Induction of systemic inflammation and thickening of subepicardial arteries in an animal model of uremia

Indução de resposta inflamatória sistêmica e espessamento de artérias subepicárdicas em um modelo animal de uremia

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ABSTRACT

Although renal dysfunction is a known risk factor for cardiovascular disease (CVD), there are few experimental studies investigating the cardiovascular consequences of this condition. **Objective:** To analyze the impact of the induction of renal dysfunction on biomarkers of cardiovascular risk and on the histology of subepicardial vessels. **Methods:** This experimental study involved thirty Wistar male rats, which were divided into two groups. One (chronic kidney disease – CKD group) underwent renal ablation, and the other (SHAM group) was submitted to kidney manipulation only. Both groups were followed up for eight weeks. During follow-up, serum levels of urea, phosphorus and TNF-α were measured. Heart tissue was processed for histological analysis. **Results:** The CKD group had increased levels of urea and phosphorus, in comparison with the SHAM group. The levels of TNF-α were increased in the CKD group and undetectable in the SHAM group ($p < 0.05$). Thickness of the middle layer of the subepicardial vessels of the CKD group was significantly higher than that of the SHAM group ($p = 0.011$). **Conclusion:** Induction of renal dysfunction in rats increased the biomarkers of cardiovascular risk and led to a thickening of the subepicardial vessels when compared with normal controls,

Keywords: Coronary Disease. Kidney Failure, Chronic. Uremia. Models, Animal.

RESUMO

A disfunção renal é um fator de risco para doença cardiovascular (DCV). Estudos experimentais controlados que possam analisar o impacto da disfunção renal no sistema cardiovascular, isolando esses fatores relacionados à uremia dos fatores de risco tradicionais, que são altamente prevalentes na população com doença renal crônica (DRC), ainda são escassos. **Objetivo:** Analisar o impacto cardiovascular em ratos com disfunção renal, analisando biomarcadores de risco cardiovascular e a histologia das artérias subepicárdicas desses animais. **Métodos:** Estudo experimental envolvendo trinta ratos machos Wistar, divididos em dois grupos. Um grupo foi submetido à ablação renal e o outro grupo SHAM (grupo controle) à manipulação do pedículo renal. Ambos os grupos foram acompanhados por oito semanas, período em que foram feitas dosagens de ureia, fósforo e do fator de necrose tumoral alfa (TNF-α). Lâminas obtidas de cortes do miocárdio foram confeccionadas para análise das características das arteríolas subepicárdicas. **Resultados:** O grupo DRC apresentou níveis elevados de uréia e fósforo em relação ao grupo SHAM. Já os níveis de TNF-α, em todas as análises, foram indetectáveis nos animais do grupo SHAM, em contraste com o grupo DRC, onde se observaram elevados níveis de TNF-α ($p < 0,05$). A espessura da camada média dos vasos subepicárdicos do grupo DRC foi significativamente maior do que em relação ao grupo SHAM ($p = 0,011$). **Conclusão:** A indução de disfunção renal determinou alterações em biomarcadores de risco cardiovascular e um aumento na espessura dos vasos subepicárdicos estudados em comparação aos animais com função renal normal.

Palavras-chave: Doença das Coronárias. Falência Renal Crônica. Uremia. Modelos Animais.

Introduction

Cardiovascular disease (CVD) is the main cause of morbidity and mortality among patients with renal dysfunction, cardiomyopathy and accelerated atherosclerotic arterial disease being its main pathophysiological translations.[1-3] Uremia-associated risk factors, such as anemia, volume overload, uremic toxicity, systemic inflammation and disturbances of the mineral metabolism are thought to be closely involved in the pathophysiology of renal dysfunction-related CVD.[2] Few controlled experimental studies analyzing the impact of renal dysfunction on the cardiovascular system have isolated uremia-related factors from traditional risk factors, which are very prevalent in the renal dysfunction population.

Renal dysfunction-related systemic inflammation has been characterized in clinical studies by increased levels of C-reactive protein (CRP) and pro-inflammatory cytokines in up to 50% of the patients on chronic dialysis therapy.[4,5] This state of systemic inflammation and consequent increase of the oxidative stress are believed to contribute to the acceleration of the cardiovascular alterations seen in uremia.[6]

As for the myocardium, the three lesions making up the structural remodeling seen in chronic kidney disease (CKD) are: myocardial hypertrophy, interstitial fibrosis and arterial and arteriolar intramural thickening.[7] Uremia-provoked vascular disease is both macrovascular and microvascular, being associated with reduced arterial compliance and altered vessel permeability and adhesion molecule expression.[8] Higher expression of leukocyte adhesion molecules on the surface of endothelial cells favors the phenomena leading to progressive arterial occlusion.[9] Subepicardial vessels are particularly susceptible to metabolic disturbances that accelerate CVD progression.

