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The High Refined Carbohydrate Diet Promotes Renal Injury in Mice Exposed to Cigarette Smoke

Laís Fernandes, Júlia Pereira Agulhari, Ana Beatriz Farias de Souza, Frank Silva Bezerra, Sílvia Dantas Cangussú*

Department of Biological Sciences, Laboratory of Experimental Physiopathology, University of Ouro Preto, MG, Brazil

***Corresponding author:** Silvia Dantas Cangussú, Department of Biological Sciences, Laboratory of Experimental Physiopathology, Federal University of Ouro Preto, Campus Universitário s / n, Morro do Cruzeiro, Ouro Preto, MG, Brazil. Email: cangussu@ufop.edu.br /cangussusilvia@gmail.com

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Abstract

This study aimed to evaluate the effects of a high refined carbohydrate diet (HRC diet) and renal response in C57BL/6 mice exposed to Cigarette Smoke (CS). Twenty-four male mice were divided: Control Group (CG), Cigarette Smoke Group (CSG) which received standard diet; HRC diet group (RG) and HRC diet and cigarette smoke group (RCSG) which received the HRC diet. After twelve weeks, CSG and RCSG were exposed to CS for five days. Twenty-four hours after the last exposure all animals were euthanized. There was an increase of inflammatory cells in the RCSG and of interstitial edema and hyaline glomeruli in the RG and RCSG. Hyperemia, membranous glomerulopathy and glomerulosclerosis were frequent lesions in the CS, RG, and RCSG. There was a decrease in the glomerular area of the RG and increase in Bowman's space of the CSG, RG, and RCSG. The glomerular densities in CSG, RG, and RCSG were lower than of the CG. There was lower plasma urea concentration in CSG, RG, RCSG and higher plasma alkaline phosphatase in RG. Our data demonstrate that the HRC diet, cigarette smoke and the association of both factors promote renal damage and are risk factors for the functioning of the renal system.

Keywords: Cigarette smoke; Histopathology; Kidney; Mice; Overweight

Abbreviations: ALP: Alkaline Phosphatase; ANOV: Analysis of Variance; CG: Control Group; CSG: Cigarette Smoke Group; RCSG: High Refined Carbohydrates Diet and Cigarette Smoke Group; RG: High Refined Carbohydrate Group

Introduction

Obesity, particularly the accumulation of visceral fat, is considered as chronic low-grade inflammation [1] and presents important factors in the initiation and progression of metabolic disorders [2]. This pathogenesis is associated with an increased risk of morbidity and mortality due to hypertension, cardiovascular diseases, type 2 diabetes, cancer, chronic liver and kidney diseases [3,4]. Several alterations in renal structure and function have been associated with obesity, such as nephrolithiasis, increased glomerular size, and glomerular function abnormalities, in addition to a number of malignancies including kidney cancer [5-7]. Glomerular abnormalities were noted in the renal biopsy specimens from patients with massive obesity by Weisinger, et al. [5]. These authors noted that some of the glomeruli contained segmental, amorphous, eosinophilic material and proposed that the cause of the reversible proteinuria seen in these obese patients was renal venous hypertension.

The obesity-related glomerulopathy, a clinical entity manifested by proteinuria, glomerulomegaly and focal and segmental sclerosis, could also contribute to increasing the prevalence of Chronic Kidney Disease (CKD) [8]. In addition to causing renal damage, obesity may accelerate renal functional loss in patients with glomerulopathy. Bonnet, et al. [9], assessing patients with biopsy-proven Immunoglobulin A (IgA) nephropathy, observed that patients with a body mass index greater than 25 kg/m^2 were more likely to develop more complex histological lesions,

systemic arterial hypertension and of progression of chronic renal failure, compared with those with normal weight. They concluded that excessive body weight may represent a new independent risk factor for disease progression in IgA nephritis.

