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Original Paper

Glutathione S-Transferase and Clusterin, New Players in the Ischemic Preconditioning Renal Protection in a Murine Model of Ischemia and Reperfusion

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Key Words
Glutathione S-transferase • Clusterin • Ischemia and reperfusion • Ischemic preconditioning

Abstract
Background/Aims: Renal ischemia and reperfusion injury (IRI) involves oxidative stress, disruption of microvasculature due to endothelial cell damage, loss of epithelial cell polarity secondary to cytoskeletal alterations, inflammation, and the subsequent transition into a mesenchymal phenotype. Ischemic preconditioning (IPC) has been proposed as a therapeutic strategy to avoid/ameliorate the IRI. Since previous results showed that IPC could have differential effects in kidney cortex vs. kidney medulla, in the present study we analyzed the effectiveness and molecular mechanisms implicated in IPC in both kidney regions. ***Methods:*** We evaluated 3 experimental groups of BALB/c male mice: control (sham surgery); renal ischemia (30 min) by bilateral occlusion of the renal pedicle and reperfusion (48 hours) (I/R); and renal IPC (two cycles of 5 min of ischemia and 5 min of reperfusion) applied just before I/R. Acute kidney injury was evaluated by glomerular filtration rate (GFR), Neutrophil Gelatinase-Associated Lipocalin (NGAL) blood level, and histologic analysis. Oxidative stress was studied measurement the Glutathione S-Transferase (GST) activity, GSH/GSSG ratio, and lipoperoxidation levels. Inflammatory mediators (IL-1β, IL-6, Foxp3, and IL-10) were quantified by qRT-PCR. The endothelial (PECAM-1), epithelial (AQP-1), mesenchymal (Vimentin, Fascin, and Hsp47), iNOS, clusterin, and Hsp27 expression were evaluated (qRT-PCR and/or Western blot). ***Results:*** The IPC protocol prevented the decrease of GFR, reduced the plasma NGAL, and ameliorated morphological damage in the kidney cortex after I/R. The IPC also prevented the

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downregulation of GST activity, lipoperoxidation and ameliorated the oxidized glutathione. In addition, IPC prevented the upregulation of vimentin, fascin, and Hsp47, which was associated with the prevention of the downregulation of AQP1 after I/R. The protective effect of IPC was associated with the upregulation of Hsp27, Foxp3, and IL-10 expression in the renal cortex. However, the upregulation of iNOS, IL-1β, IL-6, and clusterin by I/R were not modified by IPC. ***Conclusion:*** IPC conferred better protection in the kidney cortex as compared to the kidney medulla. The protective effect of IPC was associated with amelioration of oxidative stress, tubular damage, and the induction of markers of Treg lymphocytes activity in the cortical region. Further studies are needed to evaluate if lower tubular cell stress/damage after I/R may explain the preferential induction of Treg response in the kidney cortex induced by IPC.

Introduction

Acute Kidney Injury (AKI) is defined as a sudden and sustained fall of renal function, which is associated with poor outcomes such as prolonged length of both intensive care and hospital stays, advanced chronic kidney disease (CKD), and death [1]. The kidney is one of the most susceptible organs to ischemia, and renal ischemia/reperfusion represents a leading cause of AKI [2-5]. Ischemia and reperfusion injury (IRI) is a two-step condition: an initial imbalance of blood and oxygen supply followed by subsequent restoration of perfusion and re-oxygenation. Renal damage following IRI includes cell stress, a significant burst of free radicals, tubular damage (Acute Tubular Necrosis, ATN), endothelial activation associated with an inflammatory immune response leading to extensive cell injury, necrosis, and fibrosis [6]. The susceptibility to IRI can be attenuated by ischemic preconditioning (IPC) [7]. The IPC is defined as brief and intermittent ischemia and reperfusion episodes applied before prolonged ischemia. The degree of IPC-induced cytoprotection in the kidney depends on the duration of ischemia and reperfusion periods of each specific protocol [8, 9]. Microvascular, functional, and metabolic differences among renal cortex and medulla determine the differential regional effects of ischemia in kidney tissue [10] and may also determine the protective effects of IPC.

Both ischemia and the reperfusion phases are associated with the production of reactive oxygen species, inducing endoplasmic reticulum (ER) stress, and impairing the structure and function of renal tubular cells and glomerular cells [11-16]. Hsp27 is a stress protein that shows an early and transient increase after acute ischemia [17], inhibiting apoptosis by decreasing intracellular reactive oxygen species and the mitochondrial caspase-dependent apoptotic pathway [18]. Selective renal overexpression of Hsp27 in mice through lentiviral gene delivery protects against ischemic renal injury [19]. Cellular autophagy is activated in response to misfolded or aggregated proteins following ER stress [20], as is observed during renal IRI [21], proving a protective mechanism in the kidney for cell survival during IRI [22, 23].

Clusterin (CLU) is a chaperone-like glycoprotein [24] that promotes pro-survival autophagy [25], CLU suppresses macrophage infiltration and proinflammatory M1 during the recovery period following IRI, which may be partly responsible for tissue repair in the kidneys of WT mice after injury [26-28]. Interestingly, upregulation of CLU seems to be protective because CLU knockout (KO) mice showed increased susceptibility to renal IRI and impaired renal repair by IRI [29, 30]. However, the differential effects in renal cortex vs. medulla in Hsp27 and clusterin of IPC immediately before I/R are unknown.

Neutrophil gelatinase-associated lipocalin (NGAL) is a protein expressed by kidney tubular cells in response to kidney injury [31, 32]. Among several potential protective mechanisms at the cellular level [33-35], it has been proposed that IPC may attenuate IRI by decreasing the NF-κB DNA binding [36] and downregulating the NGAL expression [37].

Available evidence indicates that the development of vascular rarefaction and fibrosis determining potential recovery or progression to chronic renal failure is somehow dependent on the differentiation of endothelial and epithelial tubular into mesenchymal cells. These

processes are called epithelial-to-mesenchymal transition (EMT) and endothelial-to-mesenchymal transition (EndMT), respectively [38, 39]. EMT is reversible, leading to the loss of cellular polarity, development of migratory capacity, resistance to apoptosis, and extracellular matrix synthesis [40-42]. EndMT resembles EMT [43, 44] and leads to the loss of cellular adhesion molecules, cytoskeletal reorganization, change of the compacted cobblestone-like into spindle-shaped phenotype without polarity, associated with reduced expression of endothelial markers (e.g., vascular endothelial-cadherin, CD31/PECAM-1, and von Willebrand Factor). Both EMT and EndMT show increased expression of mesenchymal cell markers such as vimentin, fascin, and Hsp47. Renal fibrosis is mainly associated with activation of renal resident fibroblast, which transdifferentiate into myofibroblast. However, through EMT or EndMT, a significant number of cells differentiate into fibroblasts [45-48].

