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Nephroprotective Effect of a Low-Molecular-Weight Peptide Fraction from Common Bean (*Phaseolus vulgaris*) against Renal Ischemia and Reperfusion

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ABSTRACT: Ischemia−reperfusion (I/R) injury contributes to acute kidney injury. Reactive oxygen species produced during tissue reoxygenation may be targeted by antioxidant molecules obtained from dietary sources. We previously showed that peptides smaller than 3 kDa (PV3) from common hardened bean *(Phaseolus vulgaris)* exert antioxidant and renal vasodilator effects. We next evaluated whether PV3 may mitigate the ischemic damage related to oxidative stress in the kidney. The diphenyl picrylhydrazyl method was used to evaluate the PV3 antioxidant activity *in vitro*. Human endothelial cells were incubated with diaminofluorescein diacetate and dihydroethidium to probe nitric oxide (NO) and reactive oxygen species, respectively; fluorescence was analyzed by confocal microscopy. In our *in vivo* tests (CEUA 057/22), we used Wistar rats that underwent a sham surgery or an I/R procedure and were treated with PV3 (50 μg/kg). Phosphate-buffered saline 1× was used to control for hypercapnia-related metabolic acidosis, and the control group received NaCl 0.9%. Ingestive parameters, metabolism, and renal function were evaluated in metabolic cages. PV3 exerted antioxidant effects and increased NO production in endothelial cells. In rats, PV3 reverted the deleterious effects of I/R on renal function and attenuated the I/R-evoked reductions in the activity of renal superoxide dismutase and catalase enzymes in a copper- and iron-dependent manner. In conclusion, PV3 attenuates the detrimental effects of I/R on renal function, most likely through its antioxidant and oxidonitrergic properties, thereby exerting nutraceutical benefits on pathophysiological processes driven by oxidative stress.

KEYWORDS: *common beans, kidney injury, oxidative stress, antioxidant, chelate, PV3*

1. INTRODUCTION

Acute kidney injury (AKI) is a relevant public health problem and is correlated with an increase in the patient's length of hospitalization, therefore increasing morbidity and mortality rates.[1] Among the several causes of AKI, low extracellular fluid volume, insufficient cardiac output, and systemic vascular resistance are noteworthy, culminating in ischemia, acute tubular injury and vascular nephropathy, among others.[2] AKI associated with renal ischemia and reperfusion (I/R) injury may happen following kidney transplantation, surgical interventions in aorta and renal arteries, sepsis, and burns.[3,4] AKI can evolve into acute renal failure likely found after a toxic or ischemic injury, leading to cellular and tissue damage.[4]

The literature states high levels of free radicals during I/R as the mechanism in charge of generating impairments in nitric oxide (NO) production and release, which in turn reacts in the presence of oxygen generating peroxynitrite, thus diminishing the vasodilation and recruiting vasoconstricting factors, hydrolytic enzymes, and cytokines with an additional endothelial injury.[5−7] Considering that free radical scavenging is a fundamental element of the intricate physiological network sustaining homeostasis, the engagement of endogenous antioxidant defense systems becomes crucial for limiting oxidative damage and preserving cellular regulatory integrity.[9] The defense mechanisms protecting against free radical insult can be enzymatic and nonenzymatic. While catalase (CAT) and superoxide dismutase (SOD) are some of the enzymes able to prevent and control oxidative damage,[8] the nonenzymatic system relies on the ingestion of antioxidant compounds such as vitamin C (ascorbic acid) and others.[8−10]

Among the several potential candidates for mitigating I/R-related kidney injury, bioactive peptides stand out. Many of these protein fragments (2−30 amino acids) have a positive impact on human health.[11−13] Bioactive peptides are present in a wide range of dietary sources, including foods of animal origin such as eggs, milk, meat, and fish, as well as plant-derived sources, particularly legumes.[11] Since beans have protein, carbohydrates, fibers, vitamins, and minerals in meals,[14] it is considered a nutraceutical food, modulating

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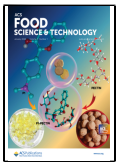

physiological functions through interactions with several proteins and receptors, resulting in chelating, antimicrobial, antihypertensive, hypoglycemic, antithrombotic, and antioxidant effects.[15,16] However, bean grains may suffer a hardening process known as hard-to-cook (HTC) that impairs their sensorial quality and consequently their commercial value, although HTC grains keep almost all nutritional facts.[11,14,17,18]

We previously demonstrated that the low-molecular-weight peptide fraction (<3 kDa) extracted from HTC *Phaseolus vulgaris* (PV3) exerts oxidonitrergic, vasodilator, and cytoprotective effects.[15] More recently, we showed that the same PV3 produces antihypertensive and natriuretic effects and improves renal function in an experimental model of arterial hypertension. PV3 improved renovascular conductance, revealing therapeutic potential for cardiovascular and renal comorbidities.[14] Nevertheless, it remains to be elucidated whether PV3 exerts a nephroprotective effect against I/R-induced renal injury. Therefore, this study was aimed at investigating the PV3 effects against the renal consequences of kidney I/R and the mechanisms involved.

2. MATERIALS AND METHODS

2.1. Materials. The beans were supplied by the Brazilian Agricultural and Research Agency (EMPRAPA) Rice and Beans, located in Santo Antônio de Goiás, Brazil. The beans are from the Pontal cultivar, belonging to the *P. vulgaris* species, from the Carioca commercial group. The methods employed to extract peptides from HTC beans and the yield content were as previously described.[15] Flour was produced from hardened grains and was stored in sealed plastic bags under refrigeration (4 °C) for subsequent peptide extraction.

2.2. Extraction of Peptide Fractions. After fractionation, the samples were freeze-dried and stored at room temperature until further use. For the extraction procedure, 1 g of flour was mixed with 5 mL of a previously prepared solvent solution composed of acetonitrile, water, and formic acid in a 25:24:1 (v/v/v) ratio. The mixture was subjected to orbital shaking for 1 h at room temperature. Subsequently, the sample was centrifuged at 28 °C for 10 min. The resulting supernatant was collected and concentrated to a final volume of 1.5 mL using a Vacufuge Plus Concentrator (Eppendorf). The concentrated extract was then stored at a temperature of −80 °C for further analysis.

2.3. Fractionation of the Extract and Quantification of Protein Content. The extract obtained went through an ultrafiltration process in a 10 kDa membrane (Amicon Bioseparations), and the filtrate obtained went through ultrafiltration again in a membrane with a cutoff of 3 kDa (Amicon Bioseparations). To carry out this process, nitrogen gas was used at a pressure of 50 kgf/cm^2 to the system. The obtained peptide fraction with a molecular weight of less than 3 kDa (named PV3) was lyophilized and stored. For the tests, it was solubilized with a sterile saline solution (NaCl 0.9%).

