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Original Paper

Maternal Hypercholesterolemia Associated with Nicotine Exposure in Adulthood May Induce Kidney Injury in Male Rats if Hypomagnesemia Occurs

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Key Words
Fetal programming • Hypercholesterolemia • Hypomagnesemia • Kidney injury • Nicotine • Renal function

Abstract
Background/Aims: Maternal hypercholesterolemia is a risk factor to renal injury in rat pups at adulthood, especially if they feed a cholesterol-enriched diet after weaning. However, the renal function of male pups of dams with hypercholesterolemia (PH) that were fed a regular chow from weaning to adulthood needs investigation, particularly those exposed to an adverse risk such as nicotine. ***Methods:*** We evaluated the renal function of PH animals and we compared the data with those found in male pups of control dams (PC) at 3- and 6-month-old by inulin clearance. Moreover, we investigated the effect of nicotine treatment for 8 days in both PH and PC animals at 6 months old via metabolic function studies and by renal histological analysis. ***Results:*** Inulin clearance and other renal function parameters were similar in PH and PC animals at 3 and 6 months old. Nevertheless, the PH group showed significant differences with regard to histological analysis despite a similar number of glomeruli. The glomerular area of PH animals was significantly smaller than that measured in PC animals, and the fractional interstitial area was significantly larger in PH animals than that measured in PC animals at 3 months old. With regard to nicotine treatment, we observed a trend for a reduction in creatinine clearance in both PC and PH groups, but only PH animals showed hypomagnesemia and the highest fractional interstitial area. ***Conclusions:*** The offspring exposed to a high cholesterol milieu during intrauterine and neonatal life may show a silent kidney injury at adulthood that may be aggravated by nicotine exposure if hypomagnesemia occurs.

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Introduction

Hypercholesterolemia can induce renal injury due to its direct action on vascular reactivity and by triggering oxidative stress and inflammation in non-vascular tissue [1-3].

In previous studies, we evaluated the renal function of rats fed a cholesterol-enriched diet for one week [4-6]. Although cholesterol levels have doubled in the serum, the glomerular filtration rate and blood pressure values have remained within normal range. However, hypercholesterolemic rats developed renal tubular dysfunction manifested by magnesuria owing to a reduction in the expression of the epithelial Mg^{2+} channel (TRPM6) in the renal cortex [5]. In addition, hypercholesterolemic rats treated with rosiglitazone showed renal injury [6], whereas hypercholesterolemic rats treated with nicotine have demonstrated evidence of impairment in their renal autoregulation [4]. Therefore, we may hypothesize that hypercholesterolemia acted as a 'first hit' to renal dysfunction resulting in magnesium deficiency and that the treatment with rosiglitazone or nicotine may provoke a 'second hit', allowing the development of renal injury. This hypothesis is in agreement with the literature that reports that a 'single hit' may not be enough to induce renal dysfunction [7].

Many experimental and human studies have linked environmental and maternal influences during fetal and perinatal life to cardiorenal diseases in adulthood [8-18]. As preterm births often occur in these cases, a recent study using metabolomics profile identified a biomarker in the blood of pregnant women predicting who might be in risk group [18]. Previously, we have also evaluated the influence of maternal hypercholesterolemia in rat offspring. We found low body weight at birth correlates with high mortality at birth and during neonatal life. Additionally, the pups exposed to maternal hypercholesterolemia and fed a cholesterol-enriched diet after weaning had a reduction in their renal function at 3 months old [15]. However, the effects in the renal function of offspring exposed to maternal hypercholesterolemia that were fed regular rat chow from weaning to adulthood are not clear yet.

Hence, we propose to evaluate whether maternal hypercholesterolemia may act as a 'first hit' to cause offspring renal dysfunction at adulthood, even if they were fed a regular rat chow after weaning. We also propose to investigate whether another risk factor to cardiovascular disease can act as a 'second hit' to renal dysfunction at adult life. Thus, we chose to treat the sibling male rats exposed to a high cholesterol milieu during intrauterine and neonatal life with nicotine at 6 months old.