In addition to obesity, it was described that cigarette smoke manifest impairment of renal function, suggesting that smoke may have a detrimental effect on renal function [10]. Moreover, cigarette smoking is associated with accelerated progression of renal disease in patients with diabetic and non-diabetic nephropathy [11]. Smoking has vasoconstrictor, thromboembolic, and direct effects on the vascular endothelium and is an independent risk factor of renal failure in males with kidney diseases [12]. Cigarette Smoke (CS) is one of several agents and environmental factors that can trigger renal damage or accelerate pre-existing renal diseases [13]. CS is composed of approximately 5,000 substances, which makes it an exogenous source of free radicals capable of initiating chemical reactions that cause cellular dysfunction and cytotoxicity [14].

Studies have shown that the risk of some diseases increases when obesity and smoking are present [15,16]. In a context of the worldwide obesity epidemic and the prevalence of smokers, studies on the consequences of the interaction obesity and smoking become relevant for global public health. However, few studies, and only in humans [17,18] have suggested a possible association between obesity, smoking, and renal disease. Therefore, this study aimed at evaluating the effects of a High Refined Carbohydrates diet (HRC diet) and renal inflammatory response in C57BL/6 mice exposed to CS.

Materials and Methods

Experimental Animals

Twenty-four male C57BL/6 aged between 5 - 7 weeks' male were supplied by the Laboratory of Experimental Nutrition (LABNEX), School of Nutrition -Federal University of Ouro Preto (UFOP). The mice were kept in plastic cages and maintained under controlled conditions of temperature, light and humidity (22±2°C, 12 hours' light/dark, 50 ± 10%, respectively) with access to food and water ad libitum. All of the animal experiments were performed according to the rules of animal protection and the ethical principles of the Brazilian Society of Science in Laboratory Animals (SBCAL) and was approved by the Ethics Committee on Animal Use of the Federal University of Ouro Preto (CEUA-UFOP) (protocol number 2014/06).

Diet, Food Intake and Body Mass Protocol

We used the diet, food intake and body mass protocols as described recently [16]. Initially, the animals were divided into four groups (n=6): Control Group (CG), Cigarette Smoke Group (CSG), High Refined Carbohydrate Group (RG) and High Refined Carbohydrates Diet and Cigarette Smoke Group (RCSG). Animals from CG and CSG received standard chow for rodents of Labina-Purina® by 12 weeks (Evialis Group, São Paulo, Brazil), and animals from RG and RCSG received a diet with high content of refined carbohydrate (10% refined sugar, 45% of standard chow, and 45% of Nestlé® condensed milk -São Paulo, Brazil) by 12 weeks. The control of food intake and body mass gain was carried out once a week. For the control of body weight gain, the animals were weighed individually using a digital balance (Mark®; M Series/B Analytical Equipment LTDA, São Paulo, Brazil). Diets were weighed before being fed to the animals and after one week of consumption, and the feces were also weighed once a week.

Cigarette Smoke (CS) exposure

After the twelve weeks of the nutritional protocol the animals of CSG and RCSG were exposed to smoke generated by 12 commercial Marlboro Red Label Box (Philip Morris International) containing 10mg of tar, 0.9 mg of nicotine, and 10 mg of carbon monoxide per cigarette administered three times a day (morning, afternoon, and night) for five consecutive days as described previously (Ramos, et al. 2018, Pena, et al. 2016) [16,19]. Initially, mice were placed in the inhalation chamber (40 cm long, 30 cm wide and 25 cm high) inside an exhaustion chapel. Each cigarette was coupled to a plastic 60 mL syringe and the smoke was injected inside the inhalation chamber. The mice were maintained in this smoke-filled atmosphere (± 3% CS concentration) by 6 minutes. Then the chamber was opened for 1 min for total exhaustion of cigarette smoke, and this procedure was repeated for each cigarette. Animals from CG and RG were exposed to ambient air under the same conditions.