We hypothesized that renal dysfunction and the consequent accumulation of uremic compounds with activation of the systemic inflammatory response lead to alterations in the arterial wall of experimental animals undergoing renal mass reduction. The purpose of this study was to analyze the behavior of cardiovascular risk biomarkers and the histological alterations of subepicardial arteries in an animal model of uremia.

Methods

We used Wistar male rats weighing between 200 and 260 g. Renal dysfunction (CKD group) was achieved through ablation of 5/6 of the renal mass of fifteen rats aged ten weeks, anesthetized with ketamine and xylazine hydrochloride. The median and posterior renal arteries of the left kidney were ligated, in order to assure an infarction of at least 2/3 of this kidney. A right total nephrectomy was then performed.[10,11] Fifteen rats (SHAM group) underwent anesthesia, ventral laparotomy and manipulation of the renal pedicles, without any removal of the renal mass. After the procedure the animals were returned to their original cages, with free access to water and standard ration (0.50% sodium, 22% protein), and kept at 23 ± 1°C on controlled 12h night/day shifts, for an eight-week period.

Blood samples from both groups were collected on days 7, 30 and 60 after the surgical procedure, for determination of the serum levels of urea, phosphorus and tumor necrosis factor-alpha (TNF-α). Urea and phosphorus were determined with a colorimetric method (Labtest®). TNF-α was determined with a microplate enzyme-linked immunosorbent assay (ELISA), that is, using a polyclonal antibody against the rat TNF-α cytokine (TNF-α/TNFSF1A – Quantikine, R&D Systyems) in a sandwich immunoenzymatic technique. All samples were collected with tripotassium ethylenediamine tetra-acetate anticoagulant (K3EDTA) and stored in endotoxin-free vials at -80°C, until the analyses were performed.

After the end of follow-up, the animals were sacrificed. Asepsis of the thoracic region was performed with polyvinylpyrrolidone-iodine (PVPI). The heart was fixated in 10% formaldehyde after the performance of four cross-sectional sections with the same longitudinal diameter. The sections were mounted in paraffin and then a Lica RM 2145 microtome was used to obtain 4-micron sections. The slides were stained with hematoxylin-eosin, Gomori`s trichrome and Weigert`s elastic tissue stains. Measurement of the thickness of the middle layer of the subepicardial vessels was made with a histomorphometry device composed of an Olympus BX 50 microscope, from which the images were transferred to a microcomputer and analyzed with the Image Pro-plus 4.5 software (Media Cybernetics®) (Figure 1).

The subepicardial vessels were chosen for analysis as they are particularly susceptible to metabolic disorders that accelerate CVD progression. The vessels found in a field at 40X magnification were selected. After localization of the subepicardial vessels to be studied, micrometric measurement of

their thickness followed the same protocol shown in Figure 1.

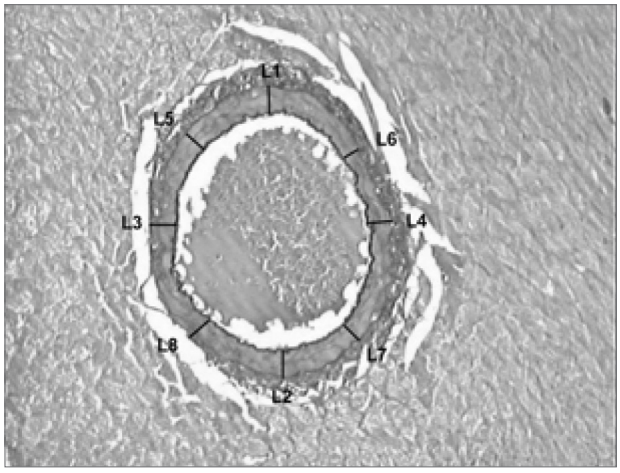
Statistical analysis

The data were expressed as means ± standard deviations. Comparisons between the means of the two groups were made with Student`s t test. Correlations between continuous variables were assessed with Pearson` s test. All analyses were made with the SPSS software (SAS Institute Inc., Berkeley (CA), USA). p values < 0,05 were considered statistically significant,

Results

Three animals of the CKD group died during follow-up and were excluded from the final analysis. Three hearts of the SHAM group were discarded during histological processing, as the material obtained was deemed inadequate. 24 animals completed the study protocol (12 CKD and 12 SHAM) and were included in the analyses.

