Original Paper

Previous Exercise Effects in Cisplatin-Induced Renal Lesions in Rats

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Key Words

Acute kidney injury • Cisplatin • Endothelium • Inflammation • Physical training

Abstract

Background/Aims: Physical training has beneficial effects on endothelial function and can influence the regeneration of the endothelial cell. We investigated the effect of physical training on cisplatin (CP)-induced acute kidney injury and assessed the impact of training on endothelial structure and function, and on the inflammatory processes in rats. **Methods:** We injected male Wistar rats subjected to previous physical training in treadmill running (trained, TR) or not (sedentary, SED) with CP (5 mg/kg) (TR+CP and SED+CP groups, respectively). Five days after the injections, blood and urine samples were collected to evaluate renal function and kidneys were harvested for morphological, immunohistochemical, enzyme-linked immunosorbent assay, and analysis of nitric oxide (NO) levels. **Results:** Rats treated with CP showed increased levels of plasma creatinine and sodium and potassium fractional excretion. These alterations were associated with increase in tubulointerstitial lesions and macrophage number, reduction of endothelial cells, and increased VEGF, vimentin, and α -smooth muscle actin expression in the outer renal medulla in the SED+CP group. We also found increased levels of renal IL-1 β and increased excretion of monocyte chemoattractant protein-1 and transforming growth factor- β compared with controls. These changes were milder in trained rats, associated with increased levels of renal tissue NO, and increased expression of p-eNOS and stromal cell-derived factor-1 α (a chemokine involved in kidney repair) in the kidneys of CP-injected trained rats. **Conclusions:** The protective effect of previous training in CP-treated rats was associated with reduced endothelial cell lesions and increased renal production of NO in trained rats.

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Introduction

Acute kidney injury (AKI) is known to be associated with high morbidity, mortality, and financial healthcare costs [1]. Acute tubular necrosis is the major cause of AKI and has been associated with inflammation and oxidative stress, which can intensify renal injury and contribute to loss of renal function [2, 3].

The chemotherapeutic drug cisplatin (CP) is widely used for the treatment of solid tumors but its most notable side effect is nephrotoxicity, which limits its use in clinical practice [4]. The primary endothelial lesion induces a sequence of events in the endothelium, including reduced vasodilation, a pro-inflammatory state, and renal capillary thrombosis with vasoconstriction, which contributes to decreased renal plasma flow. In addition, endothelial injury causes increased expression of adhesion molecules, leukocyte binding to the endothelium, vascular congestion, and cellular infiltration [4, 5]. The mechanisms associated with CP-induced kidney damage include oxidative stress, which can activate inflammation-associated pathways that include JNK [6] and p38 [7] mitogen-activated protein kinases, and the NF- κ B pathway [8]. CP injection induces neutrophil and macrophage recruitment and increases renal cortical and outer medulla tumor necrosis factor (TNF)- α and interleukin (IL)-1 β levels [9], which can intensify tubular epithelial cell loss through necrosis and apoptosis.

Unfortunately, efficient therapies to decrease CP-induced nephrotoxicity are not available. Experimental and clinical studies in the last decade have shown that physical training can enhance the endothelial function in patients with cardiovascular disease and metabolic syndrome [10, 11]. Regular moderate-intensity exercise training before induction of diabetes mellitus improved metabolic control, renal function, and structural changes, and reduced the expression of transforming growth factor (TGF)- β in renal tissue, which was associated with decreased fibronectin expression and a renoprotective effect [12]. We also observed in a recent study that previous training can reduce renal endothelial lesions and improve angiogenesis in Adriamycin-treated rats [13].

