CARACTERIZAÇÃO HEMORREOLÓGICA, BIOQUÍMICA E CARDIOVASCULAR NUM MODELO DE DOENÇA RENAL CRÓNICA MODERADA EM RATO / HEMORHEOLOGICAL, BIOCHEMICAL AND CARDIOVASCULAR CHARACTERIZATION OF A RAT MODEL OF MODERATE CHRONIC KIDNEY DISEASE

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ABSTRACT

Chronic kidney disease (CKD) is a major public health problem throughout the world. The major outcomes include a rapid progression, with development of anaemia and serious complications, namely thromboembolic and cardiovascular events. The pathophysiological alterations depend on the CKD degree, which will also determine the moment to initiate hemodialysis and recombinant erythropoietin (rhEPO) therapies. Thus, the cardio-renal complication might be better prevented or delayed if CKD patients are earlier identified and treated for the associated anaemia, which will depend on a better characterization of moderate stages of CKD. This study aimed to characterization an animal of model of moderate CKD induced by partial (¾) nephrectomy, by evaluating hemorheological, biochemical and cardiovascular profiles. Blood samples from control and CKD rats were collected at 0, 3, 9 and 15 weeks in

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order to evaluate: renal function, hemorheological parameters, iron metabolism, blood lipids, peripheral sympathetic and serotonergic systems, redox state and inflammatory markers. BP, tissues trophism indexes and kidney histomorphology were also assessed. Our data is consistent with a sustained moderate degree of CKD with a quickly compensated modest anaemia, though presenting iron metabolism disturbances. Despite the reasonable degree of functionality of the remnant kidney, as suggested by the anaemia correction and by the kidney hypertrophy, several important cardiovascular modifications were developed. Our model presented hypertension, dyslipidaemia, erythropoietic disturbances, sympathetic activation and oxidative stress. This model might be a good tool to study the cellular/molecular mechanisms underlying moderate stages of CKD and to evaluate the therapeutics efficacy for prevention, treatment/correction of cardio-renal anaemia syndromes and complications in early stages.

Key-words: Moderate chronic kidney disease**,** partial nephrectomy, rat model, hemorheological data, renal function, iron metabolism, Cardiovascular profile

INTRODUCTION

Chronic kidney disease (CKD) has been associated with a large number of alterations, namely anaemia, hypertension, inflammation, iron metabolism disturbance, white blood cell activation, oxidative stress and sympathetic overactivation[1-6]. However, the pathophysiological alterations depend on the CKD degree, which will also determine the moment to initiate hemodialysis and recombinant erythropoietin (rhEPO) therapies, for correction of anaemia, as well as its success. Therefore, cardiovascular events, renal failure, and premature death can be prevented or delayed if earlier identified and treated for CKD[6].

Animal models of CKD, achieved by a reduction in nephron number, create the possibility of testing *in vivo* the mechanisms associated to renal dysfunction, and might be essential to study the complications associated to CKD as well as the efficacy of therapeutics to prevent or delay adverse effects. The most common used techniques include surgical resection of the tissue (partial nephrectomy) and infarction[7], but there is yet limited information about its complete characterization, namely for moderate CKD stages, which will restrict the research on the molecular/cellular mechanism underlying the cardio-renal-anaemia syndrome, as well as its therapeutics. There is some evidence that the infarction model in rats presents a significant increase in proteinuria, hypertension and glomerulosclerosis when compared with the model of simple tissue excision (with equivalent reduction of renal mass)[8]. This suggests that partial nephrectomy can provide a better model of CKD in rat[7]. Furthermore, there is some evidence of a good inter-individual variability of uremia in this model of CKD. However, it is difficult to standardize it, due to the lack of consistency of nephron mass reduction, and, thus, to reach the desired degree of uremia. Without a consistent characterization

of the animal models of moderate CKD, and of a further comparison with the human pathophysiology of moderate CKD, the research on the molecular and cellular mechanisms underlying the cardio-renal-anaemia syndrome and its therapeutics will remain limited.

Since there is a lack of information in the literature concerning animal (rat) models of moderate CKD[7,9-12], we intended to perform a more complete characterization of this model, by assessing hemorheological, biochemical and cardiovascular profiles in a CKD induced by partial (¾) nephrectomy.

MATERIAL AND METHODS

Animals, diets and blood pressure measurement

Male Wistar rats (Charles River Laboratories Inc., Barcelona, Spain), 250-300g, were maintained in an air conditioned room, subjected to 12-h dark/light cycles and given standard laboratory rat chow (IPM-R20, Letica, Barcelona, Spain) and free access to tap water. Animal experiments were conducted according to the European Communities Council Directives on Animal Care.

The rats were divided into 2 groups (7 rats each): a control group and a group with surgical CKD induced by a two-stage (3/4) nephrectomy: firstly, about half of the left kidney was removed by left flank incision and, one week later, the right kidney was removed through a right lateral flank incision. All the animals from the two groups have completed the 15-week study protocol. Body weight (BW) was monitored during the experimental period.

Systolic blood pressure (SBP), diastolic blood pressure (DBP), mean blood pressure (MBP) and heart rate (HR) values were obtained using a tail-cuff sphygmomanometer LE 5001 (Letica, Barcelona, Spain) in appropriate contention cages. Before the measurements, the rats were warmed for 10-20 minutes at 25-30ºC to make the tail artery pulsations detectable and to achieve the pulse level needed. BP and HR values were obtained calculating the average of 8-10 measurements.

