demonstrated that Vit.D deficiency during pregnancy stimulates the proliferation and differentiation of pre-adipocytes, which may be associated with the alteration in the methylation of genes, such as *Vldlr* and *Hif1α*, leading to offspring obesity.[18]

The higher water intake and urinary volume in the Vit.D- animals may be a consequence of increased ANGII levels, leading to central stimuli thirst.[19] The Vit.D- animals also exhibited a decrease in urinary osmolality. These alterations were associated with the renal vessel reduction in the medulla and the tubulointerstitial compartments of the renal cortex as well as glomerular capillary rarefaction as a consequence of the reduced differentiation of renal vessels. Vessel rarefaction in the medulla, which is associated with increased ANGII due to renal development disorders, may have contributed to the changes in urinary concentration.[20,11] It has been shown that ANGII increases differentiation and branched ureteric buds *in vivo* and is responsible for renal vessel formation during kidney development.[9] Increased ANGII during renal development can lead to imbalanced angiogenesis factors for vascular branches, which impairs renal vessel maturation, promotes arteriolar wall thickening and reduces inner medulla volume.[21] The integrity of these vascular structures and the renal medulla parenchyma is necessary for the urinary concentration mechanism.

SBP was found to be increased in the Vit.D- animals. A similar result was also observed by Nascimento *et al.*, who studied the first two generations of male Swiss mouse offspring from mothers fed a Vit.D- diet.[10] These authors also observed a number of developing immature glomeruli in these animals, suggestive of the non-differentiation of these capillaries. This finding can be explained by literature findings demonstrating that calcitriol acts as a negative regulator of renin gene expression.[6,8] Thus, the upregulation of renal ANGII production in Vit.D- kidneys may lead to higher SBP and an imbalance between pro- and anti-angiogenic factors. In addition, the study by Tare *et al.* performed in female rats in the dioestrus phase showed that Vit.D- females have an additional impairment in the NO signalling pathway in arterial muscle,[22] and this effect was more intense in the dioestrus phase compared with the effect in the oestrus phase because the NO was upregulated by oestrogens.[23] These results corroborate the observation of a higher incidence of hypertension in Vit.D- rats at this stage.

The lack of differentiation of several glomeruli was evidenced by the decreased JG12 and synaptopodin staining in these glomeruli cells. These findings are consistent with those in the literature, demonstrating the role of Vit.D in cell differentiation.[24] These results together with those that showed a decrease in cubilin staining in the brush border of renal proximal tubule cells in Vit.D- animals demonstrate the lack of cell differentiation and the important role of Vit.D in renal development. Cubilin is a receptor that is expressed primarily in polarized epithelial cells and mediates the reabsorption and activation of Vit.D in the renal proximal tubule, preventing urinary loss of this vitamin. Once in the intracellular space, 25-(OH) Vit.D_3 undergoes hydroxylation to 1,25-(OH)2 Vit.D_3 to become active.[25] The lack of cell differentiation together with the decrease in cubilin expression may explain, at least in part, the lower serum levels of Vit.D in Vit.D- animals compared with those in the CG group.

Conclusion

The current findings showed that kidney development is impaired in female pups from Vit.D- mothers, with consequences in adulthood. These findings suggest that these alterations could be, at least in part, due to impairments in renal cell differentiation and angiogenesis induced by the upregulation of the RAS in Vit. D- pups.

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Conflicts of Interest. None.

Ethical Standards. The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national guides on the care and use of laboratory animals (rats and pups) and has been approved by the Brazilian College of Animal Experimentation, and the Animal Experimentation Committee of the University of São Paulo at Ribeirão Preto Medical School (COBEA/CETEA/FMRP-USP, protocol no. 190/2016).

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