To quantify the proteins and peptides in the solution, 1 mg of the lyophilized product was used and solubilized in 1 mL of saline solution, using a Qubit Protein Assay Kit and Qubit Fluorometer equipment (Invitrogen).

2.4. Copper-Peptide and Iron-Peptide Complexes. To prepare the copper-peptide complex, 8 mL of a 50 mmol/L sodium phosphate buffer solution (pH 6.0), 10 μg of copper, and 2 mL of PV3 (40 μg of protein) were used. The reaction was carried out at room temperature for 1 h under constant stirring. The pH of the medium was checked every 15 min to maintain it at pH 6.0. Subsequently, the mixture was centrifuged for 20 min at 5000 rpm, and the precipitated copper-peptide complex was lyophilized.

For the preparation of the iron-peptide complex, 2 mL of a ferric chloride tetrahydrate solution containing 1.12 mg of Fe^{2+} was added to 1.6 mL of PV3 containing 40 μg of protein and 6.4 mL of a 100 mmol/L sodium acetate buffer solution (pH 4.9). The system was incubated at room temperature for 1 h under constant stirring. The pH was checked every 15 min to maintain it at pH 4.9. Subsequently, the mixture was centrifuged for 20 min at 5000 rpm, and the precipitated iron-peptide complex was lyophilized.

2.5. Antioxidant Activity with 2,2-Diphenyl-1-picrylhydrazyl (DPPH). The antioxidant activity was determined following the methodology described by Nazeer et al.,[19] using DPPH as a free radical to assess the potential of PV3 as a hydrogen provider or free-radical scavenger. To carry out the assay, 200 μL of DPPH solution (0.15 mmol/L) was used for 50 μL of sample (concentrations ranging from 0.1 to 0.5 mg/mL). The assay was incubated at room temperature for 15 min and then read on a microplate spectrophotometer (EPOCH) at a wavelength of 520 nm. The determination of antioxidant activity was expressed through a calibration curve using Trolox as the antioxidant standard (R^2 = 0.9917).

2.6. Endothelial Cell Culture. The experiments were conducted using endothelial cells at the Multi-User Laboratory for the Assessment of Molecules, Cells, and Tissues at the School of Veterinary Medicine at the Federal University of Goiás (EVZ—UFG). The EA.hy 926 endothelial cell strain from American Type Culture Collections (ATCC—Manassas, VA, USA) was used and cultured in Dulbecco's modified Eagle's medium (DMEM) enriched with 10% fetal bovine serum (FBS), penicillin, streptomycin (10,000 IU/mL to 10 mg/mL), 1% amphotericin B, and 1% L-glutamine (reagents from Cultilab, Campinas, Brazil) and kept in a humidified incubator at 37 °C and 5% CO_2. The medium was changed as necessary until a minimum confluence of 80% was reached. In a 96-well plate, 1 × 10^4 cells/well were maintained in a humidified incubator at 37 °C and 5% O_2 for 24 h.

For the cytotoxicity assays, treatment was carried out for 24 h in a 96-well plate at PV3 concentrations [1] 50 μg/mL, [2] 150 μg/mL, and [3] 300 μg/mL, with a final volume of 200 μL. Cells were analyzed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method.[15] To check for the cytoprotective potential of PV3 against oxidative stress, after the cells reached minimum confluence in the 96-well plate, the cells were treated with PV3 [1]; PV3 [2]; and PV3 [3] or vehicle (NaCl 0.9%) for 24 h. Then, the supernatant was discarded, and the plates were washed twice with a phosphate-buffered saline (PBS) buffer. A medium containing 3% hydrogen peroxide was added and kept for 1 h and then the cytoprotection was evaluated following the MTT incubation.

To assess the levels of superoxide in the cytoplasm, a pretreatment was carried out in one group of cells incubated with PV3 for 24 h before the oxidative insult and with another group co-incubated with PV3 and hydrogen peroxide for 1 h. The Sigma-Aldrich selective fluorogenic probe method for O_2^-, dihydroethidium (DHE) was used to generate 2-hydroxide (2-OH-E+).[18] Similarly, to quantify the NO, cells underwent the same treatments described above and were exposed to a 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM) fluorescent probe (Invitrogen, Grand Island, NY, USA). The specimens were observed using confocal microscopy (TCS SP8 DMi8, Germany) with a 10× and 40× objective. For fluorescent analysis, ImageJ software (NIH, Bethesda, MD, USA) was used to quantify the percentage of the average fluorescence pixels detected in ten areas of 50 square micrometers, according to a methodology described previously.[15]

For hypoxia, cells with a confluence of approximately 80% were incubated in a hermetically sealed chamber with a Petri dish containing 10 mL of ultrapure water to maintain environmental humidity through evaporation, and the chamber was filled with a gas mixture of 1% oxygen (O_2), 10% carbon dioxide (CO_2), and 89% nitrogen (N_2) for 3 h. After the hypoxia period, the cells were removed from the chamber and fixed with 4% paraformaldehyde. They were then incubated with the probes as previously explained, and the analyses were performed using confocal microscopy (TCS SP8 DMi8, Germany) using a 10× and 40× objective.[20−23]

2.7. Experiments *In Vivo*. We used adult (12 weeks old) male Wistar rats (250−300 g) provided by the Center for Production and Science in Biomodels (CPCBio—UFG). Water and chow were given

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ad libitum and animals had a 12/12-h light/dark cycle. The experimental protocols were approved by the local ethics committee (CEUA UFG—057/22). Experiments were conducted in accordance with CONCEA (Brazil) guidelines and with the International Guiding Principles for Biomedical Research Involving Animals. Methodological choices followed Animal Research: Reporting of *In Vivo* Experiments (ARRIVE) guidelines.

Rats underwent sham surgery or an I/R procedure and were treated with PV3 (50 μg/kg) or tempol (4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl), a potent commercial antioxidant chosen as a positive control. PBS buffer 1× was also used in order to control for possible hypercapnia-related metabolic acidosis. The groups (n = 10 each) were as follows: vehicle + SHAM, vehicle + I/R, PV3 + SHAM, PV3 + I/R, PV3 + PBS + SHAM, PV3 + PBS + I/R, tempol + SHAM, tempol + I/R, tempol + PBS + SHAM, and tempol + PBS + I/R.