Sample collection and preparation

Blood samples: At the beginning of the experiments and at 3, 9 and 15 weeks after the surgical partial nephrectomy the rats were subjected to intraperitoneal anesthesia with a 2 mg/kg BW of a 2:1 (v:v) 50 mg/mL ketamine (Ketalar®, Parke-Davis, Laboratórios Pfeizer, Lda, Seixal, Portugal) solution in 2.5% chlorpromazine (Largactil®, Rhône-Poulenc Rorer, Laboratórios Vitória, Amadora, Portugal). Blood samples were immediately collected by venipuncture from the jugular vein into syringes without anticoagulant (for serum samples collection) or with the appropriate anticoagulant: ethylenediaminetetraacetic acid (EDTA), heparin or a solution of ACD (acid citrate-dextrose). Blood was centrifuged (160 *g* for 10 min. at 20ºC) to obtain the platelet rich plasma (PRP), which was then centrifuged (730 *g* for 10 min. at 20ºC) to obtain the platelet pellet and the platelet poor plasma (PPP). In order to maintain a

normal volemia, thus ensuring that results were not changed by the amount of blood collected, some parameters were analyzed only at the final time (15 weeks), namely serum oxidative and inflammatory markers and circulating catecholamines and serotonin contents.

Body and tissues weights and trophism indexes: At the end of experiments, the rats were sacrificed by cervical dislocation and the heart, the adrenals, the kidneys and the liver were immediately removed, placed in ice-cold Krebs' buffer and carefully cleaned of adherent fat and connective tissue. The BW, the whole heart weight (HW), the left ventricle weight (LVW), the adrenals (2: left plus right) weight (AW), and the kidney and liver weights were measured in all the rats under study in order to be used as trophism indexes.

Biochemical assays

Blood lipid: Serum total cholesterol (Total-c), high-density lipoprotein cholesterol (HDL-c), low-density lipoprotein cholesterol (LDL-c) and triglycerides (TGs) were analysed on a Hitachi 717 analyser (Roche Diagnostics Inc., MA, USA) using standard laboratorial methods. The main relationships between the Total-c, HDL-c and LDL-c blood concentrations were calculated to be used as atherogenic and cardiovascular risk indexes (LDL-c/HDL-c and Total-c/HDL-c).

Renal functions and hepatic enzymes: Serum creatinine, ureia and uric acid concentrations were used as renal function indexes and serum aspartate (AST) and alanine aminotransferase (ALT) levels were assessed for hepatic evaluation, through automatic validated methods and equipments (Hitachi 717 analyser).

Iron metabolism: Serum iron concentration was determined using a colorimetric method (Iron, Randox Laboratories Ltd., North Ireland, UK), whereas serum ferritin and transferrin were measured by immunoturbidimetry (Laboratories Ltd., North Ireland, UK).

Hematological data

Several hematological parameters were measured in EDTA whole blood by using an automatic Coulter Counter® (Beckman Coulter Inc., USA, CA): red blood cell (RBC) count, haematocrit, haemoglobin (Hb) concentration, hematological indices [mean cell volume (MCV), mean cell Hb (MCH) and mean cell Hb concentration (MCHC)], red cell distribution width (RDW), reticulocyte count, immature reticulocyte fraction (IRF), platelets count, platelets indices [mean platelet volume (MPV), platelet distribution width (PDW) and plaquetocrit (PCT)], and total and differential white blood cells (WBC) counts.

Oxidative equilibrium status

Thiobarbituric acid reactive-species (TBARs) assay: The blood serum was used to determine the products of lipid peroxidation, namely malondialdehyde (MDA), according to a method optimized by Estepa *et al.* (2001)[13].

Ferric reducing antioxidant potential (FRAP) assay: The serum an-

tioxidant capacity was measured as FRAP, according to previously described[14].

Serum 3-nitrotyrosine (3-NT) assay: Serum 3-NT concentration, which is an index of peroxynitrite formation, was quantified by using an enzymatic immunoassay (HyCult biotechnology b.v.; Uden, Netherlands).

Inflammatory markers

Serum levels of interleukin 2 (IL-2), IL-1β, transforming growth factor β1 (TGF-β1) and tumour necrosis factor α (TNF-α) were measured by ultrasensitive Quantikine® ELISA kits (R&D Systems, Minneapolis, USA). Serum C-reactive protein (CRP) was determined by using an ELISA kit from Helica Biosystems, Inc. (Fullerton, CA, USA). All assays were performed in duplicate.

Catecholamine assay

The contents of the catecholamines (CAs) norepinephrine (NE) and epinephrine (E) in the plasma, platelets, adrenals and kidney tissue were evaluated by high performance liquid chromatography with electrochemical detection (HPLC-ED), according to the procedures and chromatographic conditions previously described[15].

NE and E contents were measured by using known concentrations of standards (Sigma Chemical Co., St. Louis, MO, U.S.A.) and the internal standard dihydroxybenzylamine (DHBA), through the peak/area ratio technique. Chromatograms were obtained using the appropriate Gilson 710 software. Concentrations were expressed in: ng/ml for plasma and platelets, µg/g wet tissue for adrenals and ng/g for kidney.

Platelet and plasma serotonergic measures

Platelet and plasma 5-hydroxytryptamine (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA) contents were determined by HPLC-ED, according to the preparation/extraction procedures and the chromatographic conditions previously described [15], and calculated using a known concentration of the corresponding standard. Chromatograms were obtained using the appropriate Gilson 710 software. Values were expressed in ng/ml.

Kidney histology

The left kidney tissue of the control rats and the remnant left kidney tissue of the CKD animals were immersion-fixed in 4% buffered paraformaldehyde (PFA) and processed for paraffin sectioning. Three slices from each kidney were embedded. Three micrometre thick sections were stained with the haematoxylin-eosin (H&E). At least 50 glomeruli were evaluated per kidney tissue.

Data Analysis

For statistical analysis, we used the Statview 4.53 software from Abacus Concepts Inc. (Berkeley, CA, USA). Results are presented as means ± standard error of means (s.e.m.).

Comparisons between groups and between different times of evaluation were performed using Factorial ANOVA and Fisher's test. Significance was accepted at p less than 0.05.