Figure 1. Protocol used in the study for measurement of the middle layer of the subepicardial vessels, totaling eight measurements (L1-L8) for each vessel – Representation of the measurement of arteries.



There was no difference in the weight variation between the groups along the study (Table 1). Blood analyses showing significantly higher levels of urea and phosphorus in the CKD group at days 7, 30 and 60, compared with the SHAM group, confirmed the success of the renal dysfunction induction. In the SHAM group, phosphorus and urea levels remained stable throughout the observation period (Table 1).

Renal dysfunction induced a higher inflammatory response during the observation period, with increased levels of TNF-α in the CKD group and absence of this response in the SHAM controls (Table 1). Histomorphometry showed a significantly higher thickness of the middle layer of the subepicardial arteries of the CKD group, compared with the SHAM group (18.98 ± 3.98 μm *versus* 13.90 ± 4.90 μm; p = 0.011; Figures 2A and 2B).

Discussion

Renal dysfunction is a CVD risk factor, altering the serum concentrations and expression of biomarkers of cardiovascular risk, and inducing myocardial and vascular alterations which are still poorly characterized in animal models of uremia. In this study, induction of renal dysfunction with the 5/6 nephrectomy experimental model led to uremia, maintenance of an inflammatory state and vascular alterations compatible with arteriolar thickening.

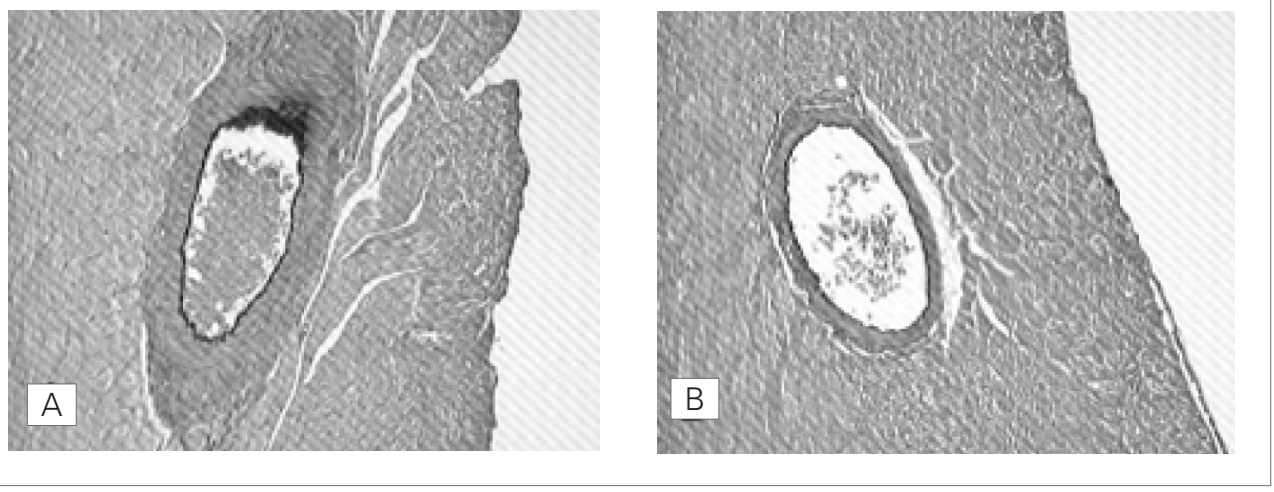
End-stage CKD frequently leads to uremia, now defined as the accumulation in the blood of organic compounds normally excreted by the kidneys,[12] specifically uremic toxins such as: organic acids, urea, beta-2 microglobulin, compounds resulting from oxidative stress and phosphorus,[13-16] which are directly related to cardiovascular morbidity and mortality in CKD. In this study, the uremia model obtained with 5/6 nephrectomy was characterized by accumulation of urea and phosphorus, two of the aforementioned uremic toxins. This model is therefore believed to be