Materials and Methods

The Research Ethics Committee of our institution (*Comissão de Ética para Análise de Projetos de Pesquisa – CAPPesq, da Diretoria Clínica do Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo*) approved this study, and we conducted all experiments according to the National Research Council Guide for the Care and Use of Laboratory Animals. Animals had free access to food and water throughout the study, and we anesthetized them for all surgical preparations with sodium thiopental (50 mg/kg b.w. i.p.).

Study design

We randomly housed 28 female Wistar rats weighing 180–220 g, obtained from the Animal Center of our institution, in individual cages one week prior to mating in our laboratory. Half of them were fed a standard diet (control dams) and the other half were fed an enriched-cholesterol diet (hypercholesterolemic dams) in the same protocol that we have previously reported [15]. In the present study, we fed both male pups of control (PC) and hypercholesterolemic (PH) dams with regular rat chow throughout the study after weaning.

Renal function study

We evaluated the renal function of both PC and PH groups at 3 and 6 months old by measuring inulin clearance as previously described [4, 6].

Briefly, we placed catheters in the trachea, jugular veins, carotid artery and bladder. Thereafter, we intravenously administered a priming dose of inulin (100 mg/kg b.w. diluted in 0.9% saline) followed by a sustained inulin infusion (10 mg/kg b.w.) at a rate of 0.04 ml/min. Prior to the start of the experiment a stabilization period of at least 30 minutes was allowed, before two urine samples were each collected at 30-min intervals. Moreover, we also collected blood samples at the beginning and at the end of the experiment. In all urine and blood samples, we measured inulin by the anthrone assay and Na^+ and K^+ by flame photometry (CELM-FC280). We also measured mean blood pressure (BP) at the beginning and at the end of the experiment by a catheter placed in the carotid artery, and we expressed the average values in mm Hg.

Nicotine exposure

In another set of PC and PH animals at 6 months old, we separated the sibling animals to treat them with or without 12.5 μg/ml nicotine (Sigma Chemical, St. Louis, MO, USA) in drinking water *ad libitum*. On the eighth day of treatment, we placed the animals in individual metabolic cages to collect urine under oil. After a 24h-period, we anesthetized the animals to catheterize the abdominal aorta to measure BP and to collect blood samples.

We quantified creatinine, magnesium (Mg^{2+}), phosphorous (P) and chloride (Cl) in urine and blood samples by colorimetric methods. We also measured total cholesterol in the serum and the protein in the urine by using test kits (Labtest; Minas Gerais, Brazil).

Renal histological study

After blood collection, we removed and fixed the kidneys of both PC and PH animals at 3 and 6 months old in 4% paraformaldehyde, post-fixed in Bouin's solution and processed for paraffin embedding. The renal tissues were sliced into 4 μm and stained with either hematoxylin and eosin or Masson's trichrome for histological examination under light microscopy. We measured the fractional interstitial area (FIA) of the renal cortex and the glomerular area stained with Masson's trichrome by the morphometry method using a light camera connected to an image analyzer (Axiovision, Carl Zeiss, Eching, Germany) [19]. We counted the number of glomeruli in each section as a whole and evaluated the glomerular area by encircling the outer edges of the glomeruli tufts in 50 sequential grid fields measuring 0.087 mm^2. For evaluation of FIA, we analyzed 20 sequential grid fields also measuring 0.087 mm^2 in the renal cortex of each kidney section. The interstitial areas were demarcated manually on a video screen, and the proportion of the outlined field was determined by computerized morphometry [20]. We used 50 grid fields to evaluate the glomerular area because this study is usually clean to be executed, that is, there is no interference of technical artifacts. On the contrary, when evaluating the FIA, it is necessary to avoid fractured areas, large vessels, presence of the glomeruli, and edges and corners of the field as well, which contribute to the exclusion of some grid fields.

Statistical analysis

We expressed data as mean ± SE. We used paired Student's t-test to compare data of the same animal and unpaired Student's t-test to compare data of histological study at 3 months old. We also used the analysis of variance (ANOVA) and the Tukey's *post hoc* test for comparisons between groups and the Dunnett's *post hoc* test for comparisons with PC rats treated with water (control group). GraphPad Prism 5.0 (La Jolla, CA) was used for statistical analyses and statistical significance was set at $P \leq 0.05$.

