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Perinatally Imposed Essential Fatty Acid Deficiency Changes Renal Function of the Adult Rat

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Abstract

This study was designed to investigate whether essential fatty acid deficiency early during development could change the content of phospholipids and cholesterol in whole membranes of the kidney and renal function at adult life. For this, female Wistar rats were maintained on a standard diet or on an essential fatty acid deficient diet (EFAD) from the age of 30 days, throughout the pregnancy, at age of 90 days and until the weaning, for evaluation of their offspring. Weanling rats were maintained on a standard diet until the age of 13 weeks. Systolic blood pressure (SBP), glomerular filtration rate (GFR), urinary sodium excretion (UNa^+V), positive cells for angiotensin II (Ang II) and cholesterol and phospholipids in whole membranes of the kidney were evaluated. Cholesterol, total phospholipids and the relative content of classes of phospholipids were unaltered in the cortex and medullary kidney. SBP, GFR and UNa^+V were also unaltered in the EFAD group. However, the number of positive cells for Ang II in the tubulointerstitial area of the renal cortex was higher in the EFAD group. Therefore, these findings indicated that although cholesterol and phospholipids were unaltered and urinary sodium excretion was unchanged, Ang II expression in the kidney was erroneously programmed and later hindering of renal function was not ruled out.

Keywords

Angiotensin II, Phospholipids, Glomerular Filtration Rate

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1. Introduction

Some features of essential fatty acid deficiency (EFAD) are the decreased levels of the n-6 and n-3 fatty acid (FA) families and an accumulation of the n-9 FA family. Linoleic acid (LA; C18:2n-6) and *α*-linolenic acid (ALA; C18:3n-3) are essential FAs (EFAs) from the n-6 and n-3 FA series, respectively, which cannot be synthesized *de novo* by animals and have to be obtained from dietary sources. LA can be converted to n-6 long-chain polyunsaturated fatty acids (n-6 PUFA), while ALA is a substrate for biosynthesis of n-3 long-chain polyunsaturated fatty acids (n-3 PUFA) [1]. All of them are membrane constituents and play several biological roles. For instance, arachidonic acid (ARA, C20:4n-6) is a precursor of second messengers which play an important role in increasing vascular resistance and, in the kidney, are inhibitors of tubular sodium reabsorption [2]. Docosahexaenoic acid (DHA, C22:6n-3) is particularly necessary for brain development and its deficiency leads to cognitive impairment [3] and other neurodegenerative diseases [4].

n-3 PUFA deficiency, in particular, during pregnancy and up to the time of weaning has been associated with a mild increase in blood pressure when the rats reach the age of 8 months [5]. When EFAD is imposed from weaning until adult age, changes in renal hemodynamics and inability to excrete an acute volume expansion [6] have been observed, as well as an increment in proximal tubule sodium reabsorption [7]. On the other hand, a multideficient diet-induced lifelong undernutrition, including in the perinatal period, where fat content provides only 4.6% of energy contrasting with 13.3% in the standard diet, leads to lowered cholesterol and phospholipids, lessened (Na^+ + K^+)ATPase activity in basolateral membranes of the renal tubules, increased fractional Na^+ excretion and unchanged blood pressure in young rats [8]. Furthermore, it is known that EFAD can reduce the activity of hepatic 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, the rate-limiting enzyme in cholesterol biosynthesis, to reduce the cholesterol synthesis in the liver [9].

Considering that malnutrition during development may imprint irreversible functional changes in the kidney, the present study investigated the hypothesis that perinatally imposed EFAD could change cholesterol and phospholipids in whole membranes of the adult rat kidney, and also whether positive cells for angiotensin II (Ang II) in the kidney, renal Na^+ excretion and blood pressure were changed.

2. Material and Methods

2.1. Ethical Considerations

The experimental procedure was approved by the Committee for Experimental and Animal Ethics at the Federal University of Pernambuco and performed in accordance with its rules.