The animals were placed in metabolic cages 24 h before the surgical procedure to acclimatize. To perform ischemia, the animals were anesthetized with 80 mg/kg of ketamine and 10 mg/kg of xylazine and injected intraperitoneally (i.p.). The left kidney was exposed and the left renal artery was isolated and occluded for 30 min, as described previously.[24] Subsequently, treatments were given (ip) according to the experimental group, and the arterial clamp was removed. The surgical field was closed with sterile sutures, and the animals received antibiotics [pentabiotico (benzathine, benzylpenicicline, sodium benzylpeniciline, potassium benzylpeniciline, procaine, benzylpeniciline and streptomycin) 5 mg/kg; 0.2 mL] and analgesic [banamine pet (flunixim meglumine) 1.1 mg/kg] intramuscularly. Rats were then set in metabolic cages for 24 h to evaluate renal function as described previously.[25] In the next day, animals were euthanized by a high dose of anesthetic (40 mg/kg, i.p.) with sodium thiopental (THIOPENTAX) (2.5%); blood was sampled through a transcardiac puncture followed by collection of urine and kidneys for further analysis.

Biochemical analyses were performed as reported by Ribeiro and co-workers.[34] Bioclin colorimetric kits were used to reveal the levels of sodium, potassium, and creatinine in plasma and urine samples. Glomerular filtration rate (GFR) was calculated from creatinine plasma and urine levels.

2.8. Markers of Oxidative Stress in Ischemic Kidney Tissue. Kidney tissue samples were homogenized in a buffer (50 mM Tris–HCl). The total homogenate (HT) was centrifuged, and the supernatant fractions (S1) were stored for testing.

2.8.1. Lipid Peroxidation Levels (LPO). To evaluate LPO levels, the thiobarbituric acid reactive substances (TBARS) method was used. LPO levels were determined spectrophotometrically, according to the method as described previously,[26] with some modifications. Kidney homogenate fractions were incubated with thiobarbituric acid, trichloroacetic acid (pH 3.4), and sodium dodecyl sulfate (SDS) at 95 °C for 60 min. The reaction product was measured at 532 nm. For interpretation of the results, a malondialdehyde (MDA) standard curve was generated, and the data are reported as MDA equivalents in nmol/mg protein.

2.8.2. Carbonylated Protein Levels (CP). CP derivatives were measured following a method described previously,[27] with some modifications. Kidney homogenate fractions were incubated with 2,4-dinitrophenylhydrazine (DNPH) prepared in 2 mol/L HCl. The mixture was kept in the dark for 1 h and vortexed every 15 min. Denaturation buffer, ethanol, and hexane were then added to each tube, vortexed for 40 s, and centrifuged at 3000g for 10 min at room temperature. The supernatant was discarded, and the pellet was washed with ethanol/ethyl acetate (1:1 v/v) and resuspended in a denaturation buffer. The sample was vortexed for 5 min, and absorbance was measured at 370 nm. Results are expressed as nmol of carbonylated protein/mg of protein.

2.8.3. Superoxide Dismutase (SOD) Activity. SOD activity was determined spectrophotometrically according to the method as described previously,[28] with some modifications. This method is based on the ability of the SOD enzyme to inhibit autoxidation of epinephrine. The S1 fractions of kidney tissue samples were incubated with 60 mmol/L epinephrine bitartrate, and the reaction intensity was measured at 480 nm. Enzymatic activity is expressed in units (U) of SOD/mg of protein.

2.8.4. Catalase (CAT) Activity. CAT activity was determined spectrophotometrically by monitoring H_2O_2 decomposition at 240 nm,[29] with some modifications. The S1 fractions of kidney tissue samples were incubated with 86 mmol/L of H_2O_2 in sodium phosphate buffer (pH 7.0). Enzymatic activity is expressed in U of CAT/mg protein.

2.8.5. Protein Content. Total protein content in kidney tissue samples was determined according to the method as described previously,[30] with some modifications. Concentrations were calculated using bovine serum albumin (BSA) as the standard.

2.9. Analyses. The results were expressed as the mean ± standard error of the mean (SEM) and analyzed using one or two-way ANOVA when appropriate (see each figure caption) with I/R and treatments as independent variables, followed by Tukey's posthoc test. GraphPadPrism 9.3 software (GraphPad Software, Inc.) was used to perform the comparisons. The significance level was set at $P < 0.05$.

3. RESULTS

3.1. Extraction and Characterization of the Antioxidant Activity of PV3. A quantitative analysis of the protein content of the low molecular weight extract (PV3) was carried out. The result obtained was 124.4 ± 1.49 mg/mL. With this result, it was possible to calculate the concentrations used in *in vitro* and *in vivo* antioxidant assays.

The *in vitro* testing indicates a half maximal inhibitory concentration (IC_{50}) of 7.74 ± 0.27 mg/mL, demonstrating the percentage inhibition of the DPPH radical as a function of the antioxidant concentration.

3.2. Viability and Cytotoxicity in Human Endothelial Cells (EA.hy926). The MTT assay assessed cytotoxicity by exposing human endothelial cells of the EA.hy 926 strain to increasing concentrations of PV3. The concentrations tested were PV3 [1] at 50 μg/mL; PV3 [2] at 150 μg/mL; and PV3 [3] at a concentration of 300 μg/mL. Although the cells were not affected by the lowest concentration of PV3, a concentration-dependent cytotoxicity was observed from 150 μg/mL after 24 h of incubation (Figure 1).

In the oxidative insult tests, the cytoprotective capacity of PV3 was evaluated in endothelial cells exposed to 3% H_2O_2 for 1 h. It was found that PV3 at a concentration of 50 μg/mL was able to maintain cell viability after exposure to oxidative stress at levels comparable to those found in control cells (not exposed to H_2O_2). It was also found that under hypoxia, cell viability was decreased. The treatment with PV3 attenuated the increases in ROS caused by hypoxia compared to the vehicle-treated group. The hypoxic environment further reduced oxidonitrergic production and release, and this effect in endothelial cells was reverted by PV3 (Figure 1).

3.3. Nitric Oxide and Free Radical Production in Human Endothelial Cells. To evaluate the PV3 effects on endothelial NO and ROS productions (Figure 2A), we chose a concentration of 50 μg/mL based on the above-described cytoprotective potential against oxidative stress found in the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The representative images depicting the effects of PV3, hydrogen peroxide, and hypoxia on the fluorescence probing NO or ROS production are shown in Figure 2B. The images obtained through DIC microscopy allowed us to observe the cell morphology, with typical features of control groups (VEH) revealed by the lower fluorescence emission of the DHE probe when compared to the same group exposed to hydrogen peroxide, as an oxidative insult.