Our previous study allowed us to standardize a protective IPC protocol and identify several molecular targets that were differentially modulated in the kidney cortex and medulla after I/R and IPC immediately before I/R. In the present study, we evaluated if IPC protective action implies an effect on GSH/GSSG (reduced/oxidized glutathione ratio), lipoperoxidation, and Glutathione S-Transferase activity (GST). We further characterized renal damage, oxidative stress, and the differential expression of proteostasis proteins, inflammation, and mesenchymal transition markers. The results show that the IPC protocol protected mainly the renal cortex by mechanisms dependent on Glutathione S-Transferase activity, clusterin, IL-10, and Hsp27 associated with improved proteostasis, lower inflammation, and reduced mesenchymal transition.

Materials and Methods

Animals

We used a validated model of renal ischemia and reperfusion injury using the BALB/c mice that were also used in our previous study [49]. Adult mice (20-25g) were housed in a 12h light/ dark cycle. The mice were maintained at the University de los Andes Animal Care Facility. Animals had food and water *ad libitum*.

Ischemia-reperfusion (I/R) and ischemia preconditioning experiments (IPC)

Animals were anesthetized with 25mg/kg *i.p.* ketamine /15mg/kg *i.p.* xylazine and maintained on a 37 °C blanket during the surgical procedure. A flank incision exposed both kidneys, and the renal pedicle was occluded for 30 min with a non-traumatic vascular clamp (cat N° 18055-02 Fine Science Tools). Renal blood flow was re-established (reperfusion stage) by clamp removal, and both incisions were sutured. The acute kidney injury is observed after 48 hours of reperfusion. According to our previous results using the murine model of IRI in BALB/c mice [49], the lower GFR values and higher values of injury and acute inflammation biomarkers are present 48 hours after reperfusion. The sham animals were subjected to the same surgery process, but they did not undergo renal pedicle occlusion [50]. In the ischemic preconditioning (IPC) protocol, animals were subjected to two cycles of 5 min of ischemia followed by 5 min of reperfusion each (IPC), immediately before the ischemia and reperfusion (I/R) [51]. Following protocols, mice were euthanized with CO_2, and kidneys were dissected and processed for Western blotting, qRT-PCR, histochemistry, and biochemical analyses. During reperfusion time, the glomerular filtration rate was measured as described below.

Glomerular filtration rate (GFR) measurement

GFR was determined in conscious unrestrained mice with 48 hours of reperfusion using the excretion kinetics of an intravenous bolus of fluorescein isothiocyanate-FITC-sinistrin (FITC-sinistrin) (6 mg/100g body weight; dissolved in NaCl 0,9% Fresenius Kabi, Austria) using a miniaturized fluorometric detector (NIC-Kidney excitation 480 nm/emission 521 nm; MediBeacon, St. Lous, USA). GFR was calculated using the half-life derived from the rate constant of the single exponential phase of the FITC-sinistrin excretion curve, as previously described [52, 53].

Oxidative stress assessment

Kidney samples were homogenized in a Dounce homogenizer in 0.15M KCl. After measuring the total protein concentration [54], aliquots were deproteinized with cold trichloroacetic acid 30% V/V for 10 min, and the pellet was removed by centrifugation. Oxidative stress was determined in kidney homogenates and evaluated as changes of the GSH pool assayed in deproteinized kidney homogenates [55] and lipid peroxidation, measured as thiobarbituric acid reactive substances (TBARS) [56]. The total enzymatic GST activity was assayed in homogenates, using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate and GSH as a cofactor.

Quantitative Real-Time PCR

Total RNA was isolated using an RNeasy Mini Kit (Qiagen) according to the manufacturer's directions. Extracted RNA was quantified at 260-nm in a NanoDrop Spectrophotometer (NanoDrop Technologies) and the integrity of the RNA was assessed by agarose gel electrophoresis. cDNA was prepared from total RNA (1μg) using a reverse transcription system (random hexamers, Improm II Reverse Transcriptase System from Promega). Then, PCR was performed duplicated for each experiment (HotStart Taq DNA polymerase from Qiagen or BRILLIANT III ULTRA-FAST SYBR GREEN QPCR (Stratagene). Amplicons were detected for Real-Time Fluorescence Detection (Rotor-Gene Q, Qiagen). The primers used are detailed in Table 1. Relative mRNA abundance was calculated using Ct values and normalized to the relative abundance of each transcript.

Histochemical analysis and tissue damage determination

Kidneys were fixed in 10% formalin, included in paraffin. The kidneys were fixed in 10% buffered formalin, embedded in paraffin, sectioned, dewaxed, rehydrated, rinsed in water, and stained with hematoxylin-eosin (HE) and periodic acid Schiff (PAS). The morphologic analysis was carried out in a blinded manner as detailed previously by Dr. Luis Contreras, pathologist [49]. The cortex and medulla were evaluated for epithelial necrosis, loss of brush border, tubular dilation, and tubular congestion, among other kidney alterations observed in AKI response.

Western blot assay

Western blot was realized as was previously published with some modifications [49]. Briefly, the renal cortex and medulla were dissected and homogenized with an Ultra-Turrax homogenizer in ice-cooled 10 mM Tris·HCl buffer at pH 7.4, supplemented with 1 mM EDTA, 1 mM EGTA, 0.25 M sucrose, 1% vol/vol Triton X-100, and a protease inhibitor cocktail (Complete Mini, Roche Applied Science). Tissue homogenates were subject to centrifugation. Step one was 900×g by 10 min. Next, the tissue was sonicated for 30 seconds