Blood Collection and Dosages of Urea, Creatinine and Alkaline Phosphatase (ALP)

Twenty-four hours after the last exposure the animals were euthanized by an overdose of ketamine (130 mg/kg) and xylazine (0.3 mg/kg). Blood was collected by cardiac puncture and placed in polypropylene tubes with 15 µl of anticoagulant. Approximately 0.8 mL was centrifuged at 10,000 rpm for 15 minutes, the plasma collected and stored in a freezer (-80 °C) for the determination of urea, creatinine, and ALP by specific commercially available kits, according to the manufacturer's instructions (Bioclin®, Quibasa).

Tissue Collection

After blood collection, the left ventricle was perfused with 20 mL saline solution (0.9%) for the removal of blood from the kidneys, later the kidneys were collected and sectioned into 2 samples. The left kidney was fixed by immersion in 10% neutral buffered formalin for at least 48 h to be used in the histological analysis, and the right one was removed and homogenized in 1,5 mL of phosphate buffer (pH 7.5) and centrifuged at 10,000 rpm

for 10 min at 4°C. The supernatant was collected and stored in a freezer at -80°C for biochemical analyses.

Histology and morphometric analyses

After fixation, kidney tissue samples were processed according to routine histological techniques and embedded in paraffin blocks. Paraffin blocks were cut into 4 μm thick sections and stained with hematoxylin and eosin (H&E) and Masson's trichrome for histopathological analyses. For semi-quantitative and morphometric analyses, we obtained twenty random images from histological slides with 40X objective (total area 1.5×10^6 μm^2) per section on a single slide per animal. These slides were photographed in Multiuser Laboratory of the Nucleus of Biological Sciences Research (NUPEB) of UFOP, using the light microscope equipped with Leica BM5000 digital camera (Leica DFC 300 FX) coupled with the software of capturing images Leica Application Suite and analyzed using the ImageJ/Fiji 1.46r software, a freeware developed by Wayne Rasband (National Institutes of Health, Bethesda, Maryland, USA).

Histological screening focused on the detection of the following abnormalities: Edema, Inflammation, Hyperemia, Hyalinized Glomerulus, Membranous Glomerulopathy, and Glomerulosclerosis. The parameters measured morphometrically were glomerular density, glomerular area, Bowman's space size and thickness of the wall of the renal tubules. The glomerular density was determined by calculating the total number of glomeruli per renal cortical area of the twenty to thirty random images, the glomerular area was defined as the area described by the outer capillary loops of the tuft, Bowman's space was calculated by the area of the Bowman's capsule and the glomerular tuft and subtracted from the second of the first area. Glomeruli were evaluated in the afferent artery measuring hence the equatorial portion of the glomerulus. The wall of the renal tubules was calculated by tracing five boundaries thereby tubule of by Image.

Data Analysis

Evaluation of data normality was performed using the Kolmogorov–Smirnov test. The degree of significance was calculated by a one-way ANOVA followed by Tukey's post hoc test. All data were normally distributed and were expressed as the mean ± standard error of the mean. The significant difference was considered when the p-value was less than 0.05. The analyses were performed with Graph Pad Prism software version 5.00 for Windows 7 (GraphPad Software, San Diego, CA).

Results

Morphological and Semi-Quantitative Analyses

The lesions found in the renal tissue of the studied groups are represented in Table 1, Figure 1. Table 1 summarizes the proportion of lesions through semi-quantitative analysis. The renal parenchyma of the Control Group (CG) presented normal morphological characteristics (Figure 1A). There was a predominance of the edematous areas and hyalinized glomeruli in animals which received a high refined carbohydrate diet (HRC diet), with or without exposure to CS (RG e RCSG) compared with CG and CSG (Table 1, Figure 1A-D). Moreover, it was more frequency of inflammatory cells in RCSG compared with CG, CSG and RG (Table 1, Figure 1E). Dilatation and congestion in the peritubular vessels (hyperemia), membranous glomerulopathy and glomerulosclerosis were very frequent in CSG, RG, and RCSG (Table 1, Figures 1B,C,D).