2.2. Animals

Thirty day old female Wistar rats maintained in a room at 22˚C ± 3˚C with a 12-h light-dark cycle, were randomly assigned to a standard (C group, n = 6) or an EFAD (EFAD group, n = 6) diet. At age of 90 days, these rats were breeding, and were maintained on their respective diets during pregnancy and lactation until weaning. Therefore, dams were submitted to a total of 102 to 112 days of either a C or an EFAD diet, with a maximal variation of 10 days for breeding, when pregnancy was confirmed by at least 10 g of body weight gain. Pregnant dams were housed in individual cages until weaning, at offspring age of 21 days. After weaning, male pups (C, n = 15 and EFAD, n = 13) were housed in collective cages with 4 animals, in accordance with perinatal dietary treatment and all of them were given a standard diet (Purina Agribands) until the age of 13 weeks. Body weight was taken at birth, weaning and weekly after weaning. Some renal function parameters were measured at age of 8 weeks. At 13 weeks animals were assigned for blood pressure measurement and creatinine clearance evaluation. After measurement of functional parameters, the animals were exsanguinated by decapitation for kidneys withdrawal to evaluate membrane cholesterol and phospholipids. Furthermore, several other organs were collected to obtain their weights.

2.3. Diets

The formulation of diets, prepared according to AIN 93 M [10] differed only by the lipid composition: 5% of soy oil for the C diet and 5% of babassu oil for the EFAD diet (**Table 1**). The soy oil shows 52.8% and 7.3% of C18:2n-6 and of C18:3n-3, respectively [11], while the babassu oil shows 1.4% - 6.6% of C18:2n-6 and lacks C18:3n-3, according to the manufacturer (Rhoster Ind. Com. LTDA, VG Paulista, SP, Brazil).

Table 1. Composition of control (C) and essential fatty acid deficient (EFAD) diets.

Diet	wt%
Casein	20.7
Starch	46.8
Sucrose	21.0
Cellulose	1.8
Oil[a]	5.0
Vitamin (AIN-93 mix)[b]	0.9
Minerals (AIN-93 mix)[c]	3.7
D, L-cystine	0.1
Butyl hydroxytoluene	0.0001

[a]The C diet contains soy oil that shows 52.84% and 7.26% of C18:2n-6 and of C18:3n-3, respectively [11], while the EFAD diet contains babassu oil that shows 1.4% - 6.6% of C18:2n-6 and lacks C18:3n-3, according to the manufacturer (Rhoster Ind. Com. LTDA, VG Paulista, SP, Brazil). [b](Rhoster Ind. Com. LTDA) containing (mg%): folic acid 20, niacin 300, biotin 2, calcium pentothenate 160, pyridoxine 70, riboflavin 60, thiamine chloride 60, vitamin B12 0.25, vitamin K1 7.5. Additionally containing (UI%): vitamin A 40,000; vitamin D3 10,000; vitamin E 750. [c](Rhoster Ind. Com. LTDA) containing (mg%): B 1.426, Ca 1.429, Cl 4.49, Cu 17.241, Cr 2.865, S 0.086, Fe 100, F 2.872, 10.593, Li 0.285, Mg 1.448, Mn 30, Mo 0.432, Ni 1.431, K 10.287, Se 0.428, Si 14.326, Na 2.938, Vn 0.287, Zn 86.

2.4. Evaluation of Blood Pressure and Renal Function

At 8 and 13 weeks animals were housed in metabolic cages (Tecniplast Gazzada, Buguggiate, Italy) for a period of 24 hours in order to measure diet and water intake, urinary flow and urinary sodium (UNa^+V). The systolic blood pressure (SBP) was measured in conscious 13 week old rats by tail-cuff plethysmography (IITC Life Science B60-7/16, Life Science Instruments, Woodland Wills, USA).

Glomerular filtration rate (GFR) was measured by evaluating endogenous creatinine clearance [7]. For this, the animals were housed in metabolic cages for 3 h with continuous urine collection. Blood samples were withdrawn at the end of this period.

The following expressions were used to calculate the renal physiological parameters: Creatinine clearance = Ucr × V/Pcr, where V is the urinary volume (in µl/min) and Ucr and Pcr are the urinary and plasma creatinine concentrations, respectively (in mmol/l). Renal function parameters were corrected to 100 g body weight, when appropriate.