RESULTS

Biochemical and hematological data

In Tables 1 and 2, we present the biochemical and hemorheological changes for CKD and control rats, before starting experiments and along the experimental period (at 3, 9 and 15 weeks after partial nephrectomy). The results were analysed in order to study the alterations associated with a moderate CKD during 15 weeks of follow-up, as compared to the control group. In CKD rats, three weeks after the partial ($^3/_4$) nephrectomy, a statistically significant increase in serum urea and creatinine concentrations were found. This increase in renal function markers remained high along the following 12 weeks of the experimental procedure (Table 1).

Concerning to hepatic function markers, a statistically significant increase was found in CKD rats for AST and ALT, particularly for the former at 9 weeks of the experimental period; at the end of experiments (15 weeks) only AST activity was still significantly higher. CKD rats presented alterations in the lipidic profile, namely, a progressively increase in Total-c, TGs and HDL-c (table 1). Iron status evaluation revealed, in CKD rats, a progressively increase in ferritin serum levels, which reached statistical significance in the last evaluation. On the contrary, transferrin serum levels were significantly lower 3 weeks after surgical procedure, and remained lower during the following 12 weeks of follow-up (Table 1).

Concerning to hematological data, 3 weeks after nephrectomy, the CKD animals showed a statistically significant decrease for RBC count, Hb and haematocrit, alongside with a significantly increase in RDW and in platelet count (table 2). These parameters were already similar to those of the control at 9 weeks after surgical intervention and remained stable until the end of the follow-up period. No statistically significant differences were found for total WBC counts; however, neutrophil and eosinophil counts increased progressively in CKD rats. In this group, basophil counts increased 3 weeks after chirurgic procedure, and decreased progressively during the remaining follow-up period, and monocyte counts increased only in the last laboratorial evaluation (Table 2).

Blood pressures, heart rate and body and tissue weights and trophism indexes

At the end of the experimental protocol (15 weeks), a statistically significant increase in SBP, DBP, MBP and HR were found in CKD rats, together with significant increases in heart and left ventricle weights, without any differences in the tissue trophism indexes (Table 3).

Oxidative equilibrium status and inflammatory markers

No statistically significant alterations were found between the two

Table 1. Biochemical changes in a rat model of moderate CKD during a follow-up period of 15 weeks

	Control and CKD rats before partial nephrectomy (n=14)	3 weeks after partial nephrectomy		9 weeks after partial nephrectomy		15 weeks after partial nephrectomy	
		Control (n=7)	CKD (n=7)	Control (n=7)	CKD (n=7)	Control (n=7)	CKD (n=7)
Renal function							
Creatinine (mg/dL)	0.41 ± 0.02	0.40 ± 0.02	0.83 ± 0.04 aa	0.33 ± 0,01	0.81 ± 0.05 aa	0.45 ± 0.02	0.91 ± 0.06 aa
Urea (mg/dL)	41.00 ± 0.68	39.20 ± 0.60	71.00 ± 2.65 a	34.50 ± 1.34	72.80 ± 2.41 aa	39.00 ± 1.58	67.83 ± 2.82 a
Uric acid (mg/dL)	0.60 ± 0.06	0.52 ± 0.05	0.46 ± 0.04	0.34 ± 0.06	0.40 ± 0.04	0.62 ± 0.16	0.48 ± 0.09
Hepatic Enzymes							
AST (IU/L)	68.25 ± 4.73	64.11 ± 3.42	56.70 ± 3.46	51.37 ± 1.21	72.00 ± 3.97 aa	81.00 ± 8.35	141.40 ± 10.83 aaa
ALT (IU/L)	34.00 ± 2.32	32.70 ± 2.31	29.33 ± 1.73	25.32 ± 1.35	39.60 ± 1.63 aaa	33.67 ± 2.32	36.00 ± 2.67
Lipid Profile							
Total-c (mg/dL)	54.33 ± 1,54	51.51 ± 1.49	71.80 ± 4.34 a	43.87 ± 3.12	84.28 ± 6.,51 aaa	55.40 ± 4.01	111.40 ± 5.22 aaa
HDL-c (mg/dL)	44.83 ± 1.22	41.77 ± 1.21	57.87 ± 2.89 a	34.37 ± 2.69	67.57 ± 4.42 aaa	42.60 ± 2.64	81.00 ± 4.18 aaa
LDL-c (mg/dL)	21.02 ± 0.78	19.31 ± 1.22	12.72 ± 1.10 aa	17.49 ± 1.00	15.81 ± 1.57	16.88 ± 1.09	16.01 ± 0.72
TGs (mg/dL)	131.66 ± 4.98	126.71 ± 8.82	84.20 ± 7.84	111.00 ± 10.28	162.40 ± 35.64	139.00 ± 21.24	207.00 ± 38.25 a
LDL-c/HDL-c	0.47 ± 0.03	0.48 ± 0.04	0.22 ± 0.02 aa	0.53 ± 0.04	0.25 ± 0.05 aaa	0.40 ± 0.05	0.20 ± 0.02 a
Total-c/HDL-c	1.21 ± 0.02	1.24 ± 0.02	1.21 ± 0.02	1.29 ± 0.04	1.24 ± 0.02	1.30 ± 0.04	1.38 ± 0.05
Iron metabolism							
Iron (µg/dL)	153.16 ± 12.89	108.91 ± 16.52	145.25 ± 8.74	194.27 ± 5.70	155.28 ± 7.51	154.20 ± 26.92	124.50 ± 11.91
Ferritin (ng/mL)	12.63 ± 1.35	12.02 ± 1.39	17.96 ± 1.12	10.01 ± 1.05	21.32 ± 2.14	12.92 ± 3.68	24.66 ± 5.32 a
Transferrin (mg/dL)	130.33 ± 3.79	130.91 ± 3.64	104.80 ± 2.79 aa	130.00 ± 2.47	106.01 ± 5.79aa	120.00 ± 7.97	81.57 ± 11.02 aaa

