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Article

Boldine Improves Kidney Damage in the Goldblatt 2K1C Model Avoiding the Increase in TGF-β

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Abstract: Boldine, a major aporphine alkaloid found in Chilean boldo tree, is a potent antioxidant. Oxidative stress plays a detrimental role in the pathogenesis of kidney damage in Renovascular hypertension (RVH). The activation of the Renin-Angiotensin System (RAS) is crucial in the development and progression of hypertensive renal damage and TGF-β is closely associated with the activation of RAS. In the present study, we assessed the effect of boldine on the progression of kidney disease using the 2K1C hypertension model and identifying mediators in the RAS such as TGF-β, that could be modulated by this alkaloid. Toward this hypothesis, rats (n=5/group) were treated with boldine (50mg/kg/day, gavage) for 6 weeks after 2K1C surgery (pressure≥180mmHg). Kidney function was evaluated by measuring of proteinuria/creatininuria ratio (U prot/U Crea), oxidative stress (OS) by measuring thiobarbituric acid reactive substances (TBARS). The evolution of systolic blood pressure (SBP) was followed weekly. α-SMA and Col III were used as markers of kidney damage; ED-1 and Osteopontin (OPN) as markers of inflammation. We also explored the effect in RAS mediators, such as ACE-1 and TGF-β. Boldine treatment reduced UProt/UCrea ratio, plasma TBARS and slightly reduced SBP in 2K1C hypertensive rats, producing no effect in control animals. In 2K1C rats treated with boldine the levels of α-SMA, Col III, ED-1 and OPN were lower when compared to 2K1C rats. Boldine prevented the increase in ACE-1 and TGF-β in 2K1C rats, suggesting that boldine reduces kidney damage. These results suggest that boldine could potentially be used as a nutraceutic.

Keywords: Renovascular hypertension, Chronic kidney disease, Oxidative Stress, Fibrosis, (S)-2,9-Dihydroxy-1,10-dimethoxy-aporphine

1. Introduction

Hypertension is one of the most common complications that predispose to other health problems, affecting several organs [1]. Human renovascular disease constitutes a relatively rare form of secondary hypertension, frequently related to the activation of the renin-angiotensin system (RAS), because of the fall in the renal blood flow and perfusion pressure due to renal artery stenosis [2,3]. The kidneys respond to this low pressure in the renal vessels producing hormones that lead to sodium and water retention, which causes a rise in blood pressure [4].

Hypertensive nephropathy begins in the glomerulus by an increase in intraglomerular pressure. These early events activate and damage mesangial cells, epithelial cells and podocytes in the glomerulus. In turn, these cells produce vasoactive and pro-inflammatory agents, which increase cell damage and promote fibrosis, reducing renal blood flow, permeability and eventually glomerular

filtration [1]. This type of hypertension is increasingly related to the pathogenesis of chronic kidney disease (CKD) [3].

CKD is a life-threatening condition characterized by progressive and irreversible loss of renal function. It is manifested by an advancing decrease in glomerular filtration rate (GFR), resulting from a rise in damaged nephrons and failure of the organ´s hormonal functions [5]. CKD has different etiologies. Independent of the cause, however, the morphological characteristics, such as tubular necrosis and glomerular sclerosis, are similar [6]

CKD is a condition whose prevalence has increased worldwide. It is estimated that over 10% of adults in developed countries suffer some degree of kidney damage [7] and has also been reported that 40% of hypertensive patients with renal damage in its terminal stage have a renal artery stenosis [3]. In this sense, animal models have emerged as important tools for understanding the mechanisms implicated in the pathogenic process, and for the assay of prospective therapies.

The Goldblatt two-kidney, one-clip (2K1C) rat hypertension model is a long-established and widely employed model in the study of renal artery stenosis and Renovascular hypertension (RVH) ([8]). In addition to providing a reproducible and clinically relevant model of systemic hypertension, the 2K1C model exhibits a prominent divergence of biological responses in the two kidneys: the stenotic kidney displays progressive atrophy, whereas the contralateral kidney exhibits a compensatory growth response [3,9]. These two facets of this model, atrophy of one kidney and increased growth of the other, are of special relevance to CKD, wherein a certain subset of nephrons exhibit injury and atrophy, whereas other nephrons display structural enlargement and hyperfunction, since the latter may serve as a precursor to chronic injury, nephron atrophy, and, ultimately, progressive kidney disease [3].

The RAS is the prototype of a classic systemic endocrine network whose actions in the kidney and adrenal glands regulate blood pressure, intravascular volume and electrolyte balance [10]. We have seen that the RAS, including angiotensin II (AngII), has a close relationship with the 2K1C model, since their levels were elevated in the development and maintenance of hypertension in this model [9].

AngII is a key mediator of CKD. It is now understood that AngII mediates renal fibrosis by stimulating endogenous synthesis of transforming growth factor-β (TGF-β) [11]. TGF-β is a cytokine that acts locally in a paracrine form, stimulating the synthesis of extracellular matrix (ECM), and inhibiting the action of matrix-degrading proteases [10]. It has been found that in the development of renal fibrosis, TGF-β is closely associated with the activation of RAS, as AngII induces the transcription and synthesis of TGF-β in damaged kidney cells. Once expressed, TGF-β induces the transformation of fibroblasts into myofibroblasts (α-smooth muscle actin-positive cells, α-SMA) and stimulates the expression of fibronectin (FN) and collagen type III (Col III). This induces the development of fibrosis in the kidney, causing deterioration of the renal function and increasing renal damage [10,12,13].