Results

Evaluation at 3 months old

Both PC and PH rats exhibited a normal range in body weight, blood pressure, renal function parameters and electrolyte homeostasis at 3 months old (Table 1). However, both PC and PH rats showed significant differences with regard to histological analysis despite

a similar number of glomeruli (Fig. 1A). The glomerular area of PH rats was significantly smaller than that measured in the PC group (Fig. 1B), and the FIA of the PH group was significantly larger than that found in the PC group (Fig. 1C). As illustrated in Fig. 1D, fibrosis is evident by the enlarged interstitial area in a representative photomicrograph of the PH group.

Evaluation at 6 months old

Both PC and PH animals at 6 months old showed higher body weight than at 3 months old, which naturally occurs during the animal's growth. Again, blood pressure, renal function parameters and electrolyte homeostasis were within normal range values in both PC and PH rats at 6 months old (Table 1).

Table 1. Renal function studies in male pups of control and hypercholesterolemic dams at 3 and 6 months old. Data are expressed as mean ± SE. PC: pups whose dams fed a standard diet; PH: pups whose dams fed a cholesterol-enriched diet; *P<0.01 vs. PH at 3 months old derived from analysis of variance followed by Tukey's test

	3 months old		6 months old	
	PC (n = 4)	PH (n = 7)	PC (n = 8)	PH (n = 6)
Body weight (g)	308±35	366±12	436±16*	443±27*
Blood pressure (mmHg)	118±4	114±2	122±2	119±4
Inulin clearance (ml/min/100g)	0.88±0.06	0.73±0.10	0.90±0.10	0.73±0.12
Plasma Na^+ (mmol/l)	138±3	133±2	133±4	138±2
Plasma K^+ (mmol/l)	5.32±0.27	4.49±0.34	3.96±0.30	3.82±0.38
Urine output (µl/min)	14.1±4.5	10.5±2.5	8.0±1.2	13.1±3.9
Na^+ excretion (µmol/min)	1.71±0.87	1.07±0.54	0.84±0.30	1.79±0.74
K^+ excretion (µmol/min)	1.70±0.19	2.49±0.82	1.68±0.44	2.02±0.61

With regard to the renal histological study, the glomerular area did not differ in PC animals at 3 and 6 months old (Fig. 2A). However, the glomerular area in the 6-month-old PH group was significantly higher than 3-month-old PH animals (P<0.01; Fig. 2 A). As illustrated in Fig. 2B, the FIA was significantly higher in PC and PH animals at 6 months old than that measured in the PC group at 3 months old. We did not detect any difference in the FIA in both 3- and 6-month-old PH animals.