2.5. Evaluation of Phospholipids in Membranes of the Kidney

One of the kidneys was collected after the rats had been killed by decapitation and was maintained in cold isotonic buffer containing 250 mmol/l sucrose, 10 mmol/l HEPES-Tris (pH 7.4), 2 mmol/l EDTA and 0.15 mg/ml trypsin inhibitor (Type II-S) supplemented with 1 mmol/l PMSF. Cortex was separated from medulla on an ice pad. The fragments were separately homogenized using a teflon/glass homogenizer. To obtain total membranes, the homogenate was centrifuged at 17,000 g for 60 min; the resulting sediment was resuspended in 250 mM sucrose, aliquoted into tubes and stored at −20˚C. Lipids were extracted from total kidney membranes as described by [12] [13]. Total membrane phospholipids (TPL), and phosphatidylcholine (PC), sphingomyelin (Spm), phosphatidylethanolamine (PE) and phosphatidylserine (PS), were separated using bi-dimensional thin-layer chromatography with silica gel H containing 2.5% of magnesium acetate. The first dimension consisted of chloroform:methanol:aqueous ammonia (65:35:5), and the second dimension consisted of chloroform:acetone:methanol:acetic acid:water (50:20:10:10:5). Iodine vapor was used to visualize the spots of individual phospholipids that were marked according to the relative mobilities of chosen standards. Individual phospholipid spots were scraped and the samples were digested with 0.3 ml of 99.9% sulfuric acid by heating at 180˚C, using a heater plate for 2 h. After the tubes were chilled, one drop of 30% H_2O_2 was added to the samples. To ensure optically clear samples the tubes were heated on a heater plate for 2 h. The phosphorus measurement to determine the TPL was performed as described previously [13] [14]. Protein concentration was determined using the Folin phenol method [15] with bovine serum albumin as the standard; 2.5% (w/v) sodium dodecyl sulphate was added to solubilize integral membrane proteins.

Evaluation of positive cells to Ang II in the kidney the immunohistochemical evaluation for Ang II positive cells in renal cortical cells was carried out as previously described [16]. Transverse slices of kidneys (3 mm) were fixed in 10% neutral-buffered formalin until being encapsulated in paraffin. After appropriate embedment in paraffin, 6-µm sections were used for incubation with antibody against Ang II (1:200 dilution) overnight at 4˚C. Sequentially, they were exposed for 1 hour to the conjugated biotin secondary antibody against rabbit (1:400 dilution), followed by 1-h incubation with avidin-biotin-peroxidase complex in a humid chamber, at room temperature and visualized by using diaminobenzidine (DAB). The sections were counter-stained by using 0.5% methyl green to count positive cells for Ang II in 60 fields, measuring 166,000 μm^2, throughout the tubulointerstitial region and in 60 glomeruli.

Analytical methods serum cholesterol, total membrane cholesterol of renal cortex and medulla, and urinary and serum creatinine were measured employing commercial kits (Labtest, Lagoa Santa, MG, Brazil). Serum and urinary Na^+ were measured by an electrolyte analyzer (AVL 9180, Roche Diagnostics GmbH, Mannheim, Germany).

Statistical analysis data is expressed as means ± SE. Statistical significance of differences ($P < 0.05$) was assessed using two-tailed unpaired Student's t-test.

3. Results

From birth to 13 weeks of age, body weight development was significantly compromised in the EFAD group (**Figure 1**). From weaning to 7 weeks of age, the body weight of EFAD was 17.9 and 9.6% ($P < 0.05$) lower, respectively, than the C group. From 8 to 13 weeks, the differences between groups were, respectively, of 8.1 to 5.4% ($P < 0.05$). At age of 13 weeks, the wet weight index (**Table 2**) of kidney, heart, testis, lungs, liver and spleen were unaffected. At ages of 8 and 13 weeks, 24 h diet and water intake, urinary flow, water balance, and the urinary density and urinary urea (**Table 3**) did not differ between the EFAD and C groups.

The EFAD did not change the levels of cholesterol or the levels of TPL in renal membranes, neither in the cortical region nor in the medullary region. The relative content of PC, PS, PE and Spm also did not change with the EFAD (**Figure 2**). Regarding blood pressure and renal function, systolic blood pressure (SBP) and GFR, measured as creatinine clearance and urinary sodium excretion (UNa^+V), were unchanged (**Figure 3**). The number of positive cells for Ang II in the glomeruli was unaltered in the EFAD group. However, the number of positive cells for Ang II in the tubule-interstitial area increased in the EFAD group (**Figure 4**).

Table 2. Effects of perinatal EFAD on wet organ mass index in 13-week-old rats.

	CON (n = 11)	EFAD (n = 10)
Spleen, %	0.11 ± 0.05	0.14 ± 0.06
Heart, %	0.30 ± 0.05	0.29 ± 0.06
Liver, %	2.50 ± 0.23	2.60 ± 0.21
Lungs, %	0.39 ± 0.03	0.46 ± 0.13
Left kidney, %	0.32 ± 0.04	0.30 ± 0.06
Testis, %	0.43 ± 0.04	0.42 ± 0.02

Values are mean ± SE.