Renal fibrosis was not observed in the animals studied (data not shown).

Lesions	CG	CSG	RG	RCSG
Interstitial edema	-	++	+++	+++
Inflammation	-	++	++	+++
Hyperemia	-	+++	+++	+++
hyalinized glomerulus	-	+	++	++
Membranous Glomerulopathy	-	+++	+++	+++
Glomerulosclerosis	-	+++	+++	+++

CG: Control Group; CSG: Control Diet And Cigarette Smoke Group; RG: High Refined Carbohydrate Group; RCSG: High Refined Carbohydrate And Cigarette Smoke Group. Very common: +++; less common ++; rarely: +; absent: -.

Table 1: Frequency of lesions in the kidney of C57BL/6 mice of the experimental groups.

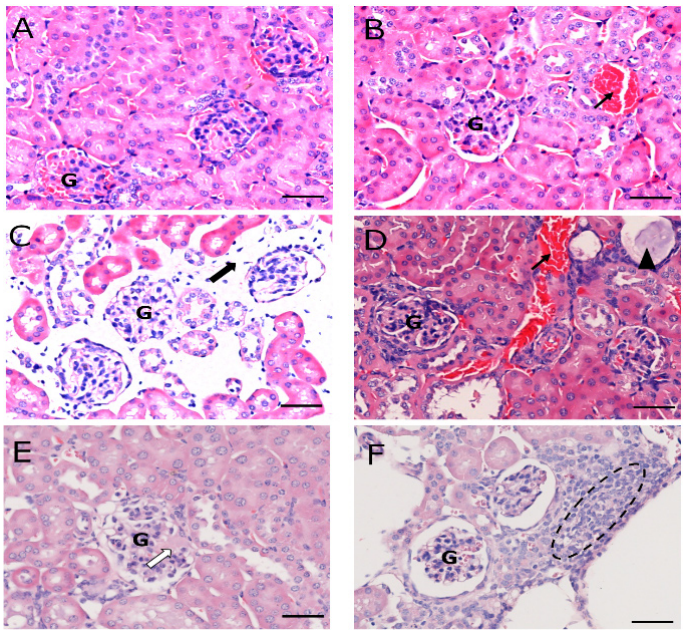

Figure 1: Comparative histopathological features in the kidneys of C57BL/6 mice that received a standard chow or diet with high content of refined carbohydrate, with or without exposure to cigarette smoke. No alterations were observed in the Control Group (CG) **(A)**: In the cigarette smoke group (CG) note dilatation and congestion in the peritubular vessels (hyperemia) (**B** - fine arrow) and interstitial edema (**C** - thick arrow). In the high refined carbohydrate group (RG) note hyperemia (**D** - fine arrow) and hyalinized glomerulus (**D** -head arrow). In the high refined carbohydrates, diet and cigarette smoke group (RCSG) observe glomerulosclerosis (**E** - white arrow) and inflammatory infiltrate intense (**F** -dotted line). Stained with hematoxylin and eosin. Barr = 100 µm.

Morphometry

We observed a significant difference in glomerular area between CG and RG groups (Figures 2A,C,F). There was a decrease in the glomerular area of the RG when compared with CG. In addition, there was an increase in Bowman's space of the CSG, RG and RCSG groups when compared with CG (Figures 2A-E). The quantitative morphometric evaluations revealed no differences in the thickness of the tubular wall between the treatments (CG, CSG, RG, RCSG) (Figures 2A-D). The glomerular density was compared between the groups. The glomerular densities in CSG, RG, and RCSG groups were considerably lower than that of the CG (Figures 3A-E).