Among the various mechanisms associated with the renoprotective effect of physical exercise is the mobilization of endothelial progenitor cells (EPCs) to the lesion site. Some studies have shown that EPCs move to neovascularization sites and differentiate into endothelial cells *in situ* [14, 15]. Stromal cell-derived factor (SDF)-1 is a chemokine that specifically binds to its CXCR4 receptor [16]. SDF-1 expression has been detected in the developing kidney [17], and the SDF-1/CXCR4 chemokine receptor system was activated in the kidney after AKI. Tögel et al. (2005) [18] showed that renal tubular cells express CXCR4, the receptor for SDF-1, with up-regulated expression after ischemia. According to the authors, this adaptive response to AKI could serve to support organ repair by renal intrinsic cells. In healthy individuals, strenuous activity leads to a time-dependent increase in circulating EPCs, associated with an increased expression of the inflammatory marker IL-6, which suggests that endothelial damage could be counteracted by the release of EPCs [19]. Nitric oxide (NO) is a key molecule for EPC mobilization during physical exercise [20]. NO is one of the most important mediators released by the endothelium and acts as a potent vasodilator and inhibits inflammation, vascular wall hypertrophy, and platelet aggregation [21]. Experimental and cell culture studies suggest that repetitive increases in shear stress caused by increased vascular blood flow increase the expression and phosphorylation of endothelial nitric oxide synthase (eNOS), thus contributing to greater bioavailability of NO [22]. Physical exercise may also reduce the expression of NADPH oxidase, thus reducing the generation of reactive oxygen species [23]. The effects of an acute period of exercise differ from those observed with a regular exercise program for a certain period. In acute exercise, oxidative stress might play a significant role, but under conditions of regular physical training, NO is the main molecule that regulates the mobilization of EPCs [24]. However, the mechanisms related to the influence of physical exercise on the maintenance of the endothelium structure are not yet fully understood. Because the severity of AKI is a

major factor in mortality, the effects of previous physical training can be important in the prevention of death and renal lesions.

In this study, we evaluated the effect of previous moderate exercise training on renal function and structure in CP-treated rats and the association of this training with inflammation, endothelial function, and angiogenesis.

Materials and Methods

Animals and experimental protocols

Male Wistar rats (70-80 g) were provided by the Animal House of Ribeirão Preto Campus, University of São Paulo (Ribeirão Preto, Brazil), and housed in polycarbonate cages at standard room temperature (22°C) and under a 12-h light/dark cycle with free access to standard rat chow and water.

First, the rats were selected based on their ability to run on a treadmill (EPR model; Gesa, São Paulo, Brazil) with 0% incline [13]. Rats were subjected to daily sessions of increasing duration (5-20 min) and intensity (running velocity: 5-20 m/min) over 5 days. The maximum velocity intensity testing (V_{max}) was then determined by subjecting the rats to graded races (increments of 3 m/min every 3 min), starting at 6 m/min up to the maximum intensity attained for each animal [25, 26]. Then, the rats were divided into two groups: those subjected to exercise training (TR, $n = 15$) and those that was sedentary (SED, $n = 12$). After V_{max} testing, we established the initial velocity (80% of V_{max}) to determine the maximal lactate steady state (MLSS), which is the benchmark test in determination of aerobic capacity [27]. The rats were then subjected to continuous running (30 min) with a 48-h rest interval. Subsequently, the distal tail was pricked with a needle and 25 μ L of blood was collected using heparinized capillary tubes at rest and after 10, 20, and 30 min of exercise. Samples were transferred to 0.6-mL microcentrifuge tubes containing 50 μ L of 1% sodium fluoride. Lactatemia was measured using a Blood Lactate Analyzer (YSI 2300 STAT PLUS, Yellow Springs, OH). MLSS was considered as the highest exercise intensity where the increase in blood lactate did not exceed 1 mM/L from the 10th to the 30th min of exercise.

The physical training program lasted for 4 weeks (5 days/week), with alternating training sessions in terms of volume and intensity during the week. Intensity and duration varied according to two grades of aerobic training/resistance: easy (continuous exercise for 60 min using 50% of MLSS intensity) and moderate (continuous running lasting 30 min using 100% of MLSS intensity) [13, 28].