Table 1. Real-time PCR primer sequences

Gene	Sequence	Type	MT (°C)	Species	Amplicon size (bp)
IL-1β	5´-CAAATCTCGCAGCAGCACA-3´	Forward	56.2	Mouse	121
	5´-TCATGTCCTCATCCTGGAAGG-3´	Reverse	56.2	Mouse	
IL-6	5´-TACCATAGCTACCTGGAGTAC-3´	Forward	52.6	Mouse	150
	5´-AATTGGGGTAGGAAGGACTAT-3´	Reverse	52.7	Mouse	
IL-10	5´-GGT TGC CAA GCC TTA TCG GA-3´	Forward	57.7	Mouse	191
	5´-ACC TGC TCC ACT GCC TTG CT-3´	Reverse	61.9	Mouse	
Foxp3	5´-AACCTGAGCCTGCACAAGTGCTTT-3´	Forward	61.6	Mouse	134
	5´-TGAGGTCAAGGGCAGGGATTGGA-3´	Reverse	62.4	Mouse	
Fascin	5'-AAGCTGATTAACCGCCCCAT-3'	Forward	57.0	Mouse	157
	5'-TGCCCGTGGAGTCTTTGATG-3'	Reverse	57.6	Mouse	
Vimentin	5'-GTGGATCAGCTCACCAACGA-3'	Forward	57.2	Mouse	160
	5'-AAGCATTGTCAACATCCTGTCTG-3'	Reverse	55.6	Mouse	
Hsp47	5´-TGCAGAAACATCTGGCAGGAC-3´	Forward	57.8	Mouse	167
	5´-CCCGTAGATGTCTTGGTCAAAGG-3´	Reverse	57.4	Mouse	
Clusterin	5'-TTGACTCTGACCCCATCACA-3'	Forward	55.7	Mouse	110
	5'-GCTTTTCCTGCGGTATTCCT-3'	Reverse	55.4	Mouse	
iNOS	5'-TCCTGCCTCATGCCATTGAGTT-3'	Forward	59.0	Mouse	81
	5'-GCCTGGCCAGATGTTCCTCTATTT-3'	Reverse	59.1	Mouse	
18S	5'-CTCTAGATAACCTCGGGCCGATCG-3'	Forward	60.2	Mouse	170
	5'-GATGTGGTAGCCGTTTCTCAGGCT-3'	Reverse	60.9	Mouse	

on ice, vortexed, and centrifugated again by 2400 × g and 17000 × by 5 min. All procedure was at 4°C. Total proteins in supernatants were measured using the BCA Protein Assay Kit (ThermoFisher Scientific), and samples were stored at -80°C. The antibodies were: rabbit anti-PECAM (77699, Cell signaling), rabbit anti-AQP-1 (ab168387-Abcam). Vimentin (clone 40E-C), and fascin (CPTC-Fascin1-3) antibodies were obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the National Institute of Child Health and Human Development (NICHD) and maintained by the University of Iowa, Department of Biological Sciences, (Iowa City, IA). The anti-Hsp27 (sc-13132; Santa Cruz) and mouse anti-β-actin (A5441; Sigma) antibodies. Secondary antibodies were anti-mouse or anti-rabbit IgG conjugated with Alexa Fluor-750 (Thermo Scientific). The resulting band intensities were quantified using Odyssey equipment and Image Studio Lite software (version 5.25; Li-Cor).

Statistical analysis

We use GraphPad Software to run the statistical analysis. The GFR and NGAL levels were studied using non-parametric Kruskal-Wallis analysis of variance (ANOVA). The GST activity, GSH/GSSG ratio, and thiobarbituric acid reactive substances levels were analyzed in the studied group by non-parametric Mann-Whitney analysis (t-Test). Also, the same criteria for t-Test generate the differences in the IL-1β, IL-6, Foxp3, and IL-10, AQP-1, PECAM-1, iNOS, Hsp27, clusterin, vimentin, fascin, and Hsp47 levels belong to sham, I/R, and IPC.

Results

IPC ameliorated the renal function impaired by I/R

We used a validated protocol of murine renal I/R [49]. Kidney injury was assessed through the serum NGAL (tubular damage biomarker), GFR, and kidney histology. The NGAL serum concentration was significantly upregulated (2.6-fold) in plasma samples from mice subjected to I/R compared with sham mice (111.0±22.7 for I/R and 43.3±6.3pg/mL for sham; $p<0.05$). The IPC protocol completely prevented the NGAL upregulation (41.8±12.0pg/mL) produced by I/R (Fig. 1A). After 48 hours of I/R, the GFR was significantly decreased by 50% of sham levels (869.2±62.0 to sham and 476.7±56.0 to I/R; µL/min/100g body weight; $p<0.05$). The IPC protocol significantly prevented the GFR loss observed by I/R (721.0±75.0 µL/min/100g body weight; $p<0.05$), reaching 83% of the sham levels (Fig. 1B). The Kidney histological analysis revealed normal kidney morphology in the cortex and medulla of the control animals. Glomeruli and tubules had normal structures (Fig. 1C sham arrows). In contrast in the I/R group, acute tubular necrosis (ATN) was present in the kidney, characterized by loss of the brush border and intratubular cellular detritus (Fig. 1C I/R, arrows). Morphological changes included other tubular epithelial alterations such as loss of the nucleus in epithelial cells and vascular congestion (Fig. 1C). In the medulla, was also observed ATN (arrows). In contrast, moderate degeneration and tubular necrosis were observed in the renal cortex from mice exposed to IPC, but in the medulla still was observed some areas of ATN and intratubular cellular detritus (arrow). Therefore, the preconditioning protocol improved the GFR and reduced the NGAL in blood but had a better histological sign of protection in the cortex than the medulla caused by the I/R.

IPC protects the Epithelial and Endothelial I/R-damage in the cortex kidney

The AQP-1 is an essential protein located in the proximal and descending thin limb epithelium tubule and is implicated in the water body homeostasis [57]. We previously published the kidney AQP-1 downregulation in the cortex and medulla by renal ischemia (30 min) and reperfusion (48 hours) [49]. Here we confirmed those results (Fig. 2A-B). We found for the first time that IPC protocol significantly prevented the loss of AQP-1 protein abundance observed during I/R in the cortex, but not in the medulla. We also studied the expression of endothelial marker PECAM-1 (platelet/endothelial cell adhesion molecule 1 or CD31), a transmembrane adhesion protein expressed in kidney endothelial cells [58-60].

We found decreased PECAM-1 expression in the medulla but not in the cortex by renal I/R (Fig. 2C-D). However, the IPC did not appear to be entirely prevented the PECAM-1 downregulation (p=0.12), suggesting that IPC did not entirely protect the epithelial and endothelial cells in the medulla.