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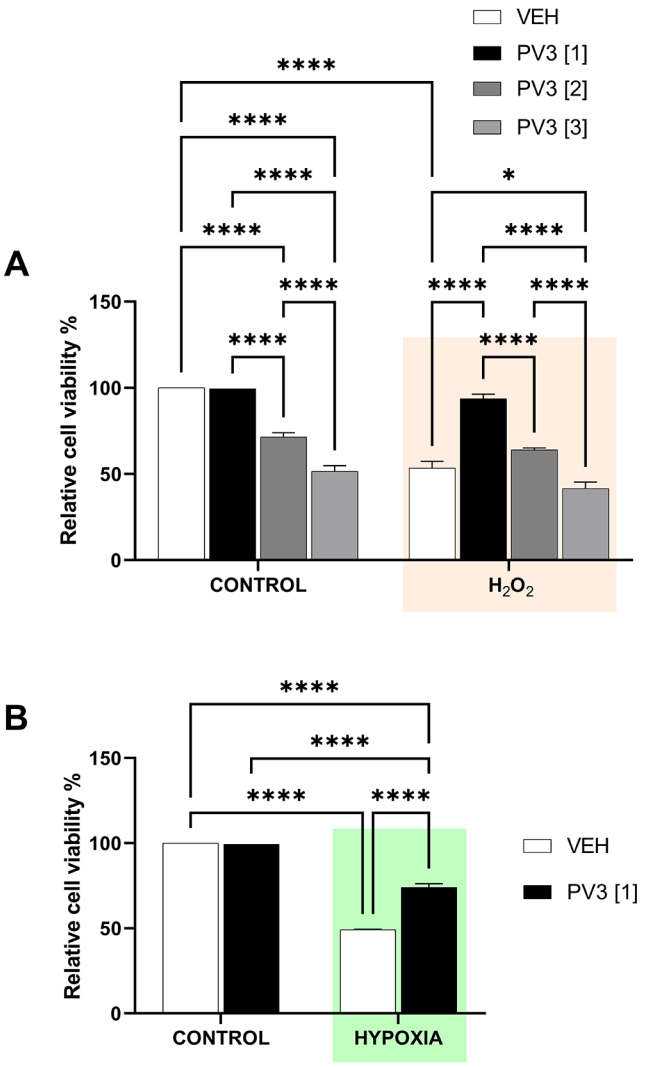

Figure 1. Panel (A) shows the relative viability of cells exposed to PV3 at different concentrations: PV3 [1] = 50 μg/mL; PV3 [2] = 150 μg/mL; and PV3 [3] = 300 μg/mL. Light orange shaded bars highlight results from experiments with cells treated with PV3 and exposed for 1 h to hydrogen peroxide in comparison to vehicle (VEH). Panel (B) shows the results from experiments with cells treated with PV3 under normoxia and exposed to hypoxia for 3 h (green shaded bars) in comparison to vehicle (VEH). Values are expressed as mean ± standard error of the mean (SEM) (analysis performed in triplicate). Two-way ANOVA analysis followed by Tukey's post-test. *P < 0.05.

Figure 2C shows the fluorescent intensity proportionally probing ROS levels. While PV3 alone was able to reduce baseline ROS levels, either the preincubation (24 h) or the coincubation of PV3 and with H_2O_2 showed that the peptide fraction attenuated ROS production evoked by the oxidative insult. Exposure to a hypoxic environment increased ROS levels, and this effect was smaller when cells were incubated with PV3, showing the potential of PV3 to mitigate hypoxia-induced oxidative stress.

Figure 2D shows that H_2O_2 and PV3 (alone, pretreated, or cotreated) increased the NO production, but the magnitude of these effects was greater during the coincubation of PV3 with H_2O_2. The group treated with PV3 in the hypoxic situation decreased oxidative stress when compared to that of VEH in the same situation. In the hypoxic scenario, the levels of NO produced by endothelial cells were higher in the PV3 group when compared to those of the VEH in the same experimental situation.

3.4. Metabolism and Food Consumption. As expected for an acute 24 h experiment, the procedures performed and the treatment with PV3 could not affect the body mass of the animals (Figure 3A). This lack of difference was also seen in food and water intakes (Figure 3B,C) that is also reflected in diuresis (Figure 3D).

3.5. Urinary Metabolism. Plasma and urinary creatinine levels were used to calculate GFR. Plasmatic and urinary creatinine levels were significantly increased in animals submitted to I/R as compared to those submitted to SHAM, while clearance was dramatically reduced in untreated ischemic kidneys (Figure 4A,B). Increased plasma and urinary levels indicate that ischemia impairs GFR and such undesired effects were reverted by treatments with the antioxidant tempol or with PV3 (Figure 4C). The combination of treatments with PBS did not aid the PV3 effects.<

The comparison between I/R and SHAM groups injected with vehicle revealed that transient ischemia increases the urinary excretion of sodium and potassium, and this effect is completely attenuated by treatments with PV3, tempol, and their combination with PBS (Figure 5A,C). Similarly, the effects of I/R on the plasmatic levels of these ions were reversed by PV3, tempol, and their combination with PBS (Figure 5B,D).

3.6. Oxidative Stress Markers in Kidney Tissue. In the biochemical analyses, the levels of the MDA metabolite showed that I/R increased TBARS levels, and this effect was reversed by all treatments in the I/R groups (Figure 6A). While acute I/R did not alter CP levels (Figure 6B), SOD enzyme activity was reduced in I/R animals injected with vehicle (*vs* SHAM-vehicle) and increased by tempol or unchelated PV3. In contrast, iron- and copper-chelated PV3 did not modify the effects of I/R on SOD kinetics (Figure 6C). CAT activity was decreased by I/R, and this effect was reversed by tempol or iron-chelated PV3 (Figure 6D).

4. DISCUSSION

The main results of this study show that PV3 (i) displayed an antioxidant activity *in vitro*; (ii) exerted cytoprotection, reducing cell damage caused by oxidative stress; (iii) attenuated the increases in reactive oxygen species and the decreases in NO caused by hypoxia; (iv) improved SOD and CAT activities in acute I/R kidneys; (v) reduced the ischemic consequences on SOD activity in an iron-and copper-dependent-manner, as revealed by experiments with chelate complexes; (vi) did not alter water balance, but increased the glomerular filtration rate, demonstrating its renoprotective potential against the deleterious effects caused by the I/R *in vivo*; (vii) modulated the sodium (and potassium) reabsorption changes evoked by I/R.