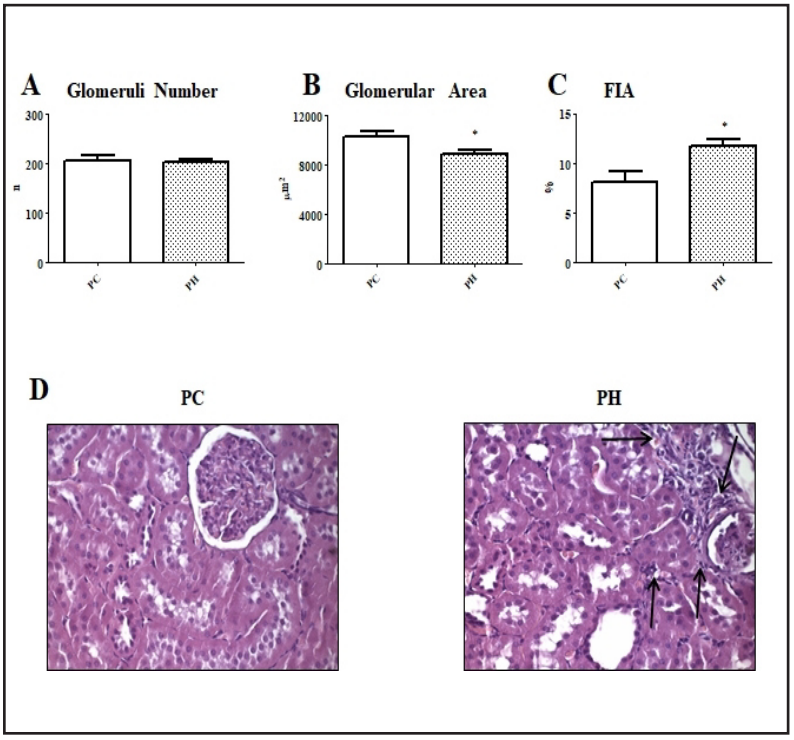

Fig. 1. Renal histological analysis in male pups of control (PC) and hypercholesterolemic (PH) dams at 3 months old. A: Glomeruli number count is similar in both PC and PH groups. B: Glomerular area is smaller in PH than in PC group. C: FIA is larger in PH than in PC group. D: Representative photomicrographs of cortical kidney sections of PC and PH groups stained with hematoxylin and eosin, x400 magnification. Fibrosis is seen clearly in enlarged interstitial area of the PH group (arrows). Columns and error bars represent mean ± standard error *P<0.05 for PH vs. PC, derived from Student's unpaired t-test.

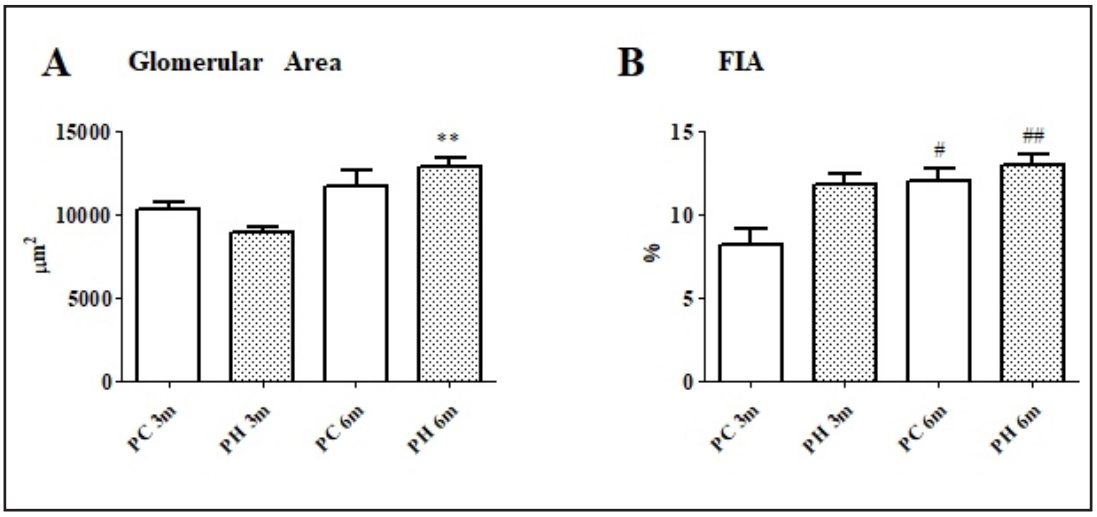

Fig. 2. Renal histological analysis in male pups of control (PC) and hypercholesterolemic (PH) dams at 3 and 6 months old. A: Glomerular area is similar in both 3- and 6-month-old PC animals, and it is significantly higher in 6- month-old PH animals than in 3-month-old PH animals. B: FIA is significantly higher in both PC and PH animals at 6 months old than that measured in the PC group at 3 months old; and we did not detect any difference in the FIA in both groups of PH animals at 3 and 6 months old. Columns and error bars represent mean ± standard error. **P<0.01 vs. PH animals at 3 months old derived from ANOVA followed by Tukey's test. #P<0.05 and ##P<0.01 vs. PC animals at 3 months old, derived from ANOVA followed by Tukey's test.