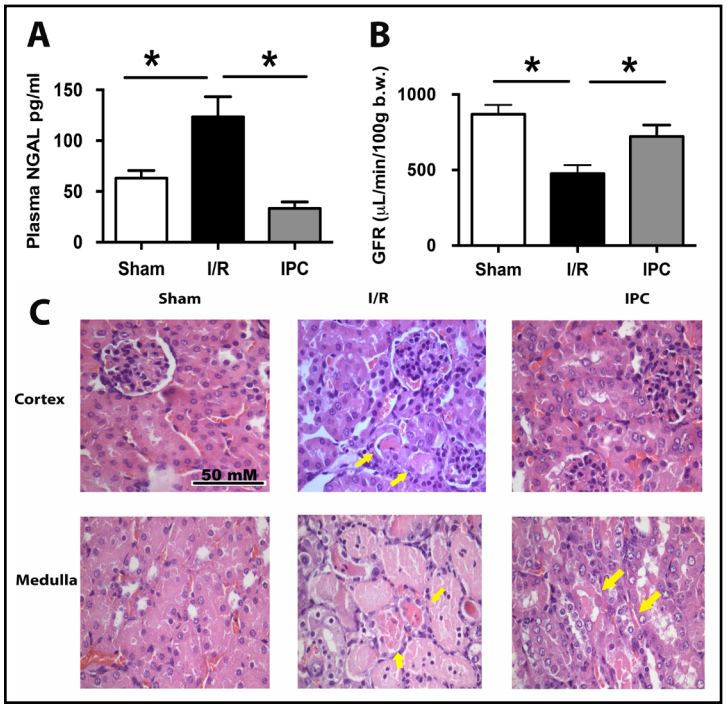

Fig. 1. IPC prevented kidney damage induced by renal I/R. BALB/c mice were subjected to sham (n=5), I/R (n=6), or IPC (n=5) protocols. A. Neutrophil gelatinase-associated lipocalin (NGAL) was determined in plasma by ELISA. B. Glomerular Filtration Rate (GFR: µl/min/100g body weight) was determined in conscious animals and calculated using the half-life derived from the rate constant of the single exponential phase of the FITC-sinistrin excretion curve. Bars graphs represent mean ± SEM. Data were tested using non-parametric Kruskal-Wallis analysis of variance (ANOVA), *p<0.05. C. Histological analysis. Representative hematoxylin/eosin (H/E) staining of the cortex and medulla kidney sections. Yellow arrows indicate areas with evident kidney acute tubular necrosis (ATN) characterized by loss of nucleus in tubules or intratubular cellular detritus. Three kidneys for each protocol were analyzed. Representative images correspond to 400X Scale bar=50 µm.

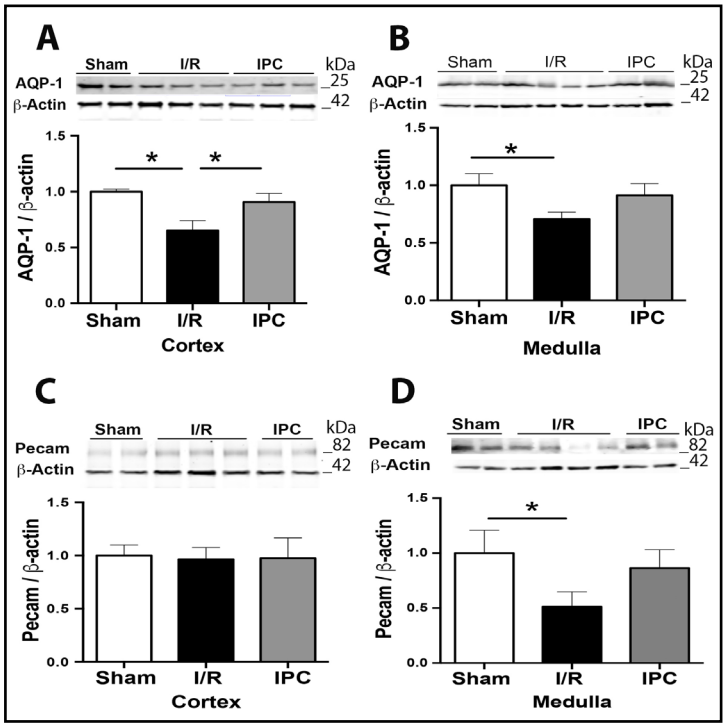

Fig. 2. IPC had a different effect in the cortex and medulla on AQP-1 and PECAM-1 protein expression. BALB/c mice were subjected to sham, I/R, or IPC protocol. The AQP-1 and PECAM-1 expression was determined by Western blot in sham (n=4), I/R (n=6), or IPC (n=8) groups in A. AQP-1 in cortex and B. AQP-1 in medulla. C. PECAM-1 in cortex. D. PECAM-1 in medulla. The upper panel shows a representative picture of AQP-1 or PECAM-1 expression, and the protein abundance was normalized by β-actin. The bar graph represents mean ± SEM and the data were analyzed using non-parametric Mann-Whitney analysis (t-test), *p<0.05.

The Glutathione S-transferase activity was lost by I/R and recovered during IPC

We studied the effect of renal ischemia and reperfusion injury in the oxidative stress level in the whole kidney, measuring the GSH-to-GSSG ratio to measure the soluble antioxidant status, Glutathione-S Transferase (GST) activity as an antioxidant mechanism, and TBARS as a readout for lipid peroxidation. In addition, we quantified the inducible form of NOS mRNA as an indicator of nitric oxide (NO) production. Mice subjected to renal I/R showed a significant decrease in the GSH/GSSH ratio compared to the sham group (50.48±11.55 for sham and 15.52±4.54 for I/R; nmol/g tissue, $p<0.05$) and IPC prevented this phenomenon (40.07±11.62 nmol/g tissue, $p<0.05$) (Fig. 3A). The renal lipid peroxidation (TBARS) status was increased 2 times during I/R compared to sham (175.8±1.7 for sham and 334.3±24.48 for I/R; nmol/g tissue, $p<0.05$). The IPC protocol reduced the TBARS at a similar level to the sham group (180.58±5.37 nmol/g tissue, $p>0.05$) (Fig. 3B), suggesting a strong antioxidant effect of the IPC protocol. We previously described that the GST activity was strongly reduced in the kidney by renal I/R in mice. Here, we reproduced those results (97.40±7.03 for sham and 4.37±0.48 for I/R; nmol/g tissue, $p<0.05$) and provided the first evidence describing the beneficial effect of IPC protocol on the GST activity (76.35±16.06 nmol/g tissue) (Fig. 3C), producing 78% of the GST activity observed in sham animals. Therefore, these data suggest that the IPC protocol prevents oxidative stress damage through an important contribution of GST activity. Interestingly, the iNOS mRNA levels increased after I/R, but the IPC did not prevent the iNOS mRNA upregulation (Fig. 3D).