By 48 h after the last training program, all rats were injected with a single intraperitoneal (i.p.) injection of CP (5 mg/kg) (Libbs, Barra Funda, Brazil) or normal saline. The SED and TR groups were further subdivided into four groups: (a) sedentary + saline (SED+SAL, $n = 6$); (b) physical training + saline (TR+SAL, $n = 6$); (c) sedentary + CP (SED+CP, $n = 7$), and (d) physical training + CP (TR+CP, $n = 9$).

Next, 24-h urine samples were collected on day 4 after either saline or CP injections for measurement of urine volume and osmolality, sodium, and potassium. On day 5 after the injections, the rats were anesthetized with sodium thiopental (40 mg/kg, i.p.). After cannulation of the aortic artery, blood samples were collected for measurement of plasma creatinine, sodium, and potassium. The kidneys were perfused with phosphate-buffered solution (0.15 M NaCl and 0.01 M sodium phosphate buffer, pH 7.4). Kidneys were harvested, one for western blot and enzyme-linked immunosorbent assay (ELISA), and the other for transverse sections, which were fixed in methacarn solution (acetic acid, methanol, chloroform), rinsed in 70% ethanol, and processed for paraffin embedding for immunohistochemical and histological analysis.

All experimental procedures were conducted in accordance with the principles and procedures outlined in the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals and were approved by the Animal Experimentation Committee of the University of São Paulo at Ribeirão Preto School of Medicine (protocol no. 006/2013).

Light microscopy study

We prepared 4-mm-thick histological sections that were stained with Masson's trichrome and examined under light microscopy. Tubulointerstitial damage was defined as the presence of tubular necrosis, inflammatory cell infiltrate, tubular luminal dilation, or tubular atrophy. Damage was graded [29] on a scale of 0-4 as follows (0 = normal; 0.5 = small focal areas; 1 = involvement of <10% of the renal outer medulla; 2

= 10-25%; 3 = 25-75%; 4 = extensive damage involving more than 75% of the renal outer medulla). Twenty grid fields measuring 0.1 mm² were evaluated in the renal outer medulla of each kidney (Axion version 4.8.3, Zeiss, Germany), and the mean values per kidney were calculated.

Antibodies

Renal tissue was evaluated for macrophage infiltration by using a monoclonal anti-rat ED1 antibody (Serotec, Oxford, UK) that selectively reacts with a cytoplasmic antigen present in macrophages and monocytes [30]. Tubular cell lesions and cell transdifferentiation were evaluated with monoclonal antibodies against vimentin or α -smooth muscle actin (α -SMA; Dako, Glostrup, Denmark) antibodies. Renal tissue angiogenesis and endothelial lesions were evaluated by using monoclonal anti-rat VEGF (Santa Cruz Biotechnology, Dallas, TX) and aminopeptidase P (JG12, a marker for endothelial cells; eBioscience, San Diego, CA) antibodies, respectively. eNOS and p-eNOS expression in renal tissue was evaluated with monoclonal anti-rat eNOS or p-eNOS antibodies (Santa Cruz Biotechnology, Dallas, TX).

Immunohistochemical analysis

For the primary antibody reaction, sections were incubated with either 1/1,000 anti- α -SMA or 1/800 anti-JG12 at 4°C overnight or with 1/1,000 anti-ED1 or 1/500 anti-vimentin antibodies for 1 h at room temperature. Detection of the reaction product was performed using an avidin-biotin-peroxidase complex (Vector Laboratories Inc., Burlingame, CA). The color reaction was developed with 3,3'-diaminobenzidine (Sigma Chemical Company, St. Louis, MO) and nickel chloride in the presence of H₂O₂. Counterstaining of the sections was then performed with methyl green, followed by dehydration and mounting. Immunoperoxidase staining for α -SMA in the tubulointerstitial area of the outer medulla was evaluated in 30 grid fields (0.1 mm²) each, which were semi-quantitatively graded and the mean score per biopsy was calculated. Each score was indicative of changes in staining intensity determined by the percentage of the grid field with positive staining: 0 = absent staining or less than 5% of stained area; 1 = 5-25%; 2 = 25-50%; 3 = 50-75%; and 4 = >75%. Immunoperoxidase staining for ED1 was determined by counting the number of positive cells (infiltrating macrophages) in the outer renal medulla tubulointerstitium; peritubular capillaries positively stained for JG12 in the outer renal medulla were evaluated by examination of 30 grid fields, and mean counts per kidney were calculated. All fields were analyzed under $\times 400$ magnification.