Nicotine exposure

Sibling PC rats treated with or without nicotine showed similar body weight, food intake and drinking intake during the treatment (Table 2). However, PH rats treated with water ate less quantities of food than PC rats treated with water. In addition, we observed a significant reduction in body weight of the sibling PH rats treated with or without nicotine (Table 2).

The nicotine treated groups had a tendency for lower creatinine clearance than their water-treated counterparts. Moreover, the treatment with nicotine did not induce any abnormality with regard to electrolyte homeostasis, except for the serum magnesium levels. Both PC and PH rats treated with nicotine showed hypomagnesemia, but they had levels of magnesium urinary excretion in the same range as the untreated sibling group (Table 2).

With regard to the renal histological study, we found similar glomerular area in both PC and PH rats treated with or without nicotine (Fig. 3A). Yet, we observed the largest FIA in PH animals treated with nicotine (Fig. 3B-F).

Discussion

Our data suggest that maternal hypercholesterolemia may predispose male adult rats to kidney injury even if they were fed regular chow after weaning. Renal injury, demonstrated by an increase in the FIA, became more evident when we exposed the animals to nicotine at 6 months old. Both PC and PH nicotine-treated animals developed hypomagnesemia due to extra-renal mechanisms. Nevertheless, we found larger zones of FIA only in PH nicotine-treated animals. Taken together, these findings suggest that maternal hypercholesterolemia may act as a 'first hit' to renal injury, and nicotine exposure associated with hypomagnesemia may trigger the 'second hit' in renal injury progression.

Fetal and neonatal exposure to adverse factors may induce changes in renal structure. Many studies describe that a reduction in nephron number may predispose to the development

of hypertension and susceptibility to renal injury in adulthood [10, 21]. In the present study, we found similar nephron number in both PH and PC animals. Nonetheless, we found a reduction in glomerular area and an increment in the FIA in the histological study of PH groups at 3 months old. These findings may indicate a 'silent' kidney injury because the animals did not show any abnormalities in their values of blood pressure, glomerular filtration rate and electrolyte homeostasis. Thus, these findings supported our proposition to investigate whether nicotine exposure in adult life may play a role in the progression of renal injury in offspring exposed to maternal hypercholesterolemia.

Table 2. Characteristics of sibling male pups of control and hypercholesterolemic dams at 6 months old treated with or without nicotine, Data are expressed as mean ± SE. PC: pups whose dams fed a standard diet; PH: pups whose dams fed a cholesterol-enriched diet; *P<0.05 vs PC treated with water derived from ANOVA followed by Tukey's test and &P<0.05 vs control group (PC treated with water) derived from ANOVA followed by Dunnett's test and #P<0.05 vs. their initial body weight, derived from Student's paired t-test

	PC		PH	
	Water	Nicotine	Water	Nicotine
Number of animals	7	7	8	8
Initial body weight (g)	488 ± 23	485 ± 22	468 ± 25	463 ± 15
Final body weight (g)	495 ± 20	479 ± 22	452 ± 21#	448 ± 12#
Body weight variation (g)	7.1 ± 12.3	-5.7 ± 4.7	-16.2 ± 7.0	-15.6 ± 6.4
Average food intake (g/day)	28.2 ± 1.3	25.4 ± 0.7	21.6 ± 1.8*&	26.8 ± 1.4
Average drink intake (ml/day)	32.4 ± 2.4	30.0 ± 1.6	28.0 ± 2.2	27.8 ± 2.9
Drink intake in metabolic cage (ml)	13.9 ± 2.1	7.6 ± 2.1	17.5 ± 3.7	8.6 ± 2.2
Urine output in metabolic cage (ml)	14.2 ± 2.2	9.2 ± 1.5	17.3 ± 2.6	10.2 ± 2.1
Total cholesterol (mmol/l)	1.74 ± 0.23	1.49 ± 0.16	1.16 ± 0.19	1.22 ± 0.16
Blood pressure (mm Hg)	111 ± 4	113 ± 6	102 ± 7	112 ± 4
Creatinine clearance (ml/min/100g)	0.39 ± 0.03	0.32 ± 0.02	0.38 ± 0.05	0.32 ± 0.03
Proteinuria/creatinine	0.85 ± 0.10	0.72 ± 0.08	0.70 ± 0.10	0.65 ± 0.08
Plasma Na^+ (mmol/l)	143 ± 3	141 ± 3	142 ± 3	140 ± 2
Na^+ excretion (µmol/day)	368 ± 59	348 ± 81	434 ± 94	378 ± 40
Plasma K^+ (mmol/l)	3.80 ± 0.20	3.46 ± 0.19	3.96 ± 0.41	3.44 ± 0.28
K^+ excretion (µmol/day)	1978 ± 207	1694 ± 97	1521 ± 188	1876 ± 218
Plasma Cl^- (mmol/l)	115 ± 8	112 ± 6	113 ± 7	109 ± 5
Cl^- excretion (µmol/day)	331 ± 98	465 ± 87	486 ± 136	713 ± 274
Plasma Mg^{2+}(mmol/l)	0.84 ± 0.04	0.68 ± 0.08	0.80 ± 0.05	0.65 ± 0.03&
Mg^{2+} excretion (µmol/day)	58 ± 11	56 ±15	49 ± 7	42 ± 10
Plasma Pi (mmol/l)	1.74 ± 0.28	1.76 ± 0.19	1.74 ± 0.15	1.82 ± 0.16
Pi excretion (µmol/day)	371 ± 70	261 ± 59	329 ± 65	365 ± 61