The effect of IPC in Hsp27 and Clusterin kidney expression

The Hsp27 (heat shock protein 27) is a chaperone and antioxidant protein whose induction follows injury protects against kidney injury [61]. We noted that the Hsp27 protein expression did not increase significantly by I/R in the cortex and medulla. However, the IPC treatment significantly increased the expression of Hsp27 when is compared with I/R in the cortex, but not in the medulla (Fig. 4A-B). On the other hand, we have previously reported that clusterin was increased in kidneys subjected to I/R, and it was downregulated by iNOS inhibition [49]. Here, we also showed that the mRNA level of clusterin was significantly increased in both cortex and medulla in the I/R compared with sham animals. Remarkably, the IPC protocol remains the clusterin upregulation observed by I/R (Fig. 4C-D).

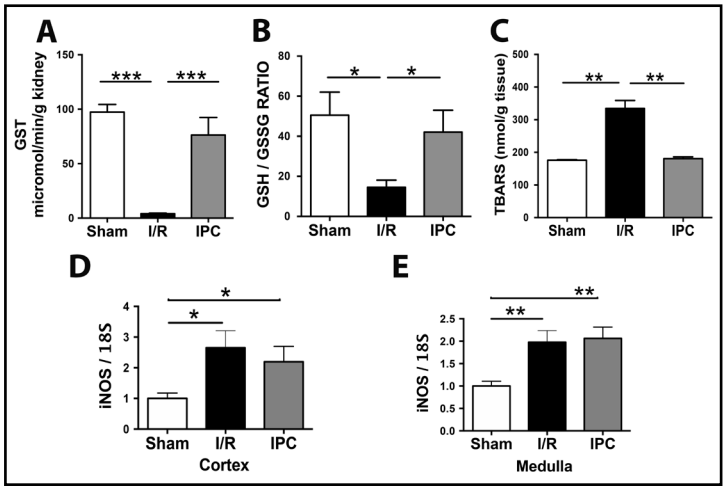

Fig. 3. IPC recovered the GST activity, reducing the oxidative stress damage, without inhibition of iNOS. Using the BALB/c mice the oxidative stress was determined in whole kidneys of sham (n=5), I/R (n=5), and IPC (n=5) group. A. GST activity. B. GSH/GSSG ratio. C. Thiobarbituric acid reactive substances (TBARS) for Lipoperoxidation measurement. D. iNOS mRNA in cortex E. iNOS mRNA in medulla The bar graph represents mean ± SEM and the data were analyzed using non-parametric Mann-Whitney analysis (t-test), *$p<0.05$; **$p<0.005$; and ***$p<0.0005$.

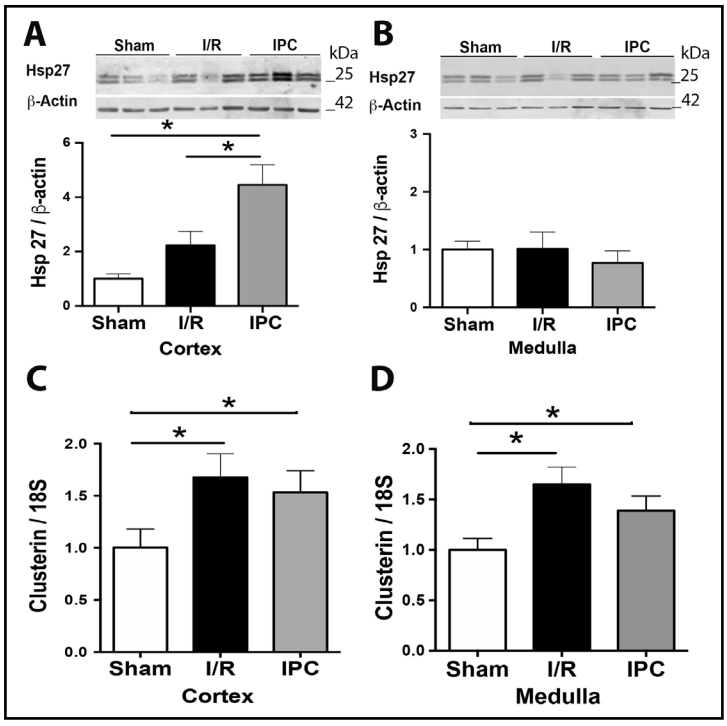

Fig. 4. IPC upregulated the expression of Hsp27 (cortex) and clusterin (cortex and medulla). BALB/c mice were subjected to sham (n=5), I/R (n=4), or IPC (n=5) surgery. The Hsp27 protein abundance was determined by Western blot (the upper panel shows a representative picture) in A. Cortex and B. Medulla. The clusterin mRNA expression was determined by qRT-PCR in C. Cortex and D. Medulla. The bar graph represents mean ± SEM and the data were analyzed by using non-parametric Mann-Whitney analysis (t-test), *p<0.05.

Effect of IPC in the inflammatory response

Inflammation is one of the most critical stages in renal injury after ischemia and reperfusion. We investigated the mRNA expression of two pro-inflammatory (IL-1β and IL-6) cytokines and two regulators of inflammation (Foxp3 and IL-10). In the kidney cortex, after 48 hours of I/R, we did not find changes in the mRNA levels of cytokines or Foxp3. Remarkably, the Foxp3 and IL-10 mRNAs were upregulated significantly by IPC in the cortex. In the kidney medulla, the IL-1β, IL-6, and IL-10 mRNAs increased after 48 hours of I/R, and IPC did not prevent the cytokines upregulation. In addition, IL-10 and Foxp3 mRNA were higher than the sham group in the medulla (Fig. 5B, D, F, and H).