The steady increase in AngII levels impairs renal function favoring the development of renovascular hypertension [14]. Moreover, if high levels of AII are kept for extended periods of time, an inflammatory response, characterized by the infiltration of macrophages (ED-1), tubular overexpression of a macrophage chemotactic and adhesion molecule such as osteopontin (OPN), and the expression of inflammatory factors such as cytokines can be induced. This response is ultimately associated with renal damage induced by hypertension [1,14].

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The activation of the RAS is crucial in the development and progression of hypertensive renal damage. Experimental evidence has demonstrated that AngII stimulates the expression of NADPH oxidase, the major enzyme for reactive oxygen species (ROS) production in the repairing tissue, thereby contributing to the appearance of oxidative stress (OS) in various organs [15]. OS is defined as tissue damage caused by an imbalance between excessive generation of oxidant compounds and insufficient antioxidant mechanisms. It has been proposed that OS is involved in several pathological conditions such as cardiovascular diseases, infections, cancer, diabetes, neurodegenerative disorders and in the course of kidney damage [16]. Experimental studies have also demonstrated that OS can induce most of the changes that are thought to contribute to hypertensive kidney disease including inflammation, endothelial dysfunction, tissue damage and hypertension [15].

As the RAS is clearly involved in the development of the pathology, antihypertensive drugs such as angiotensin-receptor antagonists or angiotensin converter enzyme (ACE) inhibitors are common therapeutic treatments employed in renovascular hypertension [4]. In addition, recent work, both in humans and in experimental renovascular hypertension animal models, demonstrate that long-term treatment with antioxidants improves both hypertension and the functional alterations in kidney and heart [4].

An important participation of oxidative events mediated by free radicals in the initiation and/or progression of kidney disease has led to the search for new antioxidant molecules [17]. Major sources of these antioxidant molecules are plants. Therefore, the potential to prevent or delay the adverse effects associated with excessive production of ROS by using previously unexplored plant products has proven to be an attractive target for investigation [17].

Peumus boldus (i.e.: Boldo) is a tree native to central and southern Chile. Dried Boldo leaves have been reported to contain alkaloids in the 0.25-0.54% or 0.4-0.5% range, of which approximately 12-19% is boldine. Boldo bark is an unusually rich source of alkaloids, of which boldine represents about 75% [18]. A large set of pharmacological activity has been attributed to boldine, such as cell protection, as well as anti-inflammatory and antipyretic effects [17,18]. In addition, boldine could scavenge highly reactive free radicals. The latter has made it possible to postulate that boldine is a nutraceutical product with the potential to be a cellular protector against oxidative damage [17]. Based on the above-mentioned evidence, this study assessed the effect of boldine on the progression of kidney disease in the renal hypertensive rat model 2K1C and identifying mediators in the RAS such as TGF-β, that could be modulated by this alkaloid.

2. Results

2.1. Boldine improves kidney function and decreases OS without decreasing SBP

Tubulointerstitial and glomerular fibrosis develops in patients and animals with hypertension causing deterioration of renal function that ultimately leads to organ damage [15]. Quantification of the proteinuria is a key element in the diagnosis and treatment of chronic kidney disease. It is also used to monitor the progress of kidney disease or response to treatment [16]. The collection of urine for 24 hours (or around this time) is considered necessary to measure proteinuria [16,23]. Therefore, we complemented the study of kidney damage by measuring urinary creatinine, this allows us to calculate U Prot / U Crea ratio a parameter used to measure renal function [23]. It has also been shown

in several hypertensive models that the renal OS may contribute to many of the changes that are associated with the development of hypertensive renal disease such as inflammation, endothelial dysfunction, tissue damage and hypertension [15]. With these antecedents, we wanted to evaluate the kidney function and oxidative stress in hypertensive 2K1C rats and evaluate if the antioxidant properties of boldine, could improve kidney function.

The U Prot / U Crea ratio (Fig. 1) increased significantly in 2K1C hypertensive rats (2K1C; 31.5 ± 8.2 AU), compared to control rats (Ctrl; 2.4 ± 1.6 AU; Ctrl+bold; 2.8 ± 1.6 AU), but these levels were significantly lower in 2K1C hypertensive rats that were treated with boldine (2K1C; 6.6 ± 4.0 AU). In Table 1, which shows the individual values of each parameter measured to assess the renal function of these rats, it can be observed that the major effect of boldine in 2K1C hypertensive rats is the reduction of proteinuria and the fractional excretion of Na^+ and K^+ and increase of creatinine clearance to similar values as those present in the control rats.

OS was evaluated by measuring TBARS, where the levels (Fig 2) in plasma of 2K1C hypertensive rats (2K1C; 22.8 ± 4.9 nmol/L) were significantly increased when compared with the control rats (ctrl; 8.4 ± 1.5 nmol/L; ctrl+bold 8.3 ± 1.0 nmol/L), but these levels were significantly lower in plasma of 2K1C hypertensive rats treated with boldine (2K1C+bold; 9.3 ± 2.3 nmol/L).