AST: aspartate aminotransferase; ALT: alanine aminotransferase; CKD: chronic kidney disease; HDL-c: high density lipoprotein-cholesterol; LDL-c: low density lipoprotein-cholesterol; TGs: triglycerides; Total-c: serum total cholesterol. Results are presented as mean ± s.e.m.: a – $p< 0.05$, aa – $p< 0.01$ and aaa – $p< 0.001$ vs the control group

Table 2. Hematological changes in a rat model of moderate CKD during a follow-up period of 15 weeks

	Control and CKD rats before partial nephrectomy (n=14)	3 weeks after partial nephrectomy		9 weeks after partial nephrectomy		15 weeks after partial nephrectomy	
		Control (n=7)	CKD (n=7)	Control (n=7)	CKD (n=7)	Control (n=7)	CKD (n=7)
RBC parameters							
Hb (g/dL)	13.82 ± 0.14	13.52 ± 0.21	11.42 ± 0.22 a	12.90 ± 0.18	13.70 ± 0.22	13.94 ± 0.36	13.44 ± 0.20
Haematocrit (%)	39.50 ± 0.49	38.61 ± 0.50	32.81 ± 0.62 a	36.34 ± 0.52	40.20 ± 0.21	40.92 ± 0.71	39.54 ± 0.57
RBC (x1012/L)	7.32 ± 0.12	7.13 ± 0.11	6.18 ± 0.12 a	6.39 ± 0.08	7.25 ± 0.19	7.44 ± 0.10	6.91 ± 0.14
MCV (fL)	53.95 ± 0.62	54.71 ± 0.53	53.09 ± 0.48	57.04 ± 0.64	55.54 ± 0.72	55.04 ± 1.32	57.27 ± 0.66
MCH (pg)	18.85 ± 0.25	19.23 ± 0.23	18.49 ± 0.17	20.20 ± 0.25	18.96 ± 0.36	18.74 ± 0.61	19.48 ± 0.28
MCHC (g/dL)	34.93 ± 0.19	34.91 ± 0.20	34.84 ± 0.13	35.43 ± 0.15	34.14 ± 0.33	34.04 ± 0.37	34.00 ± 0.30
RDW (%)	13.33 ± 0.43	13.23 ± 0.31	15.27 ± 0.35 a	12.58 ± 0.36	13.00 ± 0.20	14.88 ± 0.39	13.54 ± 0.23
Reticulocytes (x109/L)	389.01 ± 29.10	393.11 ± 22.97	423.09 ± 20.01	379.11 ± 39.95	392.09 ± 34.04	383.98 ± 28.97	326.09 ± 27.02
IRF (%)	0.38 ± 0.02	0.39 ± 0.03	0.41 ± 0.02	0.52 ± 0.07	0.42 ± 0.05	0.38 ± 0.01	0.56 ± 0.09
WBC parameters							
WBC (x109/L)	6.87 ± 1.19	6.51 ± 1.02	6.69 ± 0.38	5.14 ± 0.91	6.39 ± 0.29	5.14 ± 0.22	5.53 ± 0.85
Neutrophils (x109/L)	0.08 ± 0.01	0.07 ± 0.01	0.10 ± 0.01	0.05 ± 0.01	0.25 ± 0.17 a	0.07 ± 0.01	0.52 ± 0.31 a
Lymphocytes (x109/L)	5.82 ± 1.04	5.51 ± 0.81	4.38 ± 0.43	4.37 ± 0.79	4.86 ± 0.34	4.32 ± 0.81	4.65 ± 0.74
Basophils (x109/L)	1.15 ± 0.33	1.01 ± 0.22	1.89 ± 0.30 a	0.65 ± 0.15	1.25 ± 0.23 a	0.91 ± 0.52	0.29 ± 0.18 a
Eosinophils(x109/L)	0.012 ± 0.002	0.015 ± 0.003	0.023 ± 0.003	0.015 ± 0.003	0.028 ± 0.004 a	0.023 ± 0.007	0.064 ± 0.032 a
Monocytes (x109/L)	0.007 ± 0.002	0.006 ± 0.002	0.009 ± 0.003	0.005 ± 0.002	0.001 ± 0.001	0.004 ± 0.002	0.018 ± 0.010 a
Platelet parameters							
Platelets (x109/L)	980.50 ± 22.79	973.41 ± 22.62	1203.80 ± 47.93 a	943.87 ± 32.60	938.43 ± 49.85	980.40 ± 33.07	943.85 ± 78.49
PCT (%)	0.57 ± 0.02	0.56 ± 0.02	0.66 ± 0.02 a	0.54 ± 0.02	0.51 ± 0.02	0.58 ± 0.02	0.56 ± 0.03
MPV (fL)	5.83 ± 0.17	5.73 ± 0.12	5.53 ± 0.07	5.69 ± 0.07	5.50 ± 0.08	5.90 ± 0.14	5.70 ± 0.11
PDW (%)	16.15 ± 0.21	16.22 ± 0.19	15.99 ± 0.14	16.30 ± 0.24	16.04 ± 0.23	16.68 ± 0.26	16.60 ± 0.22

CKD: chronic kidney disease; Hb: hemoglobin; IRF: immature reticulocyte fraction; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration; MCV: mean corpuscular volume; MPV=Mean platelet volume; PCT: plateletocrit; PDW: platelet distribution width; RBC: red blood cell; RDW: red deviation weight; WBC: white blood cell. Results are presented as mean ± s.e.m.: a – $p< 0.05$, aa – $p< 0.01$ and aaa – $p< 0.001$ vs the control group

Table 3. Blood pressures, heart rate and body and tissue weights and trophism indexes in a rat model of moderate CKD after an experimental period of 15 weeks