Western blot

Tissue from the renal cortex and outer medulla was homogenized at 4°C in lysis buffer (50 mM tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 1 μ g/mL aprotinin, 1 μ g/mL leupeptin, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, pH 10, 1 mM sodium pyrophosphate, 25 mM sodium fluoride, 0.001 M EDTA, pH 8). Proteins were separated by polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes, incubated for 24 h in 30 mL of blocking buffer (TBS, 5% skim milk), washed in buffer (TBS, 0.1% Tween 20, pH 7.6) and incubated with anti-eNOS (1/500 in 5% bovine serum albumin), anti-p-eNOS (1/500), anti-VEGF (1/500), anti-CXCR4 (1/1,000), or anti-SDF-1 α (1/500) overnight at 4°C. Protein loading and/or transfer control was ensured by also incubating the membranes with anti- α 1-tubulin monoclonal antibody (1/4,000 in 5% bovine serum albumin) overnight at 4°C. Blots were washed and incubated with horseradish peroxidase-conjugated goat anti-mouse (1/20,000; Dako, Glostrup, Denmark) or goat anti-rabbit (1/5,000; Dako, Glostrup, Denmark) for 1 h at room temperature. The membrane-bound antibody was detected with Supersignal West Pico Chemiluminescent substrate (Pierce, Rockford, IL) and captured on X-ray film. The intensity of the identified lanes was quantified by densitometry with ImageJ NIH image software and was reported in arbitrary units [13].

ELISA

Urine TGF- β or MCP-1 and renal tissue IL-1 β levels were measured. Urine samples collected from the urinary bladder were immediately treated with 1 mM phenylmethylsulfonyl fluoride (Sigma Chemical Co, St. Louis, MO) and stored at -70°C until analysis. TGF- β and MCP-1 quantification was done by performing ELISA using commercial kits (Promega Corporation, Madison, MO, and Pierce Biotechnology Inc., Rockford, IL, respectively). Median values of TGF- β and MCP-1 levels were expressed in pg/mg of urine creatinine, and IL-1 β was expressed as pg/mg of protein.

Determination of renal NO levels

Renal tissue samples were homogenized in 0.1 N acetic acid (3:1), centrifuged at $10,000 \times g$ for 5 min, and then aliquoted. These samples and the plasma and urine were then deproteinized with 95% ethanol (3:1) and centrifuged at $4,000 \times g$ for 5 min. The supernatant was subjected to analysis for NO content by using the NO/O zone technique described previously [31] with the Sievers analyzer (Sievers 280 NOA, Sievers, Boulder, CO). Protein levels in renal tissue were determined by the Bradford method [32]. Median NO values were expressed in $\mu\text{g}/\text{mg}$ of urine or plasma creatinine or $\mu\text{M}/\mu\text{g}$ of protein.

Statistical analysis

One-way analysis of variance and the Tukey multiple comparison test was used for variables with normal distribution or those that showed normal distribution after $\log_e$ transformation. Normality of the dependent variables was investigated using Kolmogorov-Smirnov test. Data are expressed as median and interquartile range (25-75%), mean $\pm$ SEM, or geometric means and confidence intervals. Statistical analyses and graphics were constructed using GraphPad Prism version 6.0 for Windows (GraphPad Software, La Jolla, CA). Statistical significance was set at $p < 0.05$.