SBP was measured once a week to determine the effect of boldine in this parameter in 2K1C hypertensive rats. As shown (Fig. 3) in 2K1C hypertensive rats and in the boldine treated group (2K1C+bold) SBP was higher (≥ 200 mmHg) compared with control rats (average values between 130-140 mmHg). Boldine treatment was begun on the third week. From the third week until the sixth week of the study, 2K1C hypertensive rats treated with boldine displayed a slightly lower SBP than 2K1C hypertensive rats, that was not significantly different from the 2K1C hypertensive rats. This allows us to suggest that boldine improves renal function, reduces oxidative stress and has no effect on pressure in 2K1C hypertensive rats.

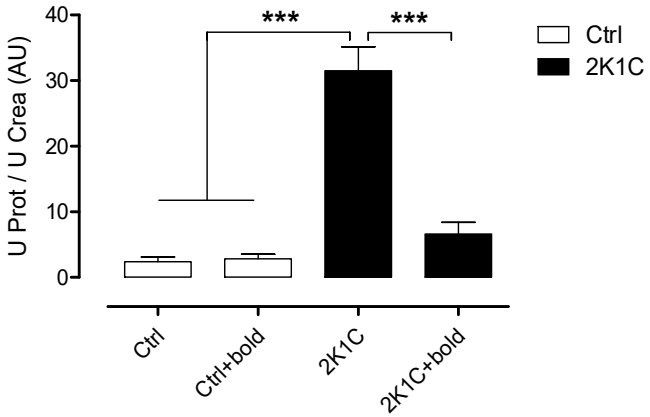

Figure 1. The renal function is improved in 2K1C hypertensive rats treated with boldine. Protein and creatinine were measured in urine samples to assess renal function. Bars represent the mean ± SE. The differences were evaluated by analysis of variance followed by Tuckey post-hoc test. ***p<0.001 vs 2K1C (n=5 per group).

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Groups	Weight (gr)	Proteinuria (mg/day)	Urine Creatinine (mg/day)	Plasma Creatinine (mg/ml)	Creatinine clearence (ml/min)	FE Na^+ (%)	FE K^+ (%)
Ctrl (n=5)	410 ± 10.5	11.6 ± 2.4***	6.4 ± 1.8	0.3 ± 0.0***	0.4 ± 0.1*	0.1 ± 0.0**	29.6 ± 6.2**
Ctrl+bold (n=5)	412 ± 20.3	15.9 ± 6.0***	6.6 ± 1.0	0.2 ± 0.0***	0.4 ± 0.1*	0.1 ± 0.0**	21.6 ± 1.1**
2K1C (n=5)	383 ± 8.7	132.2 ± 11.7	4.3 ± 0.4	0.8 ± 0.1	0.1 ± 0.0	0.6 ± 0.1	101.6 ± 23.1
2K1C+bold (n=5)	390 ± 13.4	26.4 ± 5.8 ***	4.4 ± 0.3	0.3 ± 0.1***	0.3 ± 0.1	0.2 ± 0.1*	30.6 ± 6.0**

Table 1. Values for proteinuria, creatininuria, creatininemia, creatinine clearance and fractional excretion (FE) for Na^+ and K^+ in the 4 groups. Boldine was administered by *gavage* at doses of 50mg/kg during 6 weeks after Hypertension was established in 2K1C rats (pressure≥180 mmHg). Samples of urine and plasma were obtained. Protein and creatinine were measured to assess renal function; the fractional excretion of sodium and potassium ($FENa^+$, FEK^+, [%]) was also determined. Data are expressed as mean ± SE. The differences were evaluated by analysis of variance followed by Tuckey post-hoc test. *p<0.05, **p<0.01, ***p<0.001 vs 2K1C (n=5/ all groups).

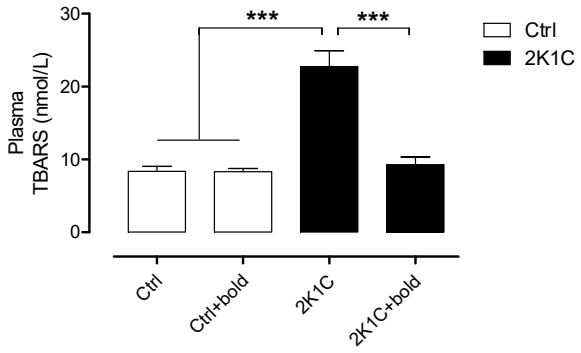

Figure 2. Boldine treatment normalizes plasma levels of TBARS in 2K1C hypertensive rats. Plasma from the four groups was used to measure reactive species of thiobarbituric acid (TBARS) as a measurement of ROS. Bars represent the mean ± SE. The differences were evaluated by analysis of variance followed by Tuckey post-hoc test. ***p<0.001 vs 2K1C (n=5/ all groups).