The low-molecular-weight peptide fraction was extracted from common beans by using an acidic solvent, thus increasing the solubility of proteins with low polarity.[10] Consequently, the extract contains peptide sequences with amphipathic characteristics, herein reported to display antioxidant activity probably raised from the modulation of electron donation.[31] Peptide's chemical structure and the amino acid sequence may hold the key: some amino acids are able to stabilize free radicals through the donation of electrons. This stabilization results in cytoprotective and tissue protective effects[15−17] similar to

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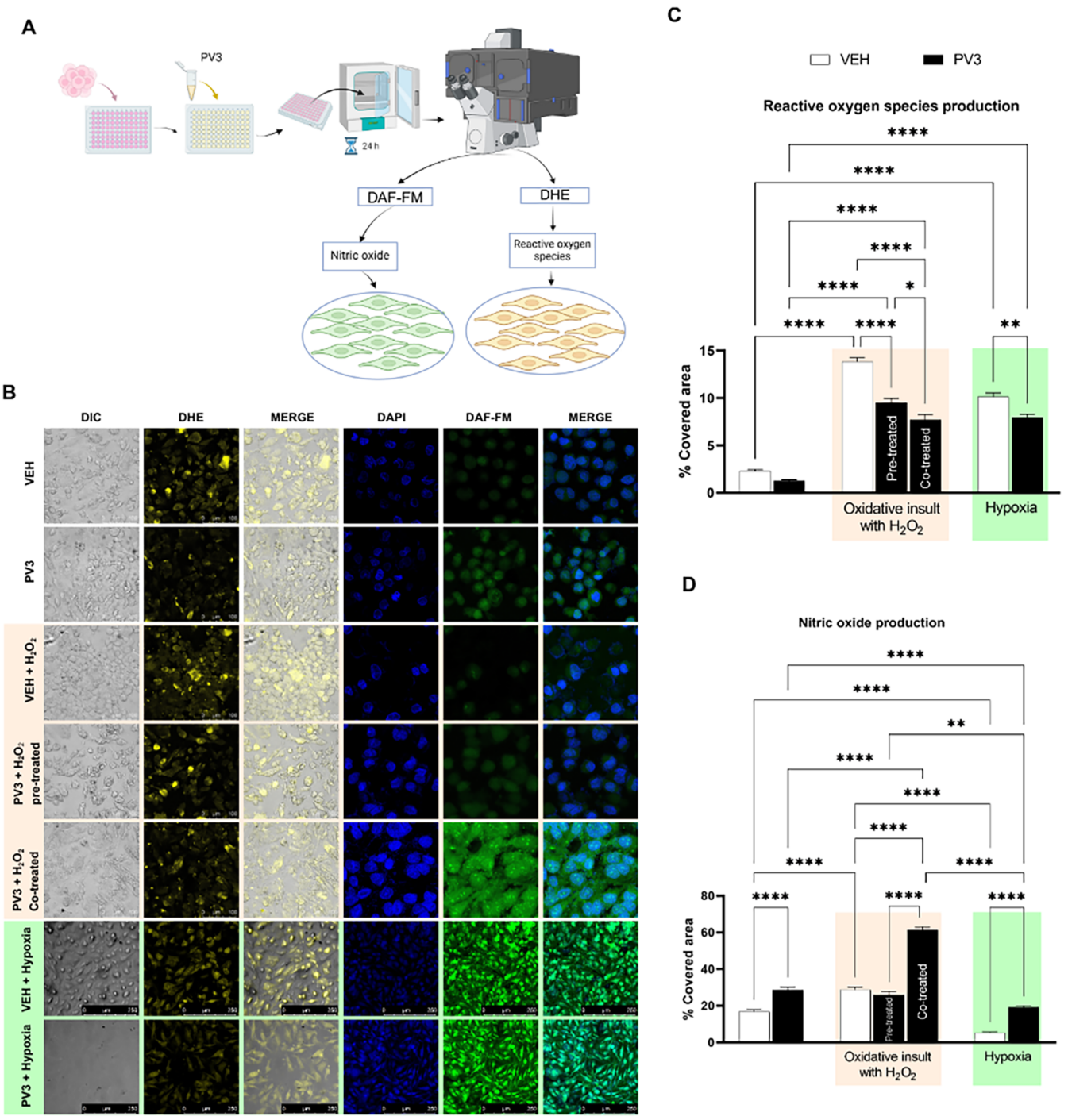

Figure 2. (A) Experimental sequence adopted in our *in vitro* cell trials. (B) Representative images sampled during confocal microscopy (DIC or fluorescence) from endothelial cells (EA.hy 926) incubated with fluorescent probes DAF-FM (probing NO) and DHE (probing ROS) and exposed to oxidative insult with H_2O_2 or hypoxic environment. (C) Quantification of oxidative stress in endothelial cells labeled with fluorescent probe DHE in all experimental conditions. (D) Quantification of NO in endothelial cells labeled with fluorescent probe DAF-FM in all experimental conditions. Values are expressed as mean ± SEM (analyses performed in 10 fields per group). Two-way ANOVA analysis followed by Tukeýs post-test. *$P < 0.05$ as delimited by the brackets.

those reported herein. The DPPH assay was attempted to follow this hypothesis and demonstrated that the PV3 peptide fraction indeed acts as an electron donor, resulting in a reaction oxidation−reduction process that reduced the DPPH radical.[15] Furthermore, previous studies[15,17,32,33] reported antioxidant activity of extracts obtained from hydrolyzed fractions of common bean and attributed this effect to the presence of amino acid residues that we found to be abundant in PV3.[15]

As expected, the body mass was unchanged in our acute experiments. It is noteworthy that the technical expertise minimized potential interferences with food intake and the hydroelectrolytic balance. Accordingly, the absence of differences between the control and SHAM groups reinforces the conclusion that the renoprotective effects attributed to PV3 in