Results

Renal function

CP-injected rats that had not been subjected to previous exercise training showed increased levels of plasma creatinine, urinary volume, sodium, and potassium fractional excretion, with associated decreased urine osmolality 5 days after injection, compared with saline-injected control rats. These CP-induced alterations were prevented in the rats that had received previous training (Table 1).

Table 1. Parameters of renal function from sedentary (SED+SAL and SED+CP) and trained groups (TR+SAL and TR+CP), 5 days after saline or CP injections. Pcreat, plasma creatinine (mg%); FE, fractional excretion (%); UV, urinary volume (mL/24 h); Uosm, urinary osmolality (mOsm/kg H_2O). The data are expressed as the median and interquartile range (25-75%) (Pcreat, UV and Uosm) or the mean $\pm$ SEM (FE). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. SED+SAL; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. TR+SAL; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ vs. SED+CP

	SED+SAL	TR+SAL	SED+CP	TR+CP
Pcreat	0.66 (0.55;0.83)	0.75 (0.61;0.89)	2.17** (1.56;4.10)	0.62* (0.57;1.91)
FE _{Na}	0.70 $\pm$ 0.01	0.56 $\pm$ 0.11	4.87*** $\pm$ 1.36	0.52## $\pm$ 0.14
FE _K	44.80 $\pm$ 6.36	46.57 $\pm$ 7.98	344.90*** $\pm$ 46.31	81.35### $\pm$ 24.86
UV	15.00 (11.00;17.00)	12.50 (8.75;14.25)	42.00** (39.50;55.75)	22.00# (15.00;55.50)
U _{osm}	1,687 (1278;1840)	1,797 (1268;1976)	516*** (454.5;572.8)	1,456# (531.5;1931)

Fig. 1. Masson's trichrome-stained histological sections from the outer renal medulla of sedentary rats injected with saline (A) or CP (B), and a trained animal injected with CP (C). Note the tubular necrosis in B, which is milder in C (bar represents 50 μm). (D) Score for tubulointerstitial lesions in the renal outer medulla from sedentary and trained rats injected with saline (SED+SAL, $n = 6$, and TR+SAL, $n = 6$) or CP (SED+CP, $n = 7$, and TR+CP, $n = 9$). Values are given as the mean $\pm$ SEM. *** $p < 0.001$ vs. SED+SAL; *** $p < 0.001$ vs. TR+SAL; ## $p < 0.01$ vs. CP+SAL.

Light microscopy

Pathological scoring for tubulointerstitial lesions (tubular cell necrosis, interstitial inflammatory infiltrate, tubular lumen dilation) showed higher scores in the outer renal medulla from CP-injected sedentary rats 5 days after injection compared with trained rats (SED+CP and TR+CP, respectively) (Fig. 1).

Fig. 2. Immunolocalization of ED1-positive cells (macrophages) in the outer renal medulla of sedentary rats injected with saline (A) or CP (B), and a trained animal injected with CP (C). Note the presence of macrophages in B, which is less intense in C (bar represents 50 μ m). (D) Number of ED1-positive cells in the outer renal medulla from sedentary and trained rats injected with saline (SED+SAL, n = 6, and TR+SAL, n = 6) or cisplatin (SED+CP, n = 7, and TR+CP, n = 9). Values are given as the mean $\pm$ SEM. ***p<0.001, *p<0.05 vs. SED+SAL; ***p<0.001, *p<0.05 vs. TR+SAL; ##p<0.01 vs. CP+SAL.

Fig. 3. Immunolocalization of vimentin in the outer renal medulla of sedentary rats injected with saline (A) or CP (B), and a trained animal injected with CP (C). Note the presence of vimentin in B, which is milder in C (bar represents 200 μ m). (D) Score for vimentin in the renal outer medulla from sedentary and trained rats injected with saline (SED+SAL, n = 6, and TR+SAL, n = 6) or CP (SED+CP, n = 7, and TR+CP, n = 8). Values are given as the mean $\pm$ SEM. *p<0.05 vs. SED+SAL; *p<0.05 vs. TR+SAL.