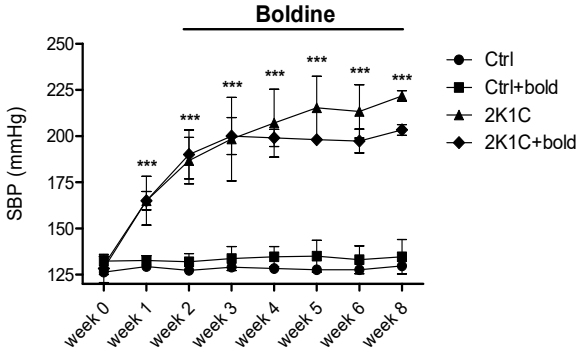

Figure 3. Boldine does not reduce SBP in 2K1C hypertensive rats. Once 2K1C animals became hypertensive (pressure ≥ 180 mmHg; week 2), a randomly selected group of animals (n=5) received the oral treatment with boldine for 6 weeks (from week 0 to week 6). Pressure was measured weekly until the day of sacrifice. Bars represent the mean ± SE. The differences were evaluated by analysis of variance followed by Tuckey post-hoc test. ***p<0.001 vs Ctrl (n=3/ all groups).

2.2. Boldine reduces renal tissue damage and inflammation in hypertensive 2K1C rats.

We wanted to evaluate the effect of boldine on morphological markers of renal tissue damage such as collagen type III (Col III) and α-smooth muscle actin (α-SMA). The distribution of collagen was assessed by Sirius Red staining quantification from semiquantitative morphometric analysis.

Evidence from several kidney diseases shows that infiltration of macrophages is closely associated with tubular expression of osteopontin (OPN). OPN is a potent chemoattractant protein that is expressed during renal damage and acts as an adhesion molecule for monocytes and macrophages [24]. For this reason, we studied macrophage infiltration (ED-1) and tubular expression of OPN in 2K1C hypertensive rats.

First, the distribution of all collagens was evaluated with Sirius Red staining. We observed an increase in amounts for this marker in 2K1C hypertensive rats (Fig 4); however, a reduced collagen distribution was observed in 2K1C rats treated with boldine, like that observed in control rats (Fig. 4). These results were complemented by semiquantitative morphometric analysis (Table 2).

Immunostaining for α-SMA and Col III was increased in 2K1C hypertensive rats, however, the amounts were lower in 2K1C+bold, like what was observed in controls rats respectively (Fig. 4). These results were also complemented by semi-quantitative morphometric analysis for α-SMA and Col III (Table 2).

The amounts of macrophages (ED-1) and tubular expression of OPN were increased in 2K1C hypertensive rats (Fig. 5). Nonetheless, lower amounts for these markers were observed in 2K1C hypertensive rats treated with boldine, like control rats and ctrl+bold rats (Fig. 5). Results obtained by immunohistochemistry were complemented by semi-quantitative morphometric analysis for OPN and ED-1 (Table 3). These results demonstrate that boldine decreases renal injury and inflammation markers reducing kidney damage in 2K1C hypertensive rats.

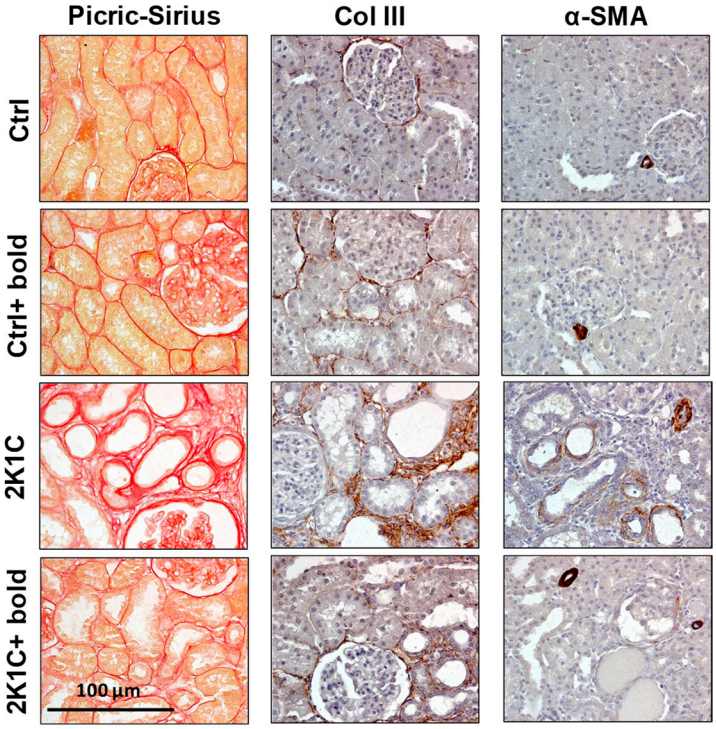

Figure 4. Boldine reduces renal tissue damage in 2K1C hypertensive rats. Sirius Red staining, α-SMA and Col III immunostaining were performed in renal samples 6 weeks after 2K1C rats were made with hypertension. Representative pictures of at least 5 different kidneys are shown. Scale bar = 100 μm. Red staining depicts collagen, while brown staining depicts α-SMA or Col III.