IPC reduced the expression of kidney mesenchymal proteins promoted by I/R

Recently, Xu-Dubois et al. [48] described a panel of endothelial-to-mesenchymal transition (EndMT) markers composed of fascin (an actin-bundling protein involved in cell motility), vimentin (an intermediate filament, a complete mesenchymal cell phenotype marker), and Hsp47 (a collagen-specific chaperone protein). These markers are expressed by microvasculature endothelial cell injury/activation, and they are detected in early posttransplant biopsies associated with poor renal graft recovery and/or late graft dysfunction. We decided to explore these three mesenchymal markers in our IPC experimental protocol. We observed that 30 minutes of ischemia and 48 hours of reperfusion upregulated the expression of fascin, vimentin, and Hsp47 (mRNA) in cortex and medulla regions (Fig. 6). Interestingly, the mRNA upregulation of vimentin and Hsp47 was reduced in the cortex and medulla when the animals were subjected to the IPC protocol (Fig. 6). Noteworthy, fascin mRNA abundance did not decrease significantly in the cortex by IPC (Fig. 6). We explored the protein abundance of vimentin and confirmed the results observed for vimentin mRNA (Fig. 6). Interestingly, the fascin protein abundance in IPC was higher in the cortex than in sham animals. Taking into account the renal protection observed in the cortex by IPC, we suggest that fascin could contribute to kidney protection. On the other hand, in the medulla, Hsp47 mRNA was significantly downregulated by IPC compared with the I/R group but still was higher than the sham group, and considering the deficient renal protection observed in the medulla by IPC we suggest that Hsp47 could contribute to the medulla kidney damage (Fig. 6).

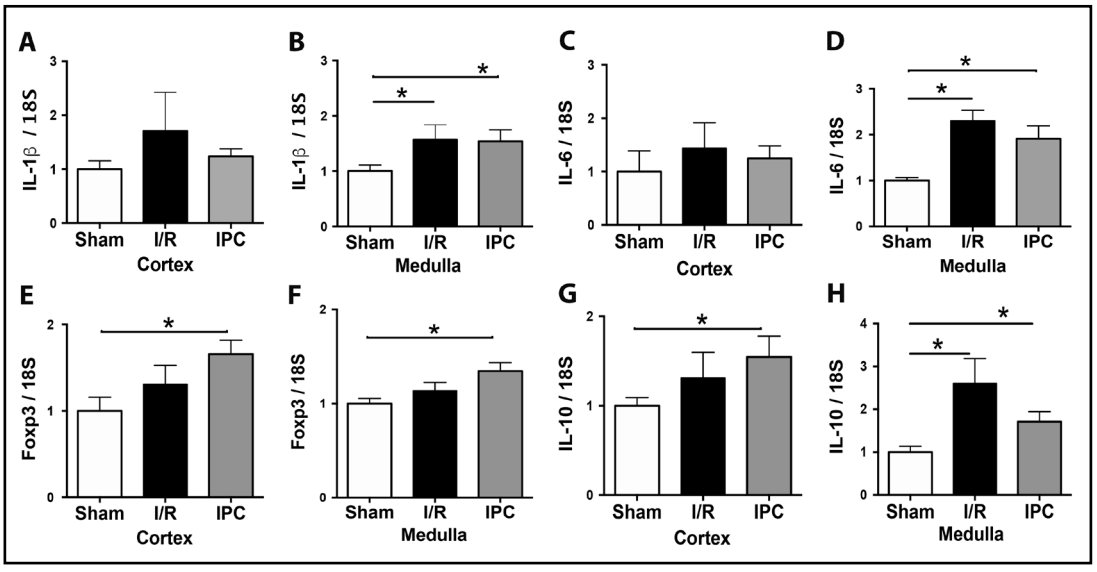

Fig. 5. IPC had a different effect in the cortex than medulla in the IL-6 and IL-10 mRNA expression. Using BALB/c mice, the mRNA expression of IL-1β, IL-6, Foxp3, and IL-10 were determined by qRT-PCR in sham (n=5), I/R (n=4), and IPC (n=5) in the A. IL-1β mRNA in cortex. B. IL-1β mRNA in medulla C. IL-6 mRNA in cortex. D. IL-6 mRNA in medulla. E.- Foxp3 mRNA in cortex. F. Foxp3 mRNA in medulla G.- IL-10 mRNA in cortex. H. IL-10 mRNA in medulla. The bar graph represents mean ± SEM and the data were analyzed using non-parametric Mann-Whitney analysis (t-test), *p<0.05.