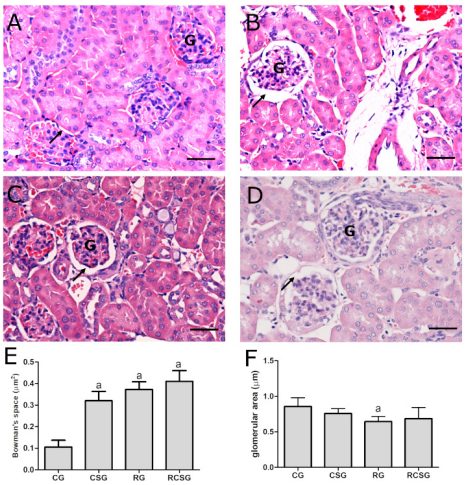

Figure 2: Photomicrographs of kidney sections stained with hematoxylin and eosin. Barr = 100 µm **(A-D)** and morphometric analyses of Bowman's space **(E)** and of the glomerular area **(F)**. For **E** and **F** data were expressed in mean ± standard error of the mean and were analyzed by one-way ANOVA followed by Tukey post-test. The letter (a) represents a significant difference between groups when compared to CG ($p<0.05$). Control Group (CG) **(A)**, Cigarette Smoke Group (CSG) **(B)**, High Refined Carbohydrate Group (RG) **(C)**, High Refined Carbohydrates Diet and Cigarette Smoke Group (RCSG) **(D)**. Bowman´s space (**A-D** -arrow), glomerulus (**A-D** -G). Both high refined carbohydrate and cigarette smoke show increased Bowman's space **(B-E)**, but there was a decrease of the glomerular area only in the high refined carbohydrate group (RG) **(C-F)**.

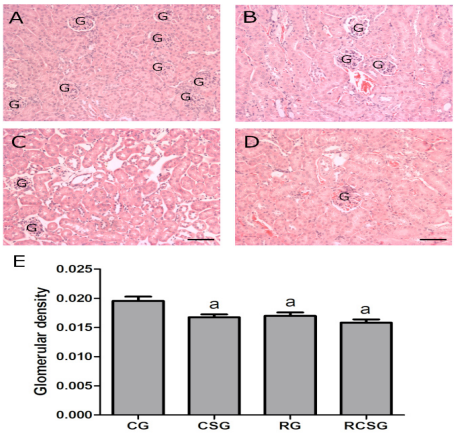

Figure 3: Photomicrographs of kidney sections stained with hematoxylin and eosin. Barr = 50 µm **(A, B, C, D)** and morphometric analyses of glomerular density **(E).** For **E** data were expressed in mean ± standard error of the mean and were analyzed by one-way ANOVA followed by Tukey post-test. The letter (a) represents a significant difference between groups when compared to CG ($p<0.05$). Control group (CG) **(A)**, cigarette smoke group (CSG) **(B)**, high refined carbohydrate group (RG) **(C)**, high refined carbohydrates diet and cigarette smoke group (RCSG) **(D)**. Glomerulus (**A-D**). The glomerular densities in CSG, RG, and RCSG groups were considerably lower than that of the CG.

Markers of renal function

There was no significant difference in the blood creatinine levels in the experimental groups. However, there was a decrease of the blood urea levels in CSG, RG, and RCSG compared with CG, and there was an increase of the blood ALP in RG compared with CSG and RCSG as can be observed in Table 2.

	CG	CSG	RG	RCSG	p
Creatinine (mg/dL)	0.5 ± 0.1	0.5 ± 0.05	0.4 ± 0.02	0.4 ± 0.02	> 0.05
Urea (mg/dL)	84.1 ± 4.4	59.0 ± 3.0[a]	61.5 ± 3.5[a]	53.9 ± 2.3[a]	< 0.05
ALP (U/L)	55.7 ± 8.6	42.5 ± 5.7	80.8 ± 3.3[b,d]	34.6 ± 6.1	< 0.05

Notes: Data are expressed as mean ± standard error of the mean and were analyzed by one-way ANOVA followed by Tukey's post-test. a Significant difference between the groups when compared with the CG. b Significant difference between the groups when compared with the CSG and d Significant difference between the groups when compared with the RCSG.