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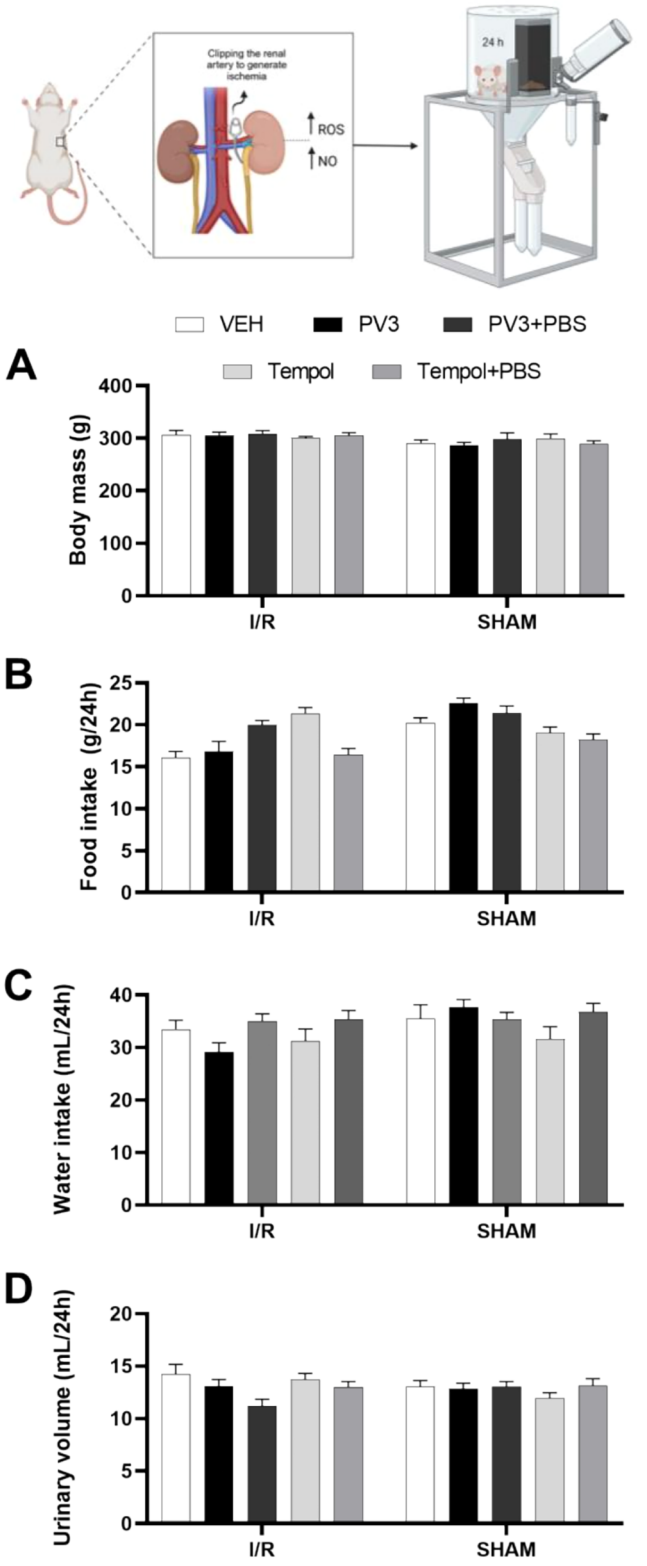

Figure 3. Ingestive parameters and diuresis of controls (SHAM) and of rats submitted to renal ischemia and reperfusion (I/R) treated with PV3 [50 μg/kg], vehicle (0.9% saline solution), PBS or tempol. (A) Body mass (g). (B) Water intake (mL/24 h). (C) Food ingestion of feed (g/24 h). (D) Urinary volume (mL/24 h) in 24 h after treatment according to the experimental group. Values are expressed as mean ± SEM (n = 10 per group). Two-way <consistency> ANOVA analysis followed by Tukeýs post-test.

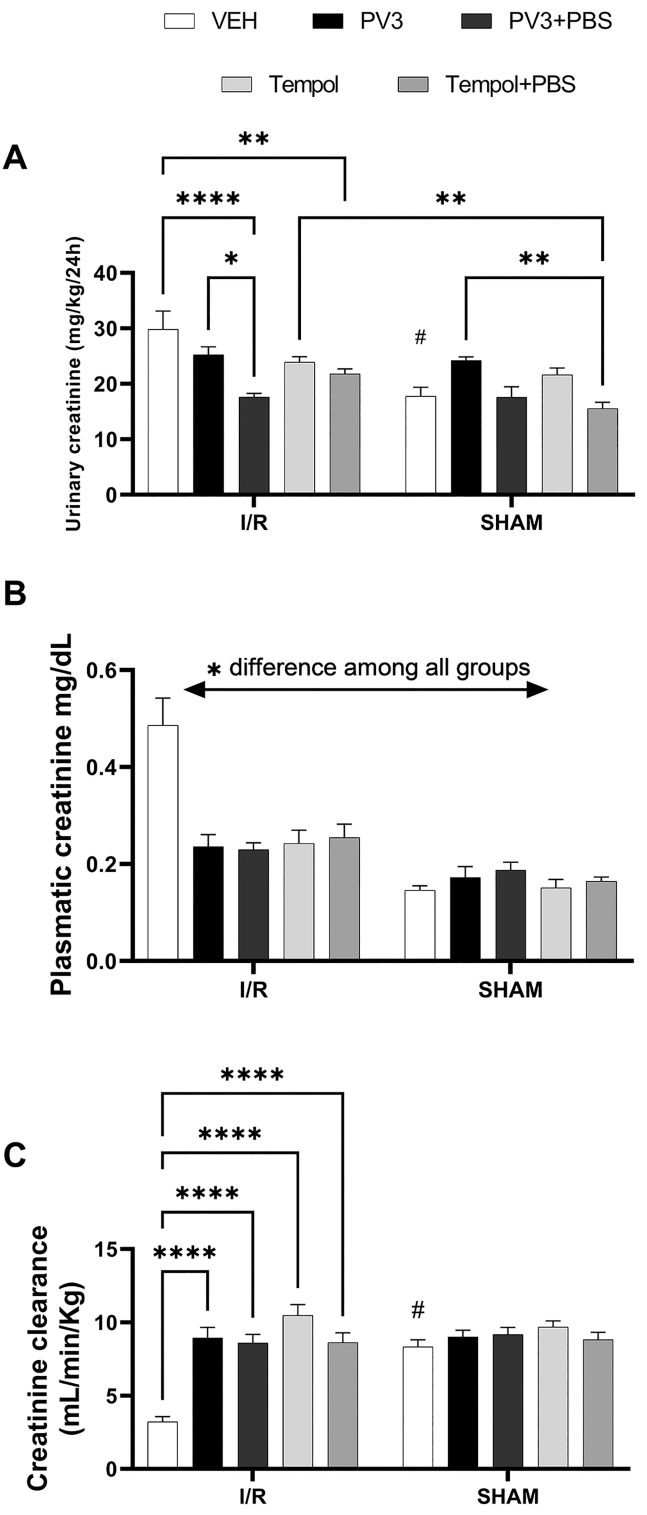

Figure 4. Comparison of urinary and plasmatic creatinine levels (A, B) and creatinine clearance (C) sampled 24 h after the ischemia−reperfusion (I/R) or SHAM procedure in rats treated with vehicle, PV3, PBS, or tempol. Values are expressed as mean ± SEM (n = 10 per group). Two-way ANOVA analysis followed by Tukey's post-test. *P < 0.05 as delimited by brackets.

ischemic kidneys were primarily driven by the bioactive molecules within the fraction, with a minimal influence of I/R surgical procedures themselves.