	Score		
	Picric-Sirius	Col III	α-SMA
Ctrl (n=5)	- (-, 5/5)	- (-, 4/5; +, 1/5)	- (-, 5/5)
Ctrl+bold (n=5)	- (-, 4/5; +, 1/5)	- (-, 4/5; +, 1/5)	- (-, 5/5)
2K1C (n=5)	+++ (+++, 4/5; ++, 1/5)	++ (+++, 2/5; ++, 2/5; +,1/5)	+ (+, 4/5; -, 1/35
2K1C+bold (n=5)	+ (+, 5/5)	- (-, 3/5; +, 2/5)	- (-, 5/5)

Table 2. Immunoreactivity score for Picric-Sirius, α-SMA and Col III from tissue-samples from rats of the 4 experimental groups. The semiquantitative morphometric analysis for Picric-Sirius, α-SMA and Col III was performed on a scale of negative to three crosses (- a +++), defined according to the degree of immunoreactivity observed in the renal tissue sample ([-] = 0-10%, [+] = 10-40%, [++] = 40-70% and [+++] = 70-100%).

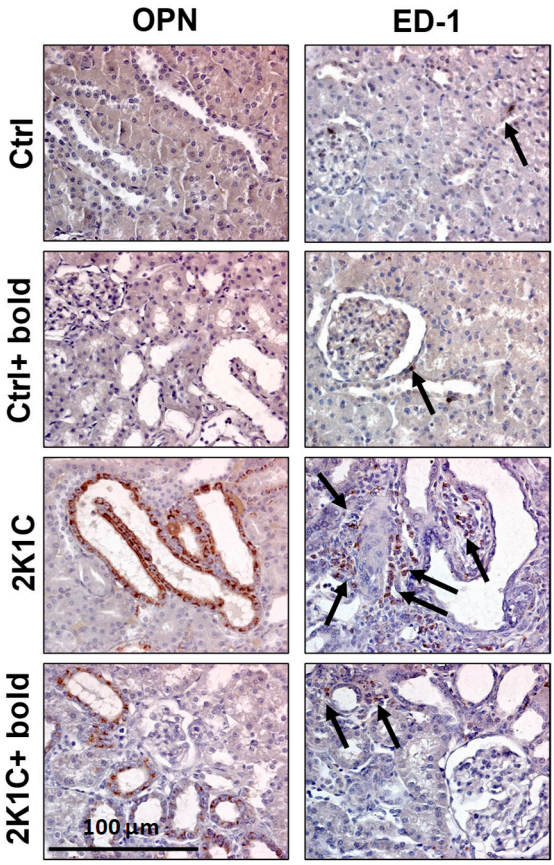

Figure 5. Boldine reduces levels of OPN and ED-1 in 2K1C hypertensives rats. Immunostaining of OPN and ED-1 were performed in renal samples 6 weeks after 2K1C rats were made hypertensive. Representative pictures of at least 3 different kidneys are shown. Scale bar = 100 μm. Brown staining represents the markers OPN and ED-1. For ED-1, the staining is indicated by black arrows.

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	Score	
	OPN	ED-1
Ctrl (n=5)	- (-, 5/5)	- (-, 5/5)
Ctrl+bold (n=5)	- (-, 3/5; +, 2/5)	- (-, 4/5; +, 1/5)
2K1C (n=5)	+++ (+++, 3/5; ++, 2/5)	+++ (+++, 3/5; ++, 2/5)
2K1C+bold (n=5)	+ (+, 4/5; ++, 1/5)	+ (-, 2/5; +, 3/5)

Table 3. Immunoreactivity score for OPN and ED-1 from the tissue-samples from rats of the 4 experimental groups. The semiquantitative morphometric analysis for OPN and ED-1 was performed on a scale of negative to three crosses (- a +++), defined according to the degree of immunoreactivity observed in the renal tissue sample ([-] = 0-10%, [+] = 10-40%, [++] = 40-70% and [+++] = 70-100%).

2.3. TGF-β, which has been linked to kidney damage, was reduced by Boldine treatment.

RAS is the prototype of a classic systemic endocrine network whose actions in the kidney and the adrenal gland regulate blood pressure, intravascular volume and electrolyte balance ([10]). Angiotensin II (AngII), a vasoactive peptide also secreted by the renal tissue, stimulates aldosterone secretion, cellular infiltration, proliferation and migration, thrombosis, reactive oxygen species (ROS) production and contributes to induce the inflammatory response characteristic of nephropathy [1]. It has been found that in the development of renal fibrosis, TGF-β is closely associated with the activation of RAS, due to AngII stimulation of TGF-β transcription and synthesis in the damaged kidney cells [13]. Therefore, AngII is a key mediator in chronic renal damage participating in the development of fibrosis through stimulation of TGF-β [11]. With these antecedents, we wanted to explore the effects of boldine on RAS mediators, such as angiotensin converting enzyme-1 (ACE-1) and TGF-β, in the 2K1C hypertensive rats.

We found that in 2K1C hypertensive rats, ACE-1 distribution was increased, however, the levels were lower in 2K1C+bold, as observed in control rats respectively (**Fig. 6A**). These results were complemented by semi-quantitative morphometric analysis for ACE-1 (**Fig. 6A**). Western blot for ACE1 (**Fig 6B**), showed similar results, where protein levels were significantly higher in 2K1C rats (0.9 ± 0.1 AU), and decreased in 2K1C rats treated with boldine (0.3 ± 0.1 AU), to control levels (in AU: Ctrl, 0.2 ± 0.0; Ctrl+bold, 0.3 ± 0.1).