	Control (n=7)	CKD (n=7)
Blood Pressures and Heart Rate		
SBP (mmHg)	114.88 ± 3.08	143.07 ± 4.61 aaa
DBP (mmHg)	99.11 ± 1.76	123.31 ± 12.38 aa
MBP (mmHg)	104.33 ± 1.89	129.64 ± 9.79 aaa
HR (beats/min.)	339.12 ± 6.28	391.89 ± 12.00 aaa
Body and Tissue Weights		
BW (Kg)	0.47 ± 0.02	0.46 ± 0.01
HW (g)	1.21 ± 0.03	1.46 ± 0.06 a
LVW (g)	0 .57 ± 0.03	0.72 ± 0.04 a
KW (g)	1.24 ± 0.04	1.94 ± 0.20
LW (g)	15.91 ± 0.58	18.23 ± 0.77
AW (g)	0.09 ± 0.01	0.10 ± 0.01
Tissue Trophism Indexes		
HW/BW (g/kg)	2.65 ± 0.07	2.98 ± 0.10
LVW/HW (g/kg)	0.47 ± 0.02	0.50 ± 0.02
LVW/BW (g/kg)	1.05 ± 0.11	1.17 ± 0.14
KW/BW (g/kg)	2.62 ± 0.05	3.96 ± 0.39
LW/BW (g/kg)	33.90 ± 0.10	38.80 ± 2.51
AW/BW (g/kg)	0.18 ± 0.02	0.20 ± 0.02

AW: adrenals weight; BW: body weight; CKD: chronic kidney disease; DBP: diastolic blood pressure; HR: heart rate; HW: heart weight; KW: kidney weight; LVW: left ventricle weight; LW: liver weight; MBP: mean blood pressure; SBP: systolic blood pressure. Results are presented as mean ± s.e.m.: a – $p< 0.05$, aa – $p< 0.01$ and aaa – $p< 0.001$ *vs* the control group

rats groups for MDA and TAS, but a significantly higher serum concentration of 3-NT was found. Concerning the inflammatory profile, we observed no significant differences between the two groups for CRP, IL-1β, IL-2 and TNF-α, excepting for TGF-β1 that augmented in CKD rats (Table 4).

Catecholamine and serotonin measures

Concerning sympathetic activity, CKD rats presented a statistically significant increase in plasma and kidney NE, and a decrease in platelet content; a significant reduction in platelet and adrenals E, and a concomitant increment in plasma E concentration were also observed (Fig. 1). Peripheral serotonergic measures in the CKD rats after the 15 weeks of follow-up showed a trend to higher values for all parameters, when compared to control animals, even though a statistically significant augment was only found for platelet 5-HIAA (Fig. 2).

Kidney morphology

The kidney morphology of the CKD rats was distinct from that of the controls (Fig. 3). The glomerular

Table 4. Redox state and inflammatory markers in a rat model of moderate CKD after an experimental period of 15 weeks

	Control (n=7)	CKD (n=7)
Redox State		
MDA (µmol/L)	0.27 ± 0.05	0.34 ± 0.06
TAS (µmol/L)	394.72 ± 51.42	408.03 ± 23.62
MDA/TAS	0.56 ± 0.05	0.73 ± 0.21
3-NT (nmol/L)	15.66 ± 1.40	50.45 ± 3.22 aaa
Inflammatory Profile		
CRP (µg/mL)	24.78 ± 1.25	25.83 ± 0.66
IL-1β (pg/mL)	26.52 ± 0.94	23.76 ± 0.99
IL-2 (pg/mL)	36.28 ± 8.70	49.34 ± 3.43
TGF-β1 (pg/mL)	358.41 ± 34.52	544.42 ± 50.43 a
TNF-α (pg/mL)	16.34 ± 1.81	15.75 ± 1.96

CKD: chronic kidney disease; CRP: C-reactive protein; IL-1β: interleukin 1β; IL-2: interleukin 2; MDA: malondialdehyde; TAS: total antioxidant status; TGF-β1: transforming growth factor β1; TNF-α: tumour necrosis factor α; 3-NT: 3-nitrotyrosine. Results are presented as mean ± s.e.m.: a – $p< 0.05$, aa – $p< 0.01$ and aaa – $p< 0.001$ *vs* the control group

capillary tufts of the CKD rats were hypercellular, with an increment of the glomerular volume. The Bowman space was also higher (Fig. 3B1). The interstitial region was lower due to tubular atrophy, together with an expansion of proximal convoluted tubules (Fig. 3B2).

DISCUSSION

Early detection of CKD and initiation of treatment should contribute to prevent or delay some of these adverse effects[16]. However, animal models of moderate CKD, which might be good tools to study the pathophysiological mechanisms underlying intermediary stages of renal disease and the efficacy of therapeutics, remain to be fully characterized. Our results have confirmed that the surgical partial (3/4) nephrectomy produces a moderate, but sustained stage of CKD. Indeed, we observed a significant (but restrained) increase in serum urea and creatinine concentrations at 3 weeks after the surgery that remained almost constant during the following period (still significantly higher than controls).

Furthermore, the results observed for RBC and reticulocyte counts, were similar to those observed for serum urea and creatinine along the follow-up period, further strengthens that a moderate stage of CKD was generated. RBC count was significantly lower 3 weeks after nephrectomy, consistent with the development of anaemia secondary to renal mass reduction; moreover, the number of reticulocytes, as well as the percentage of immature reticulocytes did not increase, further supporting a failure of the erythropoietic response mechanisms, due to insufficient erythropoietin production associated to the reduction in renal tissue. However, the anaemia was notoriously transitory, as both RBC and reticulo-