Table 3. Effects of perinatal EFAD on general parameters evaluated for 24 h in metabolic cages.

	Age, 8 weeks		Age, 13 weeks	
	CON (n = 11)	EFAD (n = 15)	CON (n = 11)	EFAD (n = 15)
Diet intake (g/100g/24h)	10 ± 1	9 ± 1	7 ± 1	7 ± 1
Water intake (ml/100g/24h)	17 ± 1	15 ± 1	11 ± 1	11 ± 1
Urinary flow (ml/100g/24h)	6 ± 1	5 ± 1	5 ± 1	5 ± 1
Urinary density (g/ml)	1.048 ± 0.001	1.049 ± 0.005	1.046 ± 0.001	1.049 ± 0.005
Water balance (ml/100g/24h)	11 ± 1	10 ± 1	7 ± 2	6 ± 2
Urinary urea (mmol/100g/24h)	109.4 ± 11.3	82.1 ± 3.0	95.00 ± 5.6	100.7 ± 6.3

Values are mean ± SE.

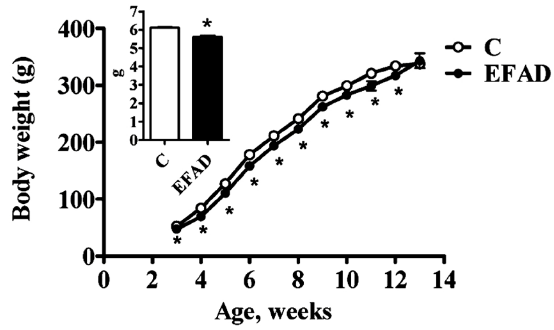

Figure 1. Effects of perinataly imposed EFAD on body weight evolution. The control (C) group (n = 36, from birth to weaning and 15 from weaning until age of 13 weeks) comprises offspring of dams maintained from age of 30 days and throughout pregnancy until weaning in a balanced diet prepared according to AIN 93 M, containing soy oil; while the EFAD group (n = 36, from birth to weaning and 13 from weaning until age of 13 weeks) comprises offspring of dams maintained in the same balanced diet, except for the replacement of babassu oil for soy oil, during the same period as the C group. Values are means ± SE. SE bars are very small to appear in the graph scale. $^*P < 0.05$ with respect to the C group.

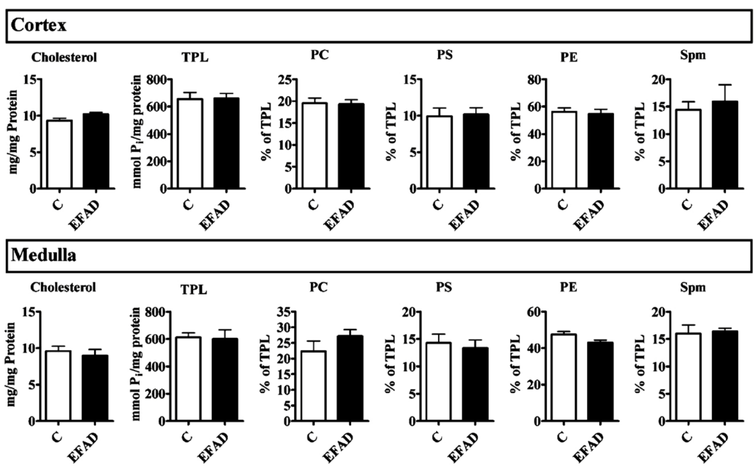

Figure 2. Effects of perinatally imposed EFAD on cholesterol and phospholipids in whole membranes of the kidney. See group description in Figure 1. The graphs are showing total phospholipids (TPL) and the relative amounts of phospholipids classes, phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylethanolamine (PE) and sphingomyelin (Spm). Results are mean ± SE of 6 essays.

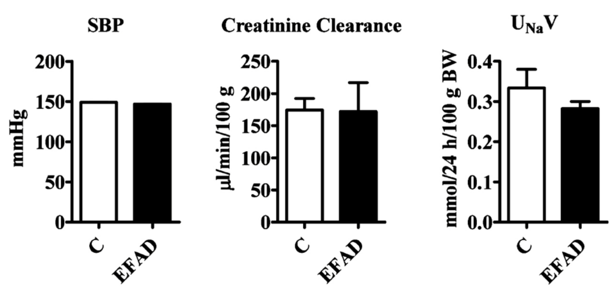

Figure 3. Effects of perinatally imposed EFAD on blood pressure and renal function. The parameters are systolic blood pressure (SBP), creatinine clearance and urinary sodium excretion (UNa^+V). See group description in Figure 1 and details for parameters calculations in Material and Methods. Results are mean ± SE of 8 animals in each group.