When we evaluated PC and PH groups treated with or without nicotine at 6 months old in metabolic cages, we observed a trend for a reduction in creatinine clearance in both PC and PH rats treated with nicotine. Creatinine clearance has some limitations as a measure of glomerular filtration rate (GFR) as rats have low amounts of muscle mass [22]. Moreover, the metabolic cage method requires a long period to collect the urine and imprecisions in urine output determinations may occur, consequently. Thus, creatinine clearance data usually present low values, which might decrease the possibility to detect significant differences in statistical analysis. In our previous study, we observed a significant reduction in GFR exerted by nicotine considering that we used the technique of inulin clearance [4]. This method shows fewer errors in accuracy and precision thus allowing many investigators to indicate inulin clearance as the gold standard method to measure GFR [23]. We did not choose inulin clearance measurement to evaluate GFR in PC and PH animals treated with nicotine because we did not have enough sibling male pups that reached the age of 6 months old to apply this method.

Nicotine may also exacerbate oxidative stress associated or not with increases in extracellular matrix and inflammation [24-26]. Oxidative stress may lead to magnesium deficiency that also increases the inflammatory response and plays a role in degenerative and chronic diseases [27-29]. Therefore, both oxidative stress and magnesium deficiency are the cause and effect of each other to increase the inflammation and fibrosis process in many organs, as for example brain, heart and kidneys.

We have previously shown in hypercholesterolemic rats treated with rosiglitazone that renal injury was likely due to hypomagnesemia [6]. Furthermore, we also demonstrated renal