Table 2: Biochemical analysis in blood from the CG, CSG, RG, and RCSG groups.

Discussion

In this study, we evaluated the association between a high refined carbohydrate diet (HRC diet) and renal inflammatory response in C57BL/6 mice exposed to Cigarette Smoke (CS). Our results demonstrate that the diet rich in refined carbohydrates, cigarette smoke and the association of both factors promote renal damage, represented by glomerular and interstitial lesions. In addition, we observed a decrease in urea levels in the groups exposed to the two insults individually and in the group in which both were associated and an increase in the peripheral blood of alkaline phosphatase in animals that received the diet rich in refined carbohydrates. In a previous study published by our research group, using the same animals was observed an increase in body mass associated with the increase in body adiposity index (BAI) in C57BL/6 mice which received an HRC diet [16].

Studies have suggested that obesity and smoking increase the risk for the development of some diseases [15,20]. However, few studies, and only in humans [17,18] have suggested a possible association between obesity, smoking, and renal disease. In a context of the worldwide obesity epidemic and the prevalence of smokers, studies on the consequences of the interaction obesity and smoking become relevant for global public health. In the present study, the HRC diet and/or the exposition to CS provided marked structural changes of the renal parenchyma that presented both glomerular lesions (membranous glomerulopathy, glomerular hypertrophy, focal and segmental glomerulosclerosis, hyalinized glomerulus) and interstitial lesions (edema, inflammation, and hyperemia). The renal corpuscle pathology, when become prevalent glomerular filtration, can be impaired, and changes in renal function may lead to CKD.

Histological findings of similar and frequent focal and segmental glomerulosclerosis, membranous glomerulopathy and hyperemia in the all experimental groups HRC diet and/or CS were consistent with experimental and human studies that considered some of these lesions common renal histological lesion in obesity [21,22] or in smoking [23]. According to Eknoyan [24], pathologic lesions of focal segmental glomerulosclerosis are common in experimental models of sustained obesity and are observed in morbidly obese humans. After consuming an HRC diet, with or without CS exposition, the mice kidneys presented more hyaline degeneration that the control diet and cigarette smoke group. The glomeruli displayed a variable degree of hyalinization, many were completely replaced by hyaline material. Another study, in humans, detected a trend toward more arterial hyalinosis in obese patients than controls [25]. Moreover, a study with smoking subjects dying of coronary heart disease also associated the smoking with arteriolar hyalinosis and thickening of small arteries in the kidney [26].

In the present study, light microscopic examination of renal tissue of animals subjected to cigarette inhalation, with or without HRC diet, and of animals only with HRC diet showed dilatation and congestion in the peritubular vessels. These results indicated that both CS and HRC diet are renal risk factors and corroborates with Ozan et al. [23] that observed dilatation and congestion in the peritubular vessels of the Wistar rats subjected to long-term cigarette inhalation. There was a predominance of the edematous areas in Cigarette Smoke Group (CSG), high Refined Carbohydrate Group (RG) and HRC diet and Cigarette Smoke Group (RCSG) compared with the Control Group (CG). According to Araujo, et al. [27], interstitial edema can occur, indistinctly, in almost all glomerulopathies. So, we can suggest that both CS and HRC diet provoked the interstitial edema observed in our study. However, Rea, et al. [25] detected no differences in edematous areas donor kidneys obtained from obese compared to non-obese individuals.