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FIGURE 1

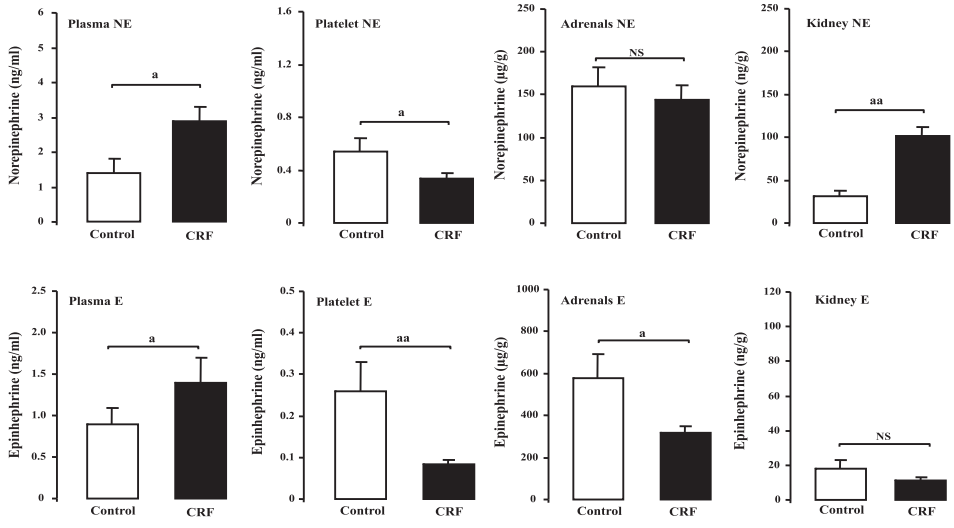

Fig. 1. Norepinephrine and epinephrine contents in plasma, platelets, adrenals and kidneys in a rat model of moderate CKD after an experimental period of 15 weeks. E: epinephrine; NE: norepinephrine. Results are presented as mean ± s.e.m.: a – $p< 0.05$, aa – $p< 0.01$ and aaa – $p< 0.001$ vs the control group. NS: non-significant

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FIGURE 2

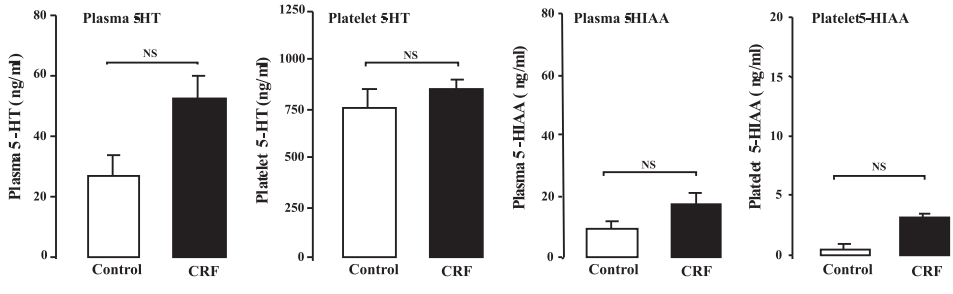

Fig. 2. Plasma and platelet 5-HT and 5-HIAA in a rat model of moderate CKD after an experimental period of 15 weeks. 5-HT: 5-hydroxy-tryptamine; 5-HIAA: 5-hydroxyindoleacetic acid. Results are presented as mean ± s.e.m.: NS: non-significant

cyte values returned to normal values in the following evaluation points (9 and 15 weeks of study), suggesting that our model is a moderate (but yet functional) CKD. This pattern is also confirmed by the low-grade histomorphological changes (lesions) found in the kidneys of CKD rats. There was also a trend to increased kidney weight (hypertrophy), consistent with a compensated renal insufficiency. Similar hypertrophy was obtained in other models of chronic renal failure induction, such as the 5/6 nephrectomized rats[9-12].

Iron-restricted erythropoiesis is a common clinical condition in patients with CKD. Several causes were proposed to underlie this situation, such as a functional iron deficiency, inadequate dietary iron intake, blood loss during the haemodialysis processes or from gastrointestinal tract (bleeding), inadequate intestinal iron ab-

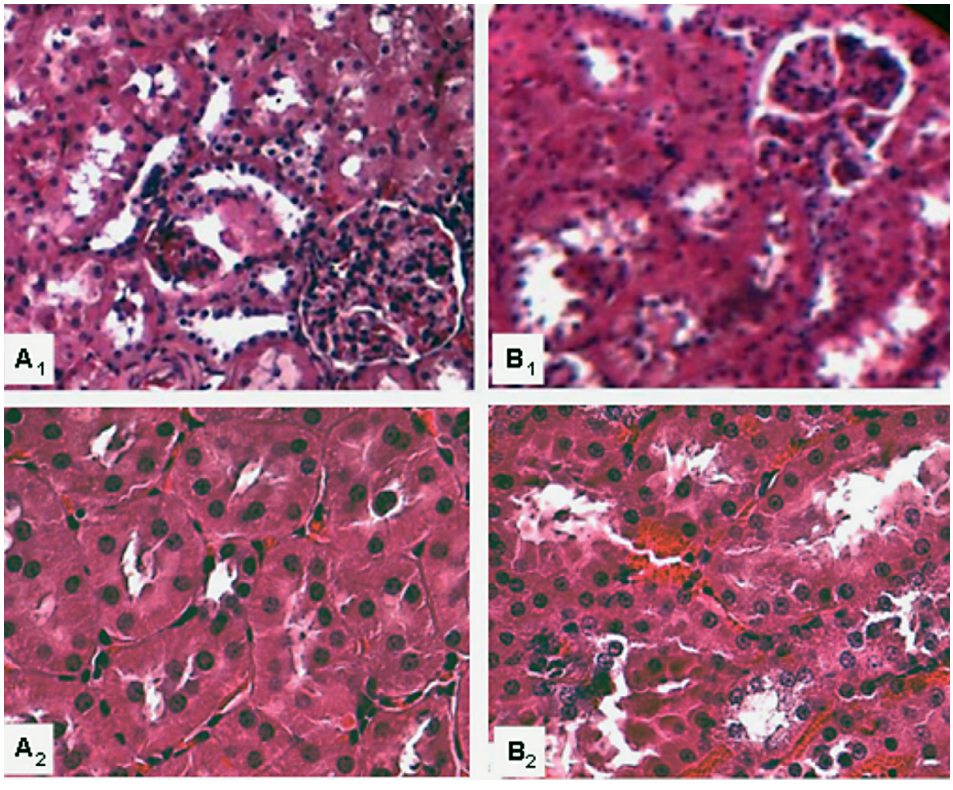

Fig. 3. Renal histology of kidneys from control (A) and moderate chronic CKD rats (B): 1 – glomerular region; 2 – proximal convoluted tubules area (original amplification x10 and x40, respectively for figures 1 and 2).