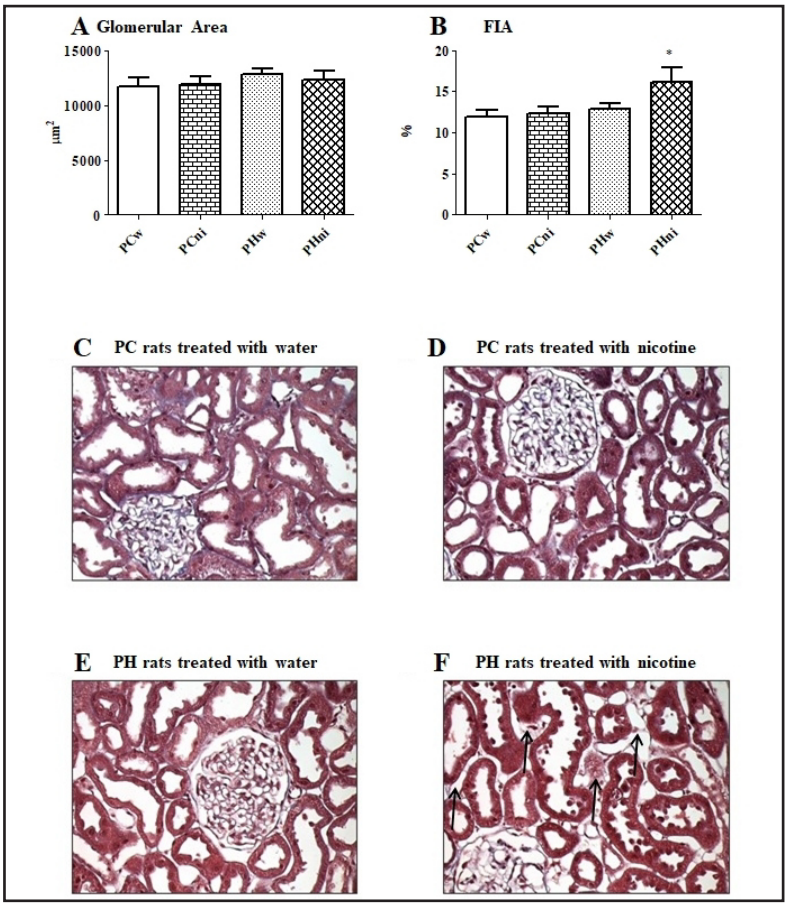

Fig. 3. Renal histological analysis in male pups of control (PC) and hypercholesterolemic (PH) dams at 6 months old treated with water (w) or nicotine (ni). A: Glomerular area is similar in both PC and PH animals treated with or without nicotine. B: FIA is significantly higher in PH rats treated with nicotine than other groups. Columns and error bars represent mean ± standard error C-F: Representative photomicrographs of cortical kidney sections of PC and PH stained with hematoxylin and eosin, x400 magnification. The arrows show the enlarged interstitial area in PH rats treated with nicotine. * $P<0.05$ for PH animals treated with nicotine vs. other groups, derived from analysis of variance followed by Tukey's test.

tubular dysfunction in hypercholesterolemic rats in another study [5]. Hypercholesterolemic rats developed hypomagnesemia due to magnesium urinary losses as a result of a reduction in expression of epithelial Mg^{2+} channels in the renal cortex (TRPM6) [5]. Hence, hypercholesterolemia, oxidative stress and hypomagnesemia are involved in a vicious cycle in which each one of them is the cause and the effect of the other.

In the present study, all animals showed total cholesterol serum levels within the normal range, and we found hypomagnesemia only in animals treated with nicotine. Both PC and PH rats treated with nicotine had similar magnesium urinary excretion rates, which may indicate an adverse effect exerted by nicotine in the gastrointestinal tract [30].

In the small intestine, large amounts of magnesium absorption occur paracellulary, whereas fine-tuning of magnesium absorption occurs in the cecum and colon by transcellular transport [29, 31]. In the distal intestine, TRPM6 channels are expressed [32]. In fact, TRPM6 interacts with a homolog protein called TRPM7 resulting in a TRPM6/7 complex that regulates the Mg^{2+} entry in intestinal cells [33]. Ryazanova et al. demonstrated the importance of TRPM7 in regulating magnesium homeostasis especially by their alpha-kinase domain. They suggested that TRPM7 kinase is involved in cell death caused by oxidative stress [34]. Thus, further studies addressing TRPM6 and TRPM7 in the gastrointestinal tract might help to elucidate the effect of nicotine in digestive system cells.

Lastly, we would like to emphasize that our study design cautiously considered evaluating only one pup per mother in each group. This criterion differs from some studies in the literature that include two or three pups in the same groups to receive the same kind of treatment. In these studies, an important bias occurs because the findings may show only an epiphenomenon. In summary, our study is really significant as it is in agreement with the foetal programming hypothesis. PH animals showed a silent renal injury at 3 months old that progresses after another trigger effect.

Conclusion

A high cholesterol milieu during intrauterine and neonatal life may act as a 'first hit' to a silent kidney injury, and nicotine exposure associated with hypomagnesemia may trigger the 'second hit' in the progression of renal injury.

Disclosure Statement

None.

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