In this study, there was a higher frequency of the inflammatory cells in renal parenchyma in animals which received an HRC diet and CS when compared with the others. Known of the correlation

between elevated expressions of pro-inflammatory cytokines or chemokine in adipose tissue with renal inflammation in rodent models of obesity [28,29]. However, this is the first study showing that exposure to CS increases the inflammatory process in the obese kidney. We did not observe morphological variations in the proximal tubules of any experimental group. However, another experimental study on rats, the long-term cigarette smoke caused a decrease in the lumen size of the proximal and distal convoluted tubules and a decrease in the height of the proximal tubule epithelium [30]. It is likely that the different results are related to other factors, such as the time of exposure to cigarette smoke and the animal species. There was an increase in the Bowman's space of the HRC diet and/or CS groups, Interestingly, the increase in the Bowman's space of these groups do associate with modifications of the glomerular area only in HRC diet group, that presented decreased of the glomerular area. Our results are according to Henegar et al. [31] that observed glomerular Bowman's space area greater in dogs fed the high-fat diet, compared with lean animals. However, these authors related their results to the expansion of Bowman's capsule, while we observed a reduction of the glomerular area, although the obesity was associated with larger glomeruli in humans [25]. Unlike us, another study showed narrowing of Bowman's space of the renal tissue of rats subjected to long-term cigarette inhalation [23].

The morphometric analysis showed a reduction in the numerical density of the renal glomeruli associated not only to HRC diet but also to cigarette inhalation. These results indicated that both CS and HRC diet are renal risk factors to renal function and corroborates with Okabayashi, et al. [22], that observed low glomerular density in patients with moderate obesity similar to that in individuals with higher obesity. Thus, these authors considered that other renal factors than body mass index (BMI) may underlie the susceptibility to glomerular disease. Biochemical examinations in experimental models are essential for estimating the health condition of the animals and provide information about changes induced by pathological processes [32,33]. In this study, creatinine levels did not show differences between the experimental groups. In this context, serum creatinine levels are dependent on several factors such as dietary intake and muscle mass, renal and extrarenal excretion. All these factors may be altered in renal disease, which makes serum creatinine an insensitive marker for assessing renal function) [34,35]. Our results corroborate with the studies of Bostom, et al. that report in patients with CKD, normal levels of creatinine, despite impaired renal function (Bostom, et al. 2002) [34].

The exposure to CS, the HRC diet and the association of both factors caused a decrease in levels of urea. At despite the reduced plasma/serum urea is less common, our results are according to Lum and Leal-Khouri [36] that observed low serum urea concentrations in a patient population with alcohol abuse. However, these authors failed to define the clinical conditions associated with low urea concentrations. A published case history [37] described a reduced urea concentration with pathological cause confined to advanced liver disease. In this study, our research group observed that HRC diet led to non-alcoholic fatty liver disease and that the cigarette smoke led to oxidative damage in hepatic tissue (unpublished data), which may explain the low serum urea concentrations. The alkaline phosphatase is a known marker of renal osteodystrophy [38]. However, there are no data in murine models of renal injury that assess the increase in ALP. In patients with chronic kidney disease, the increase in circulating ALP is related to increased mortality [38,39]. In the present study, we believe that the increase in alkaline phosphatase is a marker of the development of renal injury in the group that received the diet rich in refined carbohydrates.

The kidney is one of the most sensitive organs to the inflammation and is an important source of chemokines and cytokines in the tubular epithelium due to its close contact with high blood flow [40]. Moreover, the physical compression of the kidney by the surrounding fat and the renal structural changes associated with obesity may also play a role in the renal damage associated with obesity [41]. And considering that in the human body blood is oxygenated in the lungs in the microcirculation and pumped into the rest of the body by the large circulation to be filtered in the kidneys, it becomes important to study the renal effects of both, exposure to cigarette smoke and obesity, which in turn are scarce. Besides that, animal models remain fundamental to improving understanding of human renal disease. To our knowledge, this is the first study demonstrating the effects of high-carbohydrate diet on the kidneys of C57BL/6 mice exposed to cigarette smoke. Our study shows that the exposure to short-term CS does not exacerbates the lesions provoked by HRC diet, but both are relevant renal risk factors, conferring a substantial increase in risk for renal function deterioration. Further studies are needed to determine the mechanisms responsible for the results found. So, it should be carried out to quantify the long-term impact of the cigarette associated with carbohydrate diet in the renal parenchyma.

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