sorption and inhibition of iron mobilization from macrophages[17]. In our study, CKD rats presented serum iron values similar to those of the control and no significant changes were observed along the experiments; however, a trend towards higher values of ferritin were observed along the experiments and reached a significantly higher value at the end (15 weeks); these changes in ferritn were accompanied by a significant reduction in transferrin (observed at 3 weeks and afterwards), suggesting an inhibition in iron traffic, from macrophages to erythroid cells, leading to the progressive increase in iron storage, as showed by the progressive increase in ferritin, observed throughout the experiments. This suggests that our moderate model of CKD could also be used as a model of functional iron deficiency. Indeed, a functional iron deficiency has been reported in CKD patient under hemodialysis, namely, in moderate stages[17,18].

Liver metabolism dysfunction should plays a role in the lipid profile changes encountered in the CKD rats: increased values of total-cholesterol, HDL-c and TGs, without differences on LDL-c content, which might be explained, at least in part, by the lack in cholesteryl ester transfer protein (CETP), a characteristic of the rats[19,20]. Lipid abnormalities are found in CKD humans and the prevalence of hyperlipidaemia is higher than in the general population[21,22]. However, the risk of cardiovascular disease in CKD patients varies depending on the type of lipid abnormalities, the cause of renal disease and the degree of reduction in

glomerular filtration rate (GFR)[23]. Our rat model reproduces the changes encountered in CKD patients, in whom the main features are an increased serum level of both VLDL and IDL fractions, leading to hypertriglyceridemia and an unchanged or slightly increased LDL fraction enriched with triacylglycerols that might be attributed to the slow catabolism of the triacylglycerol-rich lipoproteins[24-26].

Besides the anaemia secondary to CKD, patients usually develop cardiac failure that further aggravates renal disease [2, 27, 28]. This triad of dysfunctions, already known as cardio-renal anaemia syndrome, is responsible for the serious complications encountered in those patients. Our study intended to clarify the degree of cardio-renal complications associated with this model of moderate CKD. Hypertension is a well established cause, a common complication, and an important risk factor for progression of the cardiovascular complications and the mortality of patients with CKD[4,5]; the pathophysiology of this hypertension is multifactorial[4]. Several authors conclude that CKD also activates (through the renin-angiotensin system participation) the sympathetic nervous system (SNS)[1]. Our results obtained using an animal model seem to be in agreement with the data from humans with CKD, despite a direct extrapolation from rat studies to humans deserves careful interpretation. In any case, an increased systolic and diastolic blood pressure was obtained, together with tachycardia and heart and left ventricle hypertrophy, which should be due to additional heart (left ventricle) effort to compensate the renal function deterioration. The kidneys, strategically positioned, have dense sensory and efferent sympathetic innervations, and can be the origin, as well as the target of overactivity of the SNS, which has been convincingly shown in CKD animal models[29]. Several studies showed that sub-totally nephrectomized rats developed a rapid increase of BP within a week after renal ablation, while totally nephrectomized rats, in which afferent sensory signals were removed, did not develop hypertension. This suggests that afferent signals from the disease kidneys are transmitted to the vasomotor control centre in the brain, thereby contributing to the increased blood pressure[1]. In our model of moderate CKD, plasma norepinephrine and epinephrine contents were increased, which might be caused by adrenal and platelet release, due to sympathetic system overactivation and platelet hyperreactivity, respectively. Moreover, plasma 5-HT was also increased, which, together with catecholamine increment, might influence platelet and vascular reactivity and, thus, contributing to cardiovascular and thromboembolic complications, as found in CKD patients[30-32].

Other factors have been studied to clarify the causes of the hypertension observed in CKD patients, including high levels of endothelin, oxidative stress or nitric oxide (NO) reduction[4,33-35]. In CKD patients, oxidative stress and inflammation could play a crucial role in the pathogenesis of the atherosclerosis, malnutrition and anaemia. In our model of moderate CKD, the redox state seems to be unaltered (MDA/TAS ratio was unchanged) which might be due to a

proper compensation of ROS formation by antioxidants. However, there was an increased serum 3-NT value, which is a marker of peroxynitrite generation, and, thus, might hypothetically reflect an increased superoxide formation and, thus, a reduced NO availability, since this dangerous oxidant is formed by the combination of both. In this model, inflammation seem to be yet less relevant, since, excepting an increment of TGF-β1, all the other markers were unchanged. The increment in ferritin, an acute phase protein, might also be viewed as part of an inflammatory state. The involvement of inflammation on this model of moderate CKD should be further confirmed, namely, by studies on the hypertrophic remnant kidney.

In conclusion, our model is consistent with a moderate but sustained degree of CKD with a compensated anaemia, though presenting a disturbance in iron metabolism. Despite the reasonable degree of functionality of the remnant kidney, as suggested by the correction of anaemia as well as by the kidney hypertrophy, several important cardiovascular modifications were developed. Therefore, our model presented hypertension, dyslipidaemia, sympathetic activation and oxidative stress. This model might be a good tool to study the cellular and molecular pathophysiological mechanisms underlying moderate stages of CKD, as occurs in humans, and, even more relevant, to evaluate the efficacy of therapeutics for prevention, treatment or correction of cardio-renal anaemia syndromes and complications in early stages.