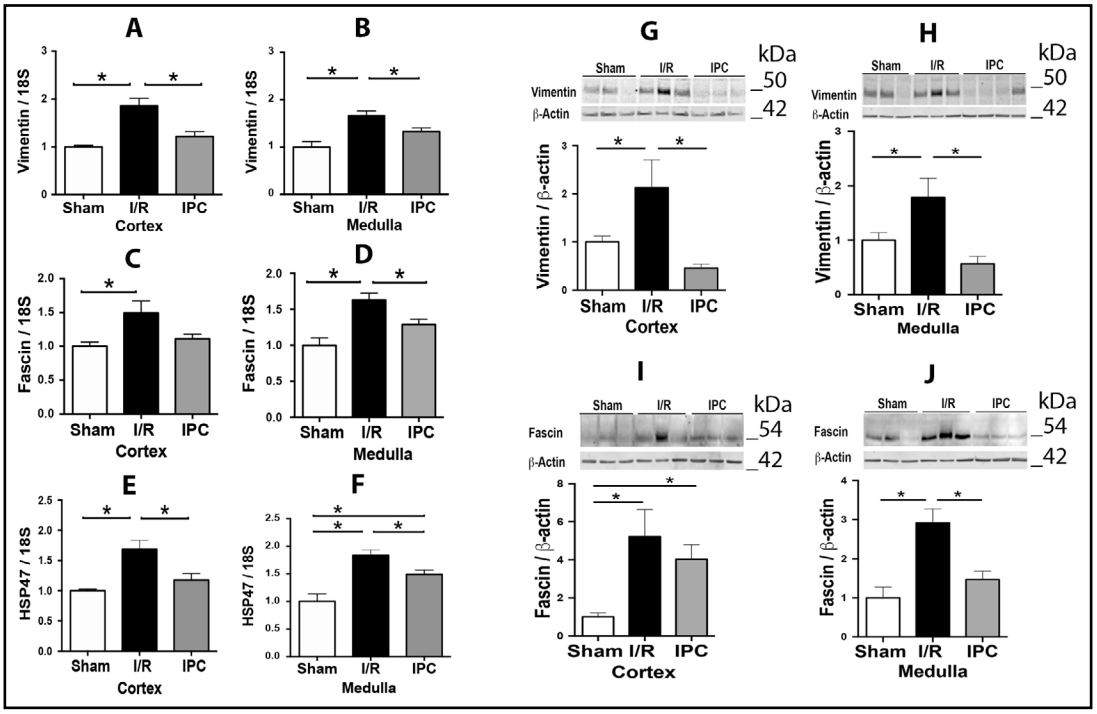

Fig. 6. IPC modified the expression of mesenchymal proteins in the kidney. Using BALB/c mice, mesenchymal markers expression were determined in sham (n=5), I/R (n=5), and IPC (n=5) in cortex and medulla by qRT-PCR and Western blot. A. Vimentin mRNA in the cortex. B. Vimentin mRNA in the medulla. C. Fascin mRNA in the cortex. D. Fascin mRNA in the medulla. E. Hsp47 mRNA in the cortex. F. Hsp47 mRNA in the medulla. G. Vimentin protein in the cortex. H. Vimentin protein in the medulla. I. Fascin protein in the cortex. D. Fascin protein in the medulla. The bar graph represents mean ± SEM and the data were analyzed using non-parametric Mann-Whitney analysis (t-test), *p<0.05.

Discussion

The major findings of this study are 1) IPC prevented the GFR reduction, the increase in NGAL blood levels, and reduced the signs of morphological damage principally in the cortex produced by I/R. 2) IPC improved the GST activity and prevented the decrease of GSH levels in the kidney, reducing lipoperoxidation. However, the IPC did not prevent the iNOS mRNA upregulation. 3) The epithelial and endothelial components were protected in the cortex by IPC, but not in the medulla. 4) Additional protective factors, such as Foxp3, IL-10 mRNA, and Hsp27 were upregulated preferentially in the renal cortex of IPC. 5) According to a panel of mesenchymal markers (vimentin, Fascin, and Hsp47), IPC reduced the upregulation of mesenchymal markers in the cortex and medulla compared to I/R. However, Fascin in the cortex and Hsp47 in the medulla remain higher in IPC than Sham group.

The effect of IPC on oxidative stress

One possible mechanism mediating the beneficial effects of IPC shown in the present study may imply metabolic protection. Recent studies in renal tubular cells subjected to ischemia *in vitro* have shown metabolic adaptation/protection from hypoxia via HIF1α due to maintenance of glycogen stores, NADH intracellular levels, and GSH//GSSG ratio [62]. Our results suggest that IPC may support the GSH levels and the GST activity.

We previously described that downregulation of GSH/GSSG ratio, GST activity, and increased lipoperoxidation observed by I/R was prevented by the pre-treatment with an iNOS inhibitor (l-NIL) [49]. In the present study, we found that IPC remains the iNOS mRNA upregulation in the same levels of I/R. Previous studies suggested a renoprotective role of iNOS during IPC. The pharmacological inhibition of iNOS or the genetic ablation of iNOS in animals resulted in the loss of the renal protective effect of IPC, indicating that iNOS activity is necessary for the development of tolerance to ischemia-induced by IPC [63]. In addition, IPC protection from cerebral ischemia was dependent on iNOS [35]. In contrast, during renal I/R, NO-derived from iNOS can dampen compensatory changes in renal hemodynamics and affect tubular reabsorption and oxygen consumption [64-67]. Thus, we speculate that the iNOS upregulation by IPC without signs of oxidative stress may explain the beneficial effects of iNOS activity during IPC.

The effect of IPC on the inflammatory response

Our results showed that IPC increased Foxp3 and IL-10 mRNA in the cortex without upregulation of IL-1β or IL-6 mRNA, suggesting that this condition may be one mechanism that confers protection to the renal cortex by IPC. These results suggest that a potential mechanism mediating the protective effect of IPC is the polarization/recruitment of Treg lymphocytes, which may ameliorate and/or limit the inflammatory response triggered by I/R. Previous studies indicated that IL-10 protects the kidney after renal ischemia injury by suppressing inflammatory mediators such as TNF-α and IL-6 [68, 69] and inhibiting leukocyte adhesion on the endothelium [70]. Compared to wild-type (WT) mice, IL-10 knockout mice with IRI showed a more marked reduction of renal function, upregulated expression of AKI biomarkers, increased expression of the pro-inflammatory cytokines, and increased expression of pro-apoptosis factors [71]. We did not observe significant changes in IL-1β or IL-6 mRNA abundance in the renal cortex after 48 hours of reperfusion. However, future studies are necessary to clarify if proinflammatory cytokines such as IL-6 presented an early increase after I/R in the renal cortex, which may have been ameliorated by IL-10 upregulation after preconditioning. Indeed, the study of Sasaki et al., showed a transient increase in renal IL-6 mRNA levels at 5- and 24-hours post-I/R, which returned at 48 hours to levels similar to those observed in sham surgery mice.

The effect of IPC on endothelial and epithelial cell alterations

Renal injury following ischemia and reperfusion provokes disruption of microvasculature due to endothelial cell damage and renal dysfunction following the loss of epithelial cell

polarity secondary to cytoskeletal alterations. AQP-1 is a protein expressed at the apical and basolateral region of the proximal tubule epithelial cells and the thin descending limb of Henle and in microvascular endothelia, including vasa recta [72]. Our study shows a differential effect of IPC in the cortex than medulla. We and others described previously that I/R decreases the expression of AQP-1 in the renal cortex and medulla. Here we observed that AQP-1 lower expression after I/R was prevented by IPC in the cortex; only a non-significant tendency to protection was observed in the medulla. PECAM-1 was reduced after 48 hours of I/R in renal medulla only, and IPC did not confer protection. In contrast, vimentin, fascin, and Hsp47 were increased by I/R, and IPC reduced this upregulation in both renal regions. However, Hsp47 in medulla during IPC was kept higher than sham animals, suggesting an association between high Hsp47 and low AQP1 or PECAM-1 expression during IPC in the medulla.