Fig. 4. Immunolocalization of α -SMA in the outer renal medulla of sedentary rats injected with saline (A) or CP (B), and a trained animal injected with CP (C). Note the presence of macrophages in B, which is less intense in C (bar represents 50 μ m). (D) Score for α -SMA in renal outer medulla from sedentary and trained rats injected with saline (SED+SAL, n = 6, and TR+SAL, n = 6) or CP (SED+CP, n = 6, and TR+CP, n = 9). All values are given as the mean $\pm$ SEM. ***p<0.001, *p<0.05 vs. SED+SAL; ***p<0.001, *p<0.05 vs. TR+SAL; ##p<0.01 vs. SED+CP.

Fig. 5. Immunolocalization of JG12 in the outer renal medulla of sedentary rats injected with saline (A) or CP (B), and a trained animal injected with CP (C). Note that the expression of JG12 is milder in B. Bar represents 50 μ m. (D) Number of PTC in outer renal medulla from sedentary and trained rats injected with saline (SED+SAL, n = 6, and TR+SAL, n = 6) or CP (SED+CP, n = 6, and TR+CP, n = 9). Western blot and densitometric analyses of

VEGF (E), CXCR4 (F), and SDF-1 α (G) in the renal cortex and outer medulla from SED+SAL (1), TR+SAL (2), SED+CP (3), and TR+CP (4). Densitometric ratios among VEGF, CXCR4 or SDF-1 α and α 1-tubulin were calculated, and data are expressed in comparison with the controls (SED+SAL), with the mean ($\pm$ SEM) control value designated 100%. All values are given as the mean $\pm$ SEM. ** p < 0.01, * p < 0.05 vs. SED+SAL; ** p < 0.01, * p < 0.05 vs. TR+SAL; # p < 0.05 vs. SED+CP.

Immunohistochemistry

Inflammatory cell infiltration in the outer renal medulla area of SED+CP rats was evidenced by increased ED1 expression (macrophage) in cells compared with controls; this increased expression was milder in the outer renal medulla of rats from the TR+CP group (Fig. 2). Compared with controls, the scores for vimentin (Fig. 3) and α -SMA were higher in the renal outer medulla (Fig. 4) of the SED+CP

group, and were also attenuated in rats from the TR+CP group. PTC density in the outer renal medulla, determined by quantification of the endothelium-specific JG12 marker, was reduced in sedentary CP-injected rats (SED+CP), and this reduction was milder in trained rats injected with CP (TR+CP) (Fig. 5).

Western blotting

Western blot analysis showed increased VEGF expression in renal tissue from rats in the sedentary group injected with CP compared with rats from the control groups injected with saline (SED+SAL and TR+SAL; p < 0.05); this increase was not observed in the trained rats injected with CP (Fig. 5E). Rats in the trained group also showed increased expression of eNOS, p-eNOS (Fig. 6D and E) and CXCR4, compared with sedentary rats, 5 days after CP

Table 2. Renal tissue levels of IL (interleukin)-1 β and the urinary excretion of MCP (monocyte chemoattractant protein)-1 and TGF (transforming growth factor)- β from sedentary (SED+SAL and SED+CP) and trained groups (TR+SAL and TR+CP), 5 days after saline or CP injections. The data are expressed as the geometric mean and confidence intervals (TGF- β) or the median and interquartile range (25;75%) (IL-1 β and MCP-1). * p < 0.051 vs. SED+SAL; * p < 0.05 vs. TR+SAL