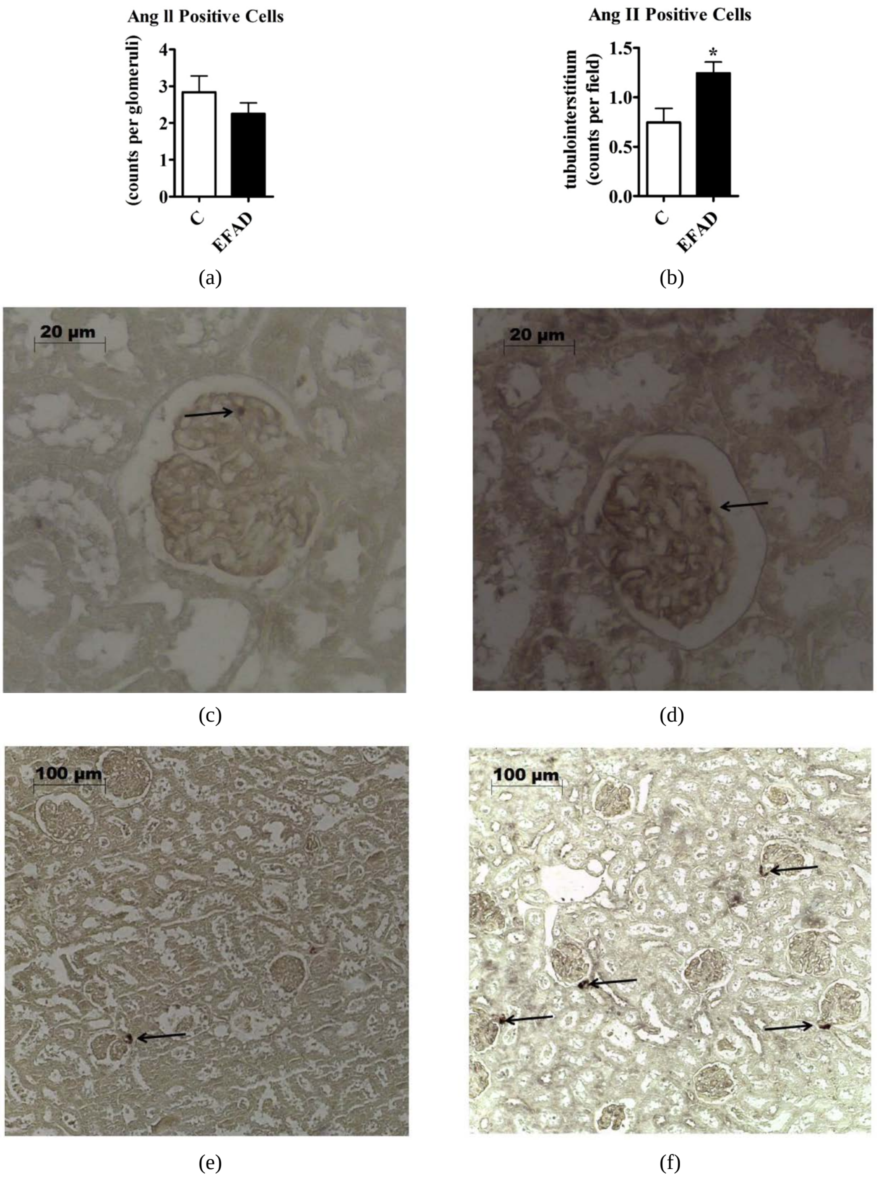

Figure 4. Effects of perinatally imposed EFAD on the number of positive cells for Ang II in the kidney. See group description in **Figure 1**. (a) The average number of cells showing Ang II per glomerulus in 60 glomeruli; (b) The average number of cells showing Ang II, counted in 60 fields measuring 166,000 μm^2. Results are mean ± SE of 6 slides in each group; (c) Representativeimmunolocalization for positive cells to Ang II, pointed by arrows, in glomeruli of C group; (d) Representative immunolocalization for positive cells to Ang II, pointed by arrows, in glomeruli of EFAD group; (e) Representative immunolocalization for positive cells to Ang II, pointed by arrows, in the tubulointerstial region of the C group; (f) Representative immunolocalization for positive cells to Ang II, pointed by arrows, in the tubulointerstial region of the EFAD group. $^*P < 0.05$ with respect to the C group.