A TGF-β ELISA assay was performed to determine plasma TGF-β amounts (**Fig 7**). We observed significantly higher amounts of TGF- β in 2K1C rats (5.2 ± 0.5 ng/ml), however, in 2K1C hypertensive rats treated with boldine, TGF-β amounts (2.5 ± 0.2 ng/ml), were closer to amounts observed in control rats (In ng/ml: Ctrl, 3.2 ± 0.3; Ctrl+bold 2.6 ± 0.3). These results suggest that boldine reduces renal damage by reducing ACE-1 and TGF-β amounts in 2K1C hypertensive rats.

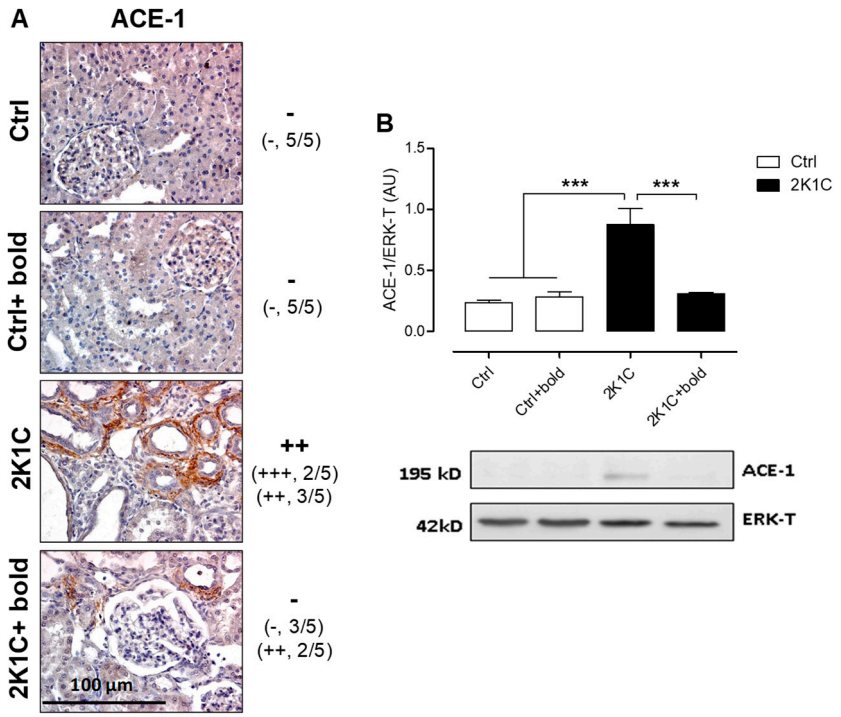

Figure 6. Boldine decreased ACE-1 expression in renal tissue of 2K1C hypertensive rats. Immunostaining for ACE-1 was performed in renal samples 6 weeks after 2K1C rats were made with hypertension (A). Representative pictures of at least 5 different kidneys are shown. Scale bar = 100 µm. Brown staining represents ACE-1 presence. Morphometric semiquantitative analysis for ACE-1 was performed on a scale of negative to three crosses (- a +++), defined according to the degree of immunoreactivity observed in the renal tissue sample ([-] = 0-10%, [+] = 10-40%, [++] = 40-70% and [+++] = 70-100%) (A). In B a Western blot for ACE-1 is shown, with pictures of ACE1 bands and T-ERK used as loading control. Bars represent the mean ± SE. The differences were evaluated by analysis of variance followed by Tuckey post-hoc test. ***p<0.001 vs 2K1C (n=5/ all groups).

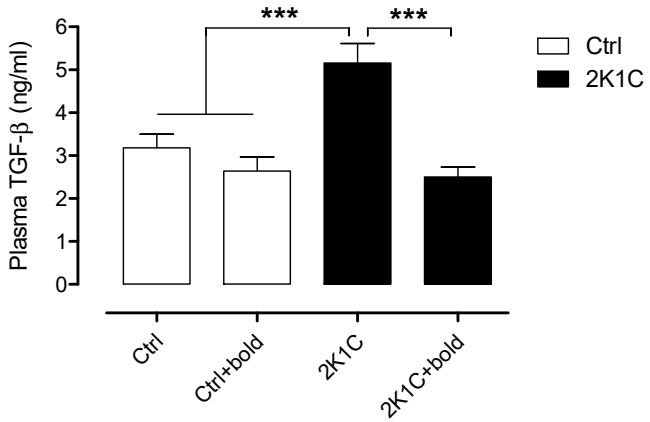

Figure 7. Boldine decreased TGF-β expression in renal tissue of 2K1C hypertensive rats. A TGF-β ELISA assay was performed to determine the TGF-β amounts secreted to plasma under different conditions, 6 weeks after rats became hypertensive. Bars represent the mean ± SE. The differences were evaluated by analysis of variance followed by Tuckey post-hoc test. ***p<0.001 vs 2K1C (n=5/ all groups).

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3. Discusion

The incidence of chronic kidney disease (CDK) is increasing worldwide and the current available therapies cannot reduce this phenomenon [25]. Current therapies focus on blood pressure control and optimizing blockade of the Renin-Angiotensin- Aldosterone System [26]. However, these treatments are partially effective in advanced and late stage of kidney disease.