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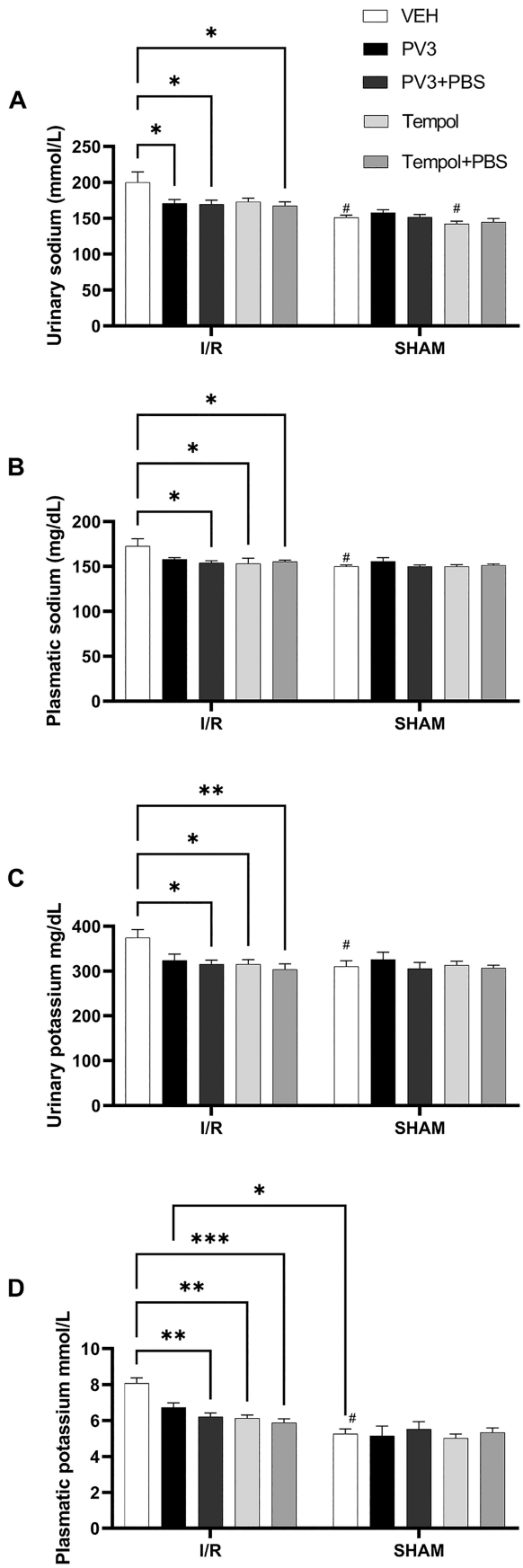

Figure 5. Comparison of urinary and plasmatic sodium (A, B) and potassium (C, D) concentrations sampled 24 h after the ischemia–reperfusion (I/R) or SHAM procedure in rats treated with vehicle, PV3, PBS, or tempol. The values are expressed as mean ± SEM (n = 10 per group). Two-way ANOVA analysis followed by Tukey's post-test. *P < 0.05 as delimited by brackets.

Creatinine is excreted by kidneys and is widely used in the clinical assessment of renal function so that measures of plasma and urinary creatinine may provide its clearance, often used to assess glomerular filtration rate.[35] In cases of renal injury due to I/R, impairments in renal function are expected, leading to a reduction in creatinine clearance.[35] Elevated creatinine in the I/R groups injected with vehicle is in accordance with that expected for an acute lesion due to ischemia and reperfusion. However, the treatment with PV3 was able to restore the changes in creatinine clearance caused by I/R to levels comparable to those of control (non-I/R) groups, indicating a nephroprotective effect displayed by PV3. Also, the presence of buffer (PBS) did not change the amplitude of the responses caused by PV3 on I/R consequences, and this suggests that PV3 acts on oxidative stress but not on the increases in acidity eventually caused by CO_2 accumulation during the ischemic period. In this regard, the capacity of PV3 in reducing the I/R consequences on renal hemodynamics and redox status can be attributed to an antioxidant effect which was already reported in cells.[14,15] Previous studies showed that changes in creatinine levels caused by I/R were restored to the homeostatic condition following treatment with *N*-acetylcysteine[36] in a similar pattern to that reported herein. Therefore, the cysteine (*N* = acetylcysteine precursor) residues profusely found in PV3 content[15] may underlie the currently described antioxidant and nephroprotective effect and the hemodynamic restoring in I/R kidneys.

Plasma potassium and sodium levels are also important as the excretion of these ions plays a crucial role in the regulation of blood pressure and extracellular fluid volume and tonicity.[35] During homeostasis, sodium is maintained in a low concentration in the tubular kidney cells, being reabsorbed by the sodium/potassium ATPase pump in the basolateral membrane. However, during I/R, significant tubular damage occurs, resulting in an increase in the excretion of sodium and potassium ions, since the reabsorption in the tubules is compromised by the reduction of the energy substrate for ATPase pumps during the hypoperfusion period.[37,38] The PV3 effects on ion handling of ischemic kidneys are additional evidence of nephroprotection against I/R that may rely on ion pump enzymatic modulation. It is important to highlight that PV3 restored plasma and urinary parameters to levels comparable to those found in untreated nonischemic groups, and this supports the assertion that the nephroprotective action exerted by PV3 may prevent tubular damage. Notwithstanding, the precise mechanisms by which PV3 modulates tubular ion transport following ischemia warrant further investigation.

PV3 was able to increase NO production in endothelial cells, which suggests a mechanism of action on the arteriolar vasomotion that is uncontroversially known to increase renovascular conductance, consequently improving renal bed perfusion. These findings are in line with those previously described by our group.[14,15] The results obtained with cells treated with PV3 and exposed to oxidative insult with H_2O_2 demonstrate that PV3 could reduce reactive oxygen species, which also corroborates with our recent results in renal