	IL-1 β (pg/mg prot)	MCP-1 (pg/mg urinary creatinine)	TGF- β (pg/mg urinary creatinine)
SED+SAL	0.63 (0.28;0.76)	314.4 (104.5;630.4)	247.6 (130.5-469.8)
TR+SAL	0.71 (0.48;0.92)	205.6 (158.6;325.1)	121.0 (70.45-207.9)
SED+CP	2.10 (1.78;3.08)*	1,887 (1,519;2,533)*	10,403 (6,574-16,462)*
TR+CP	0.73 (0.50;3.43)	824.8 (602.4;1,539)	3,081 (705.4-13,457)

injection ($p < 0.05$) (Fig. 5F). The reduction in SDF-1 α expression in renal tissue from SED+CP rats, compared with saline-injected rats ($p < 0.05$), was milder in trained rats injected with CP (Fig. 5G).

ELISA

Urinary excretion of TGF- β and MCP-1 and renal tissue levels of IL-1 β were increased in the SED+CP group compared with controls. These changes were milder in rats from the TR+CP group ($p < 0.05$) (Table 2).

NO levels in renal tissue, plasma, and urine

Renal tissue NO levels were increased in both groups injected with CP, compared to the groups of rats injected with saline. However, in the sedentary rats (SED+CP) this increase was less intense compared to trained rats injected with CP (TR+CP) ($p < 0.01$). NO levels were decreased in plasma and urine, compared with controls, in both groups treated with CP. Urine NO levels were higher in trained rats injected with saline, compared with other groups (Fig. 6A-C)

Discussion

Endothelial cells are essential for the survival of other renal cells because release oxygen and nutrients to the renal tissues and help to maintain the glomerular capillaries [33]. Endothelial lesions and dysfunction are observed in AKI, where they are involved in the establishment and evolution of renal injury [34].

AKI is characterized by a sudden drop in renal function, which can develop within hours or days after the insult and is potentially reversible [2]. Our data show that exercise training before CP-induced AKI ameliorated the decrease in renal function and the structural lesions in the kidneys of the rats injected with this drug. The rats injected with CP showed an increase in plasma creatinine levels 5 days after the injection. These rats also demonstrated increased urinary volume and sodium and potassium fractional excretions and decreased urinary osmolality. Morphological analysis showed increased tubular damage, mainly in the outer strip of the outer medulla of the kidneys, where CP is reabsorbed and concentrated [35]. These changes were associated with inflammation, evidenced by increased ED1-positive cell (macrophage) infiltration in the outer renal medulla and IL-1 β levels in renal tissue as well as the increased urinary excretion of MCP-1. All these changes were milder in rats subjected to exercise training before injection of CP.

Immunohistochemical analysis also showed increased immunostaining for vimentin and α -SMA in the outer renal medulla of all rats injected with CP, which indicated the presence

Fig. 6. NO levels in renal tissue (A), plasma (B), and urine (C) from sedentary (SED+SAL and SED+CP) and trained groups (TR+SAL and TR+CP), 5 days after saline or CP injections. Western blot and densitometric analyses of p-eNOS (D) and e-NOS (E) in the renal cortex and outer medulla from SED+SAL (1), TR+SAL (2), SED+CP (3), and TR+CP (4). Densitometric ratios among p-eNOS or e-NOS and α 1-tubulin were calculated, and data are expressed in comparison with the controls (SED+SAL), with the mean ($\pm$ SEM) control value designated 100%. The data are expressed as the mean $\pm$ SEM. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ vs. SED+SAL; *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ vs. TR+SAL; ## $p < 0.01$, # $p < 0.05$ vs. SED+CP.

of tubular cell lesions and transdifferentiation. These changes were also milder in the rats subjected to physical training (TR+CP). Under normal conditions, vimentin is present in only the glomeruli, and tubular cells only express vimentin when proliferating. Thus, the presence of vimentin in the tubular compartment is suggestive of a recent tubular lesion [36]. Increased expression of α -SMA has already been observed in several renal diseases and is a marker of cellular transdifferentiation, which is associated with renal fibrosis [37]. Tubular and interstitial cells can transdifferentiate into myofibroblasts under activation and express α -smooth muscle actin, a protein that is normally expressed in the renal tissue only by vascular smooth muscle cells and increase the production of extracellular matrix components. Macrophages are also involved in fibrosis through the release of fibrogenic peptides such as TGF- β , endothelin and angiotensin II. In addition, these cells can produce other components of the extracellular matrix, such as various types of collagen and fibronectin [38]. We also observed increased urinary levels of TGF- β (a potent fibrogenic cytokine) that were less intense in the group of rats subjected to physical exercise before CP injection (TR+CP).