The Goldblatt two-kidney, one-clip (2K1C) rat hypertension model exhibits two facets of this model, atrophy of one kidney and increased growth of the other [3]. These are of special relevance to CKD, wherein a certain subset of nephrons exhibit injury and atrophy, whereas other nephrons display structural enlargement and hyperfunction, since the latter may serve as a precursor to chronic injury, nephron atrophy, and, ultimately, progressive kidney disease [27].

The quantification of proteinuria is a fundamental element in the diagnosis and treatment of renal disease. It is also used to monitor the progress of kidney disease or response to treatment. However, it has important limitations that make this parameter is not used to assess kidney damage in advanced stages. This is complemented by the study of kidney damage by measuring creatinine, designed to measured kidney function [23].

To evaluate the degree of renal damage, which is characteristic of patients with CDK, renal function was assessed by measuring the ratio of U Prot / U Creat (**Fig. 1**), which strongly correlates with protein excretion in 24 hours [28,29]. Treating in 2K1C rats with boldine (50mg/kg/day) resulted in a significant decrease of this ratio. This allows us to suggest that boldine prevents loss of renal function, maintaining glomerular filtration and preventing kidney damage during CDK.

The pathophysiological basic mechanisms of renal disorders are associated with factors that predispose to oxidative and inflammatory imbalance [30,31]. The phenomena that occur in a CKD that can damage the tubule and the glomerulus, can lead to the generation of reactive oxygen species (ROS) such as TBARS [32–34].

Boldine has been demonstrated to be a potent antioxidant for its ability to scavenge HO· radicals in several experimental models [17]. With this background, we evaluated ROS levels in 2K1C hypertensive rats by measuring TBARS (Thiobarbituric acid reactive species) and observed a decrease in OS. The reduction in TBARS (**Fig. 2**) suggests that boldine is reducing OS, and by doing that it is reducing the amount of species that can react with Thiobarbituric acid in the plasma of 2K1C hypertensive rats. These results are consistent with those observed in other studies, where the antioxidants quercetin, resveratrol, bardoxolone methyl and gamma-aminobutyric acid were used and improved GFR in rats with renovascular hypertension [4,35–37].

Sustained high blood pressure is one of the most powerful determinants of renal hypertrophy [4]. In our study, the 2K1C rats treated with boldine showed a weak decrease in SBP as compared to the 2K1C group (**Fig. 3**). This weak reduction was far from normal values. However, this effect was accompanied by decreased lipid peroxidation and improved renal function. This fact suggests that boldine possesses antihypertrophic and renoprotector properties unrelated to its antihypertensive effect, contrary to what was observed in the work of Lau et al, where boldine treatment significantly lowered SBP in spontaneously hypertensive rats (SHR) [38].

Renovascular Hypertension is increasingly related to the pathogenesis of CKD [3]. CDK is associated with the development of renal interstitial fibrosis and is characterized by atrophy and/or tubular dilatation and increased interstitial matrix deposition [39]. In addition, in a wide range of renal

diseases, macrophage infiltration (ED-1) is closely related to the regulation of tubular expression of osteopontin (OPN). OPN is a potent chemoattractant that is expressed during kidney damage and acts as an adhesion molecule for monocytes and macrophages [12,24]. Also, it is believed that the development of interstitial fibrosis is the cause of the irreversibility of renal dysfunction [40,41]. Although the participation of different cells has been postulated, myofibroblasts (which express α-SMA and Col III) are the main cells that participate in renal fibrosis [42]. If this hypothesis is true, we could speculate that boldine, due to its antioxidant properties [17], could improve renal damage and inflammation. For this reason, we evaluated the effect of boldine on the expression of characteristics markers of kidney damage such as α-SMA and Col III, and inflammation markers such as ED-1 (macrophage infiltration) and OPN respectively. Our results (**Fig. 4** – **5**; **Table 2** – **3**) show that the increase in markers of kidney damage and inflammation were lower in 2K1C rats treated with boldine when compared to untreated 2K1C rats. Accordingly, we can postulate that boldine reduces fibrosis and inflammation in kidney damage in 2K1C hypertensive rats.

The activation of the renin-angiotensin system (RAS) is a crucial factor in the development and progression of organ damage in hypertension, diabetes and CDK [27,30,43]. Accumulating evidence over the past several years suggest that TGF-β plays a pivotal role in the progression of many immune and nonimmune-mediated renal diseases [13,41,44]. It is known that TGF-β is involved in the development of fibrosis [13], induces inflammatory response which is characterized by infiltration of macrophages (ED-1) and over-expression of OPN [12,40], and can induce the expression of the NADPH-Ox, one of the key enzymes in the ROS generation, contributing to OS [15]. In addition to TGF-β, AngII also contributes to the progression of renal disease through hemodynamic as well as nonhemodynamic mechanisms [3,13]. These two apparently diverse factors, which are involved in the deterioration of renal function and structure during CKD, may be joined together by the observation that AII induces TGF-β expression [3,13]. Ozawa et al. In 2007, observed that in a model of Sprague Dawley rats stimulated with AngII during 6 days, and then 3 or 6 days without the stimulus, respectively, they presented interstitial fibrosis, glomerular hypertrophy, infiltration of macrophage, increase in the amount of mRNA of the chemoattractant protein MCP-1 and also in mRNA quantity of transforming growth factor-β (TGF-β) [1]. It is also known that TGF-β stimulates the synthesis of extracellular matrix (ECM) and inhibits the action of proteases that degrade the matrix [10,11], influencing the development of fibrosis and inflammation in the kidney, causing deterioration of function and increasing renal damage [10,12,13]. In addition, TGF- β has been shown to have a close relationship with RAS in the development of hypertension and kidney damage in the 2K1C Goldblatt hypertensive model [3]. With this background, we wanted to study what happened to the mediators of RAS, such as ACE-1 (**Fig. 6**) and TGF-β (**Fig. 7**). According to the results the increase in ACE-1 and TGF-β were lower in 2K1C rats treated with boldine than that observed in untreated 2K1C rats.