The effect of IPC on Hsp27 and Clusterin

Hsp27 is a chaperone that confers cytoprotection from ischemia [73, 74] by stabilizing the actin cytoskeleton, displaying an anti-apoptotic function, reducing ATP depletion, and reactive oxygen species [75]. Hsp27 overexpression protects the heart and brain from ischemic injury [76], and recent studies show that Hsp27 is necessary for protection against renal IRI conferred by pharmacological A1 adenosine receptor (AR) activation before renal ischemia [77]. The upregulation of Hsp27 by renal injection of adenovirus encoding Hsp27 or by the injection of LPS before renal IRI was also protective, ameliorating the increase in creatinine/BUN and apoptosis up to 24 hours after I/R [78, 79]. Furthermore, Jo et al., in a delayed model of ischemic preconditioning and uninephrectomy (four cycles of 5/5 min of I/R applied for 24h before 30 min of renal ischemia in the left kidney), found increased expression of Hsp27, which was associated with the reduction of cellular injury [63]. In addition, the induction of Hsp27 before I/R prevented the increase in hydrogen peroxide (HPO) generation up to 24 hours post-I/R, and the knockdown of Hsp27 did not cause a further increase in HPO generation after I/R as compared with control mice with unaltered basal Hsp27 expression [78]. In the present study, we found that I/R did not significantly increase the expression of Hsp27 in the cortex. However, the IPC protocol increased the Hsp27 protein expression in the renal cortex. Thus, the induction of Hsp27 in the cortex would be implicated in preventing oxidative stress obtained by IPC and probably through GST. It is tempting to speculate that similar to LPS injection, the upregulation of Hsp27 induced by IPC could be a consequence of cortex-specific activation of Hypoxia Inducible Factor 2a (HIF-2a) pathway [78]. However, LPS-stimulated renoprotection was also dependent on the upregulation of eNOS and iNOS. Here we found the upregulation of iNOS and Hsp27 in the cortex by IPC but not in the medulla making necessary further studies to clarify if the induction of Hsp27 due to IPC is somehow dependent on NO bioavailability.

Proteins enter the ER and exit as either folded or misfolded proteins. Altered redox signaling and increased reactive oxygen species (ROS) disrupt ER function and proteostasis [80]. GSH plays a core role in maintaining ER oxidoreductases in a reduced state to catalyze reduction or isomerization reactions, and under oxidative stress conditions, GSH acts as a redox buffer source, in addition to its role in disulfide bond formation. Our results showed that the IPC protocol prevented the decrease in GSH/GSSG balance and increasing the GST activity. Thus, the IPC protocol suggests help to maintain ER function and proteostasis.

Noteworthy, in agreement with other groups, we found that clusterin is induced following renal I/R [49]. however, clusterin induction was not modified by IPC, suggesting a potential protective role during IPC.

Finally, we observed differences in the effect of IRI and IPC in the cortex and medulla. In the cortex, IRI downregulated (AQP-1) and upregulated (iNOS, clusterin, vimentin, fascin1, and Hsp47) different molecular panel than medulla. Thus, IRI induced downregulation of AQP-1 and PECAM-1, but increased the IL-1β, IL-6, IL-10, iNOS, clusterin, vimentin, fascin, and Hsp47 expression in medulla. So, the results indicate that I/R has a more marked/persistent inflammatory effect in the medulla than in the cortex. Consistent with differential injury

mechanisms in the cortex and medulla, IPC showed differential protective effects. IPC upregulated Hsp27 in the cortex but not in the medulla, and the markers of Treg lymphocytes, FoxP3 mRNA, and IL-10 mRNA, were also induced by IPC in the kidney cortex. The potential mechanisms that may potentiate Treg activity response in the renal cortex after IPC may depend on amelioration of injury due to enhanced tissular tolerance to transient hypoxia and lower cellular damage after I/R.

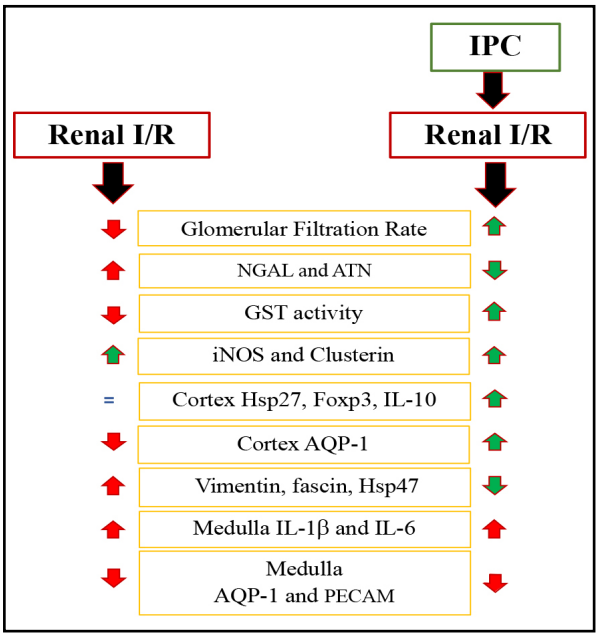

Fig. 7. Schematic representation of ischemia/reperfusion (I/R) or Ischemic preconditioning (IPC) effect. The kidney injury signals and kidney protective signals are represented in red and green arrows, respectively. The equal symbol means without significant modification.

Conclusion

In summary, we observed that IPC reduced oxidative stress and tubular damage, concomitant with the upregulation of GST activity, clusterin, Foxp3, IL-10, and Hsp27 (factors involved in cell protection) evident in the cortex. Interestingly, the increase of IL-1β and IL-6 mRNA levels and the downregulation of PECAM-1 and AQP-1 protein expression in kidney medulla during IPC shows that the IPC has a preferential better effect in the cortex (Fig. 7). In addition, during IPC the iNOS upregulation of oxidative stress, suggests a potential explication of why iNOS is good during IPC and bad during I/R. Our results provide relevant and novel evidence to understand the molecular components involved in IPC, showing that molecular mechanisms that lead to I/R protection may be cortex and medulla specific. In addition, we described new components associated with IPC, such as clusterin and GST, that are potential targets of future investigation.

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Author Contributions
Conceived and designed experiments: CEI.
Performed the experiments: CP, YH; ML, JR, CA, JL, LM.
Analyzed the data: CP, ML, JL, LM, CEI.
Wrote the paper CP, LM, CEI.
Contributed reagents/materials/analysis tools: CEI, LM, CA. JL, CP.

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Statement of Ethics
All experimental procedures were under institutional and international standards for humane care and laboratory animal use (Animal Welfare Assurance Publication A5427-01,

Office for Protection from Research Risks, Division of Animal Welfare. NIH, USA). All procedures were approved by the Committee on the Ethics of Animal Experiments of the University de los Andes, Chile.

Disclosure Statement

The authors have no conflicts of interest to declare.

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