REFERENCES

1. Rump LC, Amann K, Orth S, *et al.* Sympathetic overactivity in renal disease: a window to understand progression and cardiovascular complications of uraemia? Nephrol Dial Transplant 2000; 15:1735-1738.
2. Parmar MS. Chronic Renal Disease. BMJ 2002; 325:85-90.
3. Stenvinkel P. Anaemia and inflammation: what are the implications for the nephrologists? Nephrol Dial Transplant 2003; 18:viii17-viii22.
4. Koomans HA, Blankestijn PJ, Joles JA. Sympathetic hyperactivity in chronic renal failure: a wake-up call. J Am Soc Nephrol 2004; 15:524-537.
5. Costa E, Pereira BJ, Rocha-Pereira P, *et al.* Role of prohepcidin, inflammatory markers and iron status in resistance to rhEPO therapy in hemodialysis patients. Am J Nephrol 2008;28:677-683.
6. Bakris GL. Protecting renal function in the hypertensive patient: clinical guidelines. Am J Hypertens 2005; 18:112S-119S.
7. Liu ZC, Chow KM, Chang TM. Evaluation of two protocols of uremic rat model: partial nephrectomy and infarction. Ren Fail 2003; 25:935-943.
8. Ofstad J, Horvei G, Kvam FI, *et al.* Glomerular hemodynamics in progressive renal disease. Kidney Int 1992; 36:S8-S14.
9. Fine L. The biology of renal hypertrophy. Kidney Int 1986; 29:619-634.
10. Gretz N, Waldherr R, Strauch M. The remnant kidney model; in Gretz N, Strauch M (Eds.). Experimental and genetic rat models of chronic renal failure. Basel, Karger, 1993; 1-28.
11. Hostetter TH, Olson JL, Rennke HG, *et al.* Hyperfiltration in remnants nephrons: a potentially adverse response to renal ablation. J Am Soc Neprol 2001; 12:1315-1325.
12. Perez-Ruiz L, Ros-Lopez S, Cardús A, *et al.* A forASTten method to induce experimental chronic renal failure in the rat by ligation of the renal parenchyma. Nephron Exp Nephrol 2006; 103(3):e126-e130.
13. Estepa V, Ródenas S, Martín MC: Optimización de un método para la determinación de la peroxidación lipídica en suero humano. Anal Real Acad Farm 2001; 67:1-17.
14. Benzie IFF, Strain JJ. The Ferric Reducing Ability of Plasma (FRAP) as a Measure of "Antioxidant Power": The FRAP Assay. Anal Biochem 1996; 239:70-76.
15. Reis F, Rocha L, Ponte L, *et al.* Effect of preventive and regressive isosorbide 5-mononitrate treatment on catecholamine levels in plasma, platelets, adrenals, left ventricle and aorta in cyclosporin A-induced hypertensive rats. Life Sci 2005; 77:2514-2528.
16. Remuzzi G, Ruggenenti P, Perico N. Chronic renal diseases: renoprotective benefits of renin-angiotensin system inhibition. Ann Intern Med 2002; 136:604-615.
17. Hörl WH. Iron therapy for renal anemia: how much needed, how much harmful? Pediatr Nephrol 2007; 22:480-489.
18. Brugnara C. Iron deficiency and erythropoiesis: new diagnostic approaches. Clin Chem 2003; 49:1573-1578.

19. Oschry Y, Eisenberg S. Rat plasma lipoproteins: reevaluation of a lipoprotein system in an animal devoid of cholesteryl ester transfer activity. J Lipid Res 1982; 23:1099-1106.
20. Vaziri ND, Liang K. ACAT inhibition reverses LCAT deficiency and improves plasma HDL in chronic renal failure. Am J Physiol Renal Physiol 2004; 287:F1038-F1043.
21. Kasiske BL. Hyperlipidemia in patients with chronic renal disease. Am J Kidney Dis 1998; 32:S142-153.
22. Tsimihodimos V, Dounousi E, Siamopoulos KC. Dyslipidemia in chronic kidney disease: an approach to pathogenesis and treatment. Am J Nephrol 2008; 28:958-73.
23. Chan CM. Hyperlipidemia in chronic renal disease. Ann Acad Med Singapore 2005; 35:31-35.
24. Grundy SM: Management of hyperlipidemia of kidney disease. Kidney Int 1990; 37:847-853.
25. Attman PO, Samuelsson O, Alaupovic P. Lipoprotein metabolism and renal failure. Am J Kidney Dis 1993; 21:573-592.
26. Szolkiewicz M, Nieweglowski T, Korczynska J, *et al.* Upregulation of fatty acid synthase gene expression in experimental chronic renal failure. Metabolism 2002; 51:1605-10.
27. Silverberg D, Wexler D, Blum M, *et al.* The cardio-renal anaemia syndrome: does it exist? Nephrol Dial Transplant 2003; 18:viii7-viii12.
28. Wexler D, Silverberg D, Blum M, *et al.* Anaemia as a contributer to morbidity and mortality in congestive heart failure. Nephrol Dial Transplant 2005; 20:vii11-vii15.
29. Campese VM. Neurogenic factors and hypertension in chronic renal failure. J Nephrol 1997; 10:184-187.
30. Vanhoutte PM. Platelet-derived serotonin, the endothelium, and cardiovascular disease. J Cardiovasc Pharmacol 1991; 17:S6-12.
31. Azzadin A, Mysliwiec J, Wollny T, *et al.* Serotonin is involved in the pathogenesis of hypertension developing during erythropoietin treatment in uremic rats. Thromb Res 1995; 77:217-224.
32. Malyszko J, Malyszko JS, Pawlak D, *et al.* Homeostasis, platelet function and serotonin in acute and chronic renal failure. Thromb Res 1996; 83:351-361.
33. Ritz E, Koomans HA. New insights into mechanisms of blood pressure regulation in patients with uraemia. Nephrol Dial Transplant 1996; 11:52–59.
34. Pechter U, Aunapuu M, Riispere Z, *et al.* Oxidative stress status in kidney tissue after losartan and atenolol treatment in experimental renal failure. Nephron Exp Nephrol 2004; 97:e33-7.
35. Manning RD Jr, Tian N, Meng S. Oxidative stress and antioxidant treatment in hypertension and the associated renal damage. Am J Nephrol 2005; 25:311-317.