Table 1 General characteristics of the animals at the three assessment points of the study

	CKD (n = 12)			SHAM (n = 12)		
	7° dia	30° dia	60° dia	7° dia	30° dia	60° dia
Weight	260 ± 20	283 ± 16	322 ± 26	291 ± 26	302 ± 16	327 ± 18
Urea (mg/dL)	119 ± 44	120 ± 44	136 ± 76	46 ± 7	46 ± 8	54 ± 13
Phosphorus (mg/dL)	5.88 ± 0.32	6.71 ± 0.29	7.14 ± 0.28	5.76 ± 0.28	5.86 ± 0.27	5.49 ± 0.27
TNF-α (pg/mL)	3.18 ± 0.62*	2.58 ± 0.54*	1.86 ± 0.47*	Undetectable	Undetectable	Undetectable

*Significant difference between the levels of TNF-α of the CKD group compared with the SHAM group, at days 7, 30 and 60 (p < 0.05 for all analyses).
CKD: chronic kidney disease; SHAM: control group; TNF-α: tumor necrosis factor-alpha.

Figure 2. Representative slides of subepicardial vessels. A) (CKD group) – Artery from CKD rats. B) (SHAM group) – Artery from controls.



interesting for the study of the cardiovascular repercussions of uremia. To date, this model has been almost exclusively used to investigate the progression of renal disease.

We definitely identified a systemic and sustained inflammatory state in the nephrectomized rats, showing that, in this model, inflammation occurs alongside with the accumulation of uremic toxins, such as urea and phosphorus. Patients with renal dysfunction frequently develop systemic, sustained inflammation, documented by increased levels of CRP, inteleukin-6 and TNF-α[4,5,17], which strongly correlate with cardiovascular complications.[17] It has been observed that exposure of the endothelium to increased levels of inflammatory biomarkers leads to endothelial dysfunction and thickening and vasospasm,[18] which may underlie the ischemia of susceptible tissues, such as the myocardium. Besides these uremia-related alterations, arterial functional and structural alterations in CKD lead to impairment of arterial distensibility, with increased after-load and peripheral vascular resistance, left ventricular hypertrophy and reduced coronary perfusion due to vascular remodeling.[7,19,20]

The main finding of our study was the observation that induction of renal dysfunction leads to thickening of the middle layer of subepicardial arteries, even after a follow-up period of only eight weeks. Few studies have associated CKD with the histopathology of blood vessels. In studies with experimental animals, uremic rats were observed to develop a significant reduction of the arterial lumen and an increased wall/lumen ratio of the mesenteric arteries, in comparison with normal controls.[21] In addition, calcium-channels blockers and angiotensin-converting enzyme inhibitors prevented thickening of the wall of the intramyocardial arterioles in animal models.[22] These structural modifications, as well as their response to pharmacological therapy, could not be adequately explained by the presence of arterial hypertension and traditional CVD risk factors, suggesting the involvement of other cardiovascular risk factors, such as those linked to CKD.

The findings indicate that subepicardial vessels are affected by uremia, with specific cardiovascular alterations and peculiar clinical presentation. Clinical studies have shown that up to 50% of CKD patients with angina do not have significant coronary disease. Such patients have a myocardial microangiopathy, such as the one observed in our study, resulting from thickening of the arterial wall which, under stress, precludes vascular relaxation and predisposes to myocardial ischemia. On histopathology, there is hypertrophy of the smooth muscle cells, increased expression of endothelium-derived growth factor and deposition of type IV collagen.[7] This arteriopathy seems to present in the later stages of the disease when, among other alterations, phosphorus balance is disrupted by additional renal tissue loss and the systemic inflammatory response becomes more conspicuous. Identification of cardiovascular risk factors in uremia then becomes important, as it may reveal peculiar pathophysiological mechanisms, leading to a better understanding of the natural history of the disease and the development of therapies to reduce the risk of morbidity and mortality. Likewise, CKD-specific cardiovascular risk factors indicate a particular investigation strategy for these patients, who develop an arteriopathy with peculiar features.

In conclusion, we observed that uremia and its immunologic and metabolic alterations (systemic inflammation and hyperphosphatemia) were related to the presence of arteriopathy, specifically characterized by thickening of the middle layer of subepicardial arteries of experimental animals. This model may be used for the investigation of the mechanisms underlying the uremic arteriopathy and to test cardioprotective interventions in renal dysfunction.

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