4. Discussion

The hypothesis that perinatally imposed EFAD could change cholesterol and phospholipids in whole membranes of the adult rat kidney was not supported. However, the tubule-interstitial area in the kidney presented an increased number of positive cells for Ang II, even though the renal sodium excretion and GFR were unchanged. These findings indicate that the Ang II expression in the kidney was erroneously programmed and that later hindering of renal function is not ruled out.

Taking into account that the mothers were submitted to EFAD for 60 to 70 days before the first day of pregnancy, the offspring was effectively subjected to lower levels of n-6 and n-3 PUFA, from the conception until the weaning. ARA and DHA, respectively, products of linoleic and α-linolenic acids, essential fatty acids, are drastically reduced in plasma [17] [18] and in tissues as the kidney [17] after 8 weeks of treatment.

The reduced birth weight and the lower body weight gain during development were a characteristic effect of EFAD [19] [20]. To reduce body weight, there is evidence that EFAD leads to increased basal metabolism [21] [22], although its actual mechanism is not yet known. Respiratory frequency is increased in EFAD rats [22], but chain enzymes activity in the mitochondria are changed in the heart and skeletal muscle [21]. Undernutrition during lactation normally affects body weight development [23] more severely than undernutrition restricted to fetal life. Under EFAD, particularly during lactation, the plasma levels of IGF-I are reduced [20] contributing to the reduction in body weight. In the present study, the EFAD during prenatal and lactation periods compromised body weight gain irreversibly. However, the lessened difference of body weight between C and EFAD at adult age, compared with post-weaning, suggests that the catch up could happen at a later age. This is likely due to the fact that EFAD during lactation depresses leptin levels in the offspring [20] [24] during the early stages of development. However, lowered leptin during the perinatal period could lead to hyperleptinemia and obesity later in life [25] [26].

Considering that cholesterol was unchanged in the membranes of the kidney, the first assumption that may be taken is that HMG-CoA reductase activity was not programmed during the perinatal period, at least in the kidney. HMG-CoA reductase is the rate-limiting enzyme for cholesterol synthesis. There is evidence that EFAD decreases HMG-CoA reductase activity [9], when the animals are evaluated immediately after the diet was imposed. It is worthy to emphasize that in the present study the essential fatty acid replenishment began after the weaning, at age of 21 days, and that the animals were evaluated at age of 90 days. Regarding phospholipids, the present data does not ensure that specific PUFA, such as ARA and DHA, were recovered, something that may be considered one limitation of this study. However, the present data determines that total phospholipids are not changed in the membranes of the kidney. Increased activity of delta 9 desaturase, responsible for synthesis of monounsaturated FA, is one of the effects of EFAD [27]. The activity of this enzymeis recovered in the liver after perinatal (n-3) PUFA deficiency is followed by its repletion after the weaning [28]. However, there is evidence that in the hypothalamusan imbalance between (n-6) and (n-3) PUFA early in life is not recovered at adult age [29].

Aside from the unaltered cholesterol and phospholipids in the kidney, the urinary sodium excretion was also unchanged, as well as the glomerular filtration rate. Therefore, fractional sodium excretion was not evaluated. However, the increased number of cells positive for Ang II in the tubule-interstitial area, suggests that changes in the renin angiotensin system were caused by EFAD. The expression of Ang II in the kidney is one marker of renal development during nephrogenesis. The presence of Ang II during kidney development leads to an increase in the glial cell-derived neurotrophic factor (GDNF) [30], which is a crucial growth factor for ureteric bud proliferation [31]. Increased at adult life in the kidney, Ang II has been correlated with increased oxidative stress and increased sodium reabsorption [32], or even increased blood pressure [33]. However, in the present study the EFAD group did not show increased SBP. A previous research study showed that maintenance of an imbalance spanning the whole life of the rat, until the age of 33 weeks, leads to elevated blood pressure, while the replacement of the diet at the age of 12 weeks leads to a reduction in the levels of blood pressure, even though the animals had higher blood pressure than control rats [5]. Thus, together, this previous evidence allied to an increased number of Ang II cells in the kidney, may indicate that renal function and hypertension may occur later in life.

5. Conclusion

In summary, essential fatty acid deficiency imposed during perinatal period programmed an increase in the number of cells positive for Ang II in the kidney.

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