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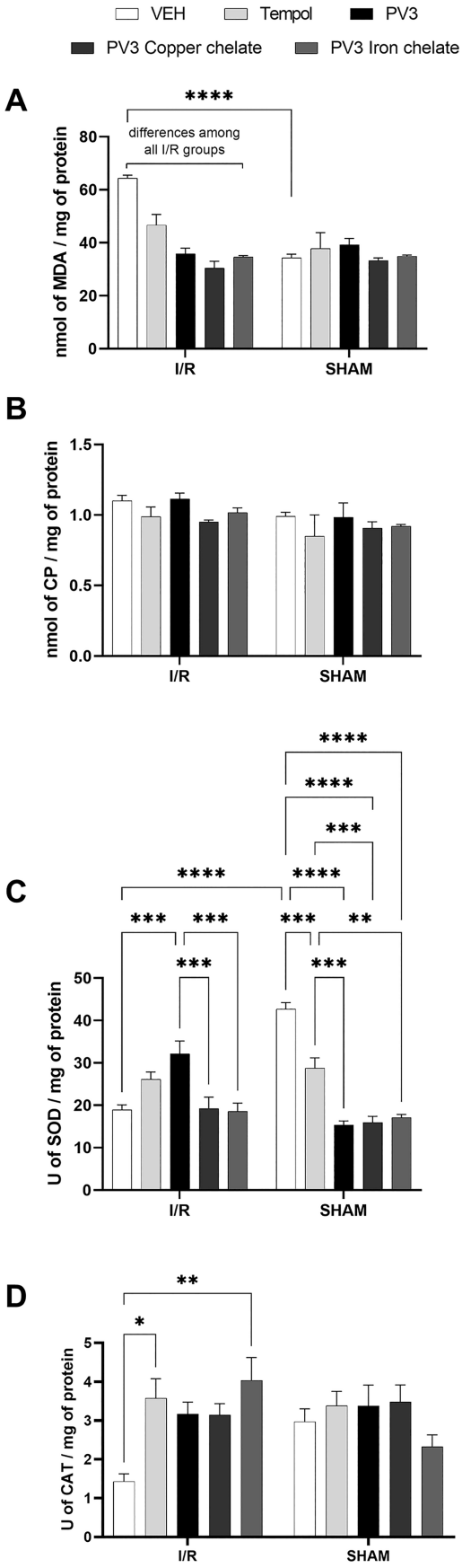

Figure 6. Comparison of malondialdehyde (MDA) metabolite reflecting thiobarbituric acid reactive substances (TBARS) levels, carbonylated protein (CP) levels, and the activity of superoxide dismutase (SOD) and catalase (CAT) enzymes in the renal tissue 24 h after ischemia/reperfusion (I/R) or SHAM surgeries sampled from rats treated with vehicle, PV3, PV3 copper chelate, or PV3 iron chelate. Values are expressed as mean ± SEM (n = 10 per group). Two-way ANOVA followed by Tukey's post-hoc test. *P < 0.05, as indicated by brackets.

function during hypertension.[14] The reduction in SOD activity found in I/R kidney samples allows a convergence of the evidence obtained in *in vitro* and *ex vivo* trials, since SOD is an enzyme responsible for removing reactive oxygen species whose levels are known to increase in ischemic situations.[39] The increases in SOD activity by PV3 during I/R may be linked to the hypoxic-related increases in H_2O_2. Conversely, the reductions in SOD kinetics in the presence of PV3 in the SHAM group may be attributed to the scavenging effects exerted by the peptide fraction that alone would reduce H_2O_2 levels; such a notion is confirmed by our experiments in endothelial cells.

The enzymatic assays in kidney tissues of rats treated with copper- and iron-chelated PV3 suggest that these metals are essential to achieve SOD-mediated nephroprotection in ischemic kidneys since such effects were abolished in groups treated with chelated PV3. These findings are consistent with the well-known essential roles of these metals and chelated peptides in SOD enzymatic kinetics.[40−42] CAT, in turn, requires iron as a cofactor to facilitate the formation of the CAT-H_2O_2 complex, which efficiently produces the non-harmful products water and O_2.[43] The fact that the activity of iron-chelated PV3 is like that of unchelated PV3 suggests that the amount of iron present in PV3 (or in beans) does not interfere with CAT during I/R. This necessitates complementary assays to determine the additional PV3 mechanisms of action on redox status. It would also be important to investigate whether PV3 interferes with the deleterious effects of the excessive iron release in ischemic tissue resulting from erythrocytic death.[44]

To deepen the understanding of the mechanisms, complementary experiments testing cells in a hypoxic environment were performed to check whether PV3 acts only on oxidative stress, as evidenced by our findings, or whether it further affects metabolism to a level that attenuates hypoxia induced by ischemia. The hypoxic *in vitro* setting reduced NO production and increased reactive oxygen species in human endothelial cells, which were similar to previously reported.[45−47] The ability of PV3 to attenuate hypoxia-induced cellular damage and the subsequent rise in oxidative stress may be explained by its modulatory effects on mitochondrial pathways, particularly those involved in the regulation of the respiratory chain and redox homeostasis. By influencing processes such as electron transport efficiency, maintenance of the nicotinamide adenine dinucleotide ($NADH/NAD^+$) balance, and the control of ROS generation, PV3 may help preserve mitochondrial integrity under hypoxic conditions, in agreement with mechanisms previously described.[48] Current results obtained through the MTT assay indeed meet this hypothesis, as they reflect the activity of mitochondrial dehydrogenases, which are directly linked to metabolic competence and bioenergetic status. Present DPPH and MTT results further corroborate with our previous wealth of evidence,[15] supporting hydrogen donation by PV3 as a

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mechanism modulating mitochondrial activity under hypoxia. Undoubtedly, peculiarities of the chemical interaction of PV3 components with mitochondrial pathways deserve further investigation.

We conclude that PV3 exerts a nephroprotective effect against renal I/R by reducing the tissue levels of oxygen reactive species, consequently attenuating ischemic/hypoxic and oxidative damage and keeping renal function. Such benefits arise from direct antioxidant effects, enzymatic modulation, and actions on mitochondrial mechanisms controlling the redox status. Our findings set PV3 as a possible source of antioxidants and regulators of renal tissue perfusion. The results also indicate that hardened beans, which are waste from agribusiness, can be used in nutraceutical formulations to collaborate in the treatment of circulatory diseases, whose pathophysiology relies on oxidative stress. Although current comprehensive data integrating cell culture, biochemical analyses, and animal models strengthens the validity of our conclusions, this study has limitations that should be considered while extrapolating to chronic and clinically heterogeneous situations of renal injury. Furthermore, it remains necessary to test the viability of orally given formulations containing PV3 on renal diseases, besides revealing peculiarities on absorption, distribution, metabolization, optimal therapeutic window, long-term safety, and excretion.

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ABBREVIATIONS

AKI, acute kidney injury; ATCC, American Type Culture Collections—Manassas, VA, USA; CAT, catalase enzyme; CP, carbonylated protein levels; DAF-FM, 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate; DHE, dihydroethidium; DMEM, Dulbecco's modified Eagle's medium; DNPH, 2,4-dinitrophenylhydrazine; DPPH, 2,2-diphenyl-1-picrylhydrazyl; FBS, fetal bovine serum; GFR, glomerular filtration rate; HT, total homogenate; HTC, hard to cook; I/R, ischemia and reperfusion; LPO, lipid peroxidation levels; MDA, malondialdehyde; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NO, nitric oxide; PV3, <3 kDa peptide fraction from *Phaseolus vulgaris*; ROS, reactive oxygen species; S1, supernatant fractions; SDS, sodium dodecyl sulfate; SOD, superoxide dismutase enzyme; VEH, vehicle

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