The increased expression of VEGF observed in the sedentary group of CP-treated rats was not observed in trained rats injected with CP. In this trained group, the expression of p-p-eNOS and eNOS was higher compared with that in sedentary rats treated with CP, possibly indicating preserved endothelial function in the CP-treated rats subjected to prior training. The increased NO levels in renal tissue, as observed in our study and a possible consequence of increased p-eNOS expression, can lead to reduction in the vasoconstriction effect of CP and the inflammatory process, contributing to renoprotection in trained rats injected with CP. Increased levels of NO are associated with inhibition of TGF- β bioactivity and fibronectin synthesis, which suppress the stimulation of mesangial cell and matrix protein synthesis [39]. Therefore, the increased NO levels in renal tissue of rats from the TR+CP group could contribute to the decreased renal expression of TGF- β induced by CP in our study.

Tubulointerstitial lesions were milder in rats subjected to exercise training before CP injection (TR+CP), compared with the sedentary group (SED+CP). At this point, the elevated VEGF expression in the SED+CP group was not observed in TR+CP rats. The decreased expression of VEGF in the TR+CP group can be explained by the finding that tubular cell injury and the regenerative process were milder in these rats. Tubular and endothelial cells could respond to injury by VEGF secretion, inducing PTC proliferation so as to overcome the tubular insult [40]. In a study of renal ischemia-reperfusion injury, Kanellis et al [41], observed that the increased VEGF receptor-2 expression in PTCs could steer the effects of VEGF released by ischemic tubular epithelial cells to adjacent endothelial cells to preserve capillary perfusion and enhance tubular cell survival and recovery. This may explain some of our results regarding the increased VEGF in SED+CP rats. The number of JG12 (endothelial cell marker) positive cells in the renal outer medulla was lower in the SED+CP group compared with controls (SED+SAL and TR+SAL) and TR+CP rats, demonstrating the protective effect of physical training on endothelial cells.

CXCR4 expression was higher in the trained group injected with CP (TR+CP), compared with sedentary rats treated with CP (SED+CP) ($p < 0.01$), and the decreased SDF-1 α expression in the SED+CP group compared with controls (SED+SAL and TR+SAL; $p < 0.05$) was milder in the TR+CP rats. The increased expression of CXCR4 and SDF-1 α could also contribute to tubular cell regeneration. In a rat study of ischemia-reperfusion injury, Togel et al [18], observed that repair of damaged renal tissue is initiated by extrinsic cells via activation of SDF-1 expression 24 h after the insult. It was already demonstrated that inflammatory cytokines, such as TNF- α and IL-1 β , could attenuate the increase in CXCR4 expression [42]. Our results showed that the high levels of IL-1 β in the kidney in the SED+CP group were not observed in the TR+CP group and were associated with the CXCR4 increase compared to sedentary rats.

Conclusion

Taken together, our data show that exercise training before CP injection reduced renal damage induced by this drug. These effects were associated with reduction of inflammatory response and amelioration of characteristic endothelial lesions.

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H.D.C.F., L.F.A., N.G.R., and C.M.F. performed the research and analyzed the data; M.P. designed the training study; R.S.C. was responsible for histological analysis; T.M.C. designed the experimental protocol. All authors read and approved the final manuscript.

Disclosure Statement

The authors declare that there are no conflicts of interest in this study.

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