In the present study, despite normalizing renal function and OS, reducing fibrosis, inflammation and RAS mediators; boldine treatment failed to completely normalize the elevated SBP in 2K1C rats. The exact mechanism(s) of this discrepancy is unclear; however, it is noteworthy to mention that several OS-independent factors, for example, alterations in sympathetic nervous system, can also contribute to the development of hypertension in 2K1C rats [38].

4. Conclusion

In conclusion, these results suggest that boldine prevents the increase in ACE-1, causing a decrease in all downstream mediators, including TGF-β and mediators downstream of this marker, suggesting that boldine reduces kidney damage affecting the RAS in the kidney of 2K1C rats (**Fig. 8**). We postulate that due to its properties, boldine has a high potential to be used as a nutraceutical compound in the treatment of renal problems.

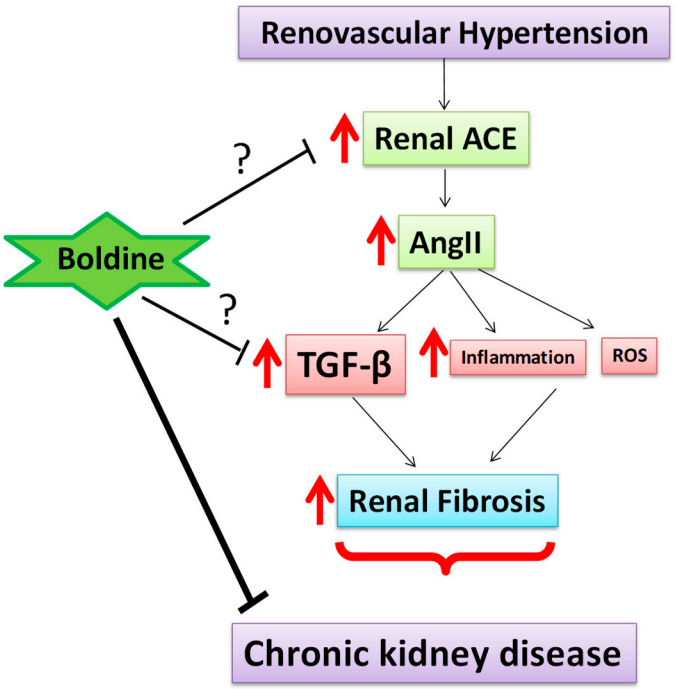

Figure 8. Mechanism in which the 2K1C hypertensive rat model leads to the development of renal damage and boldine would cause the reduction of kidney damage. RAS is a key mediator in chronic kidney damage as it participates in the development of fibrosis by stimulating the synthesis of TGF-β. In addition, high levels of Ang II maintained for long periods can induce the inflammatory response and generation of ROS, contributing to the onset of oxidative stress. Therefore, Ang II is a key mediator in chronic kidney disease as it participates in the development of fibrosis by stimulating the synthesis of TGF-β. Boldine could prevent the increase in ACE-1, causing a decrease in all downstream mediators, including TGF-β and mediators downstream of this marker; and could even prevent the same increase of TGF- β, suggesting that boldine reduces kidney damage affecting the RAS in the kidney of 2K1C rats.

5. Methods

5.1. Antibodies and chemicals.

The following primary antibodies were used: monoclonal antibodies against macrophages (clone ED-1) obtained from AbD Serotec (Kidlington, OX, UK); antibodies against α-smooth muscle actin (α-SMA) obtained from Sigma-Aldrich (St. Louis, MO, USA); antibodies against Collagen type III (Col III) and Angiotensin converting enzyme-1 (ACE-1) from Santa Cruz Biotechnology (Santa Cruz, CA,

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372 USA); and Osteopontin (OPN) antibody (MPIIIB101) was provided by the laboratory of Dr. Carlos
373 Vío (Developed by DSHB, Iowa City, IA, USA). Secondary antibodies and the corresponding PAP
374 complexes were purchased from ICN Pharmaceuticals-Cappel (Costa Mesa, CA, USA). Triton X-100,
375 3.3'-diaminobenzidine, λ – carrageenan, Tris-HCl, hydrogen peroxide, phosphate salts, and other
376 chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

377 *5.2. Animals and experimental protocol*

378 Adult male Sprague–Dawley rats (100–120 g) were maintained under a 12h light / 12h dark cycle,
379 with food (20.5% protein, 5% fiber, 4% fat; Champion, Chile) and water *ad libitum* at the University
380 animal care facilities. All procedures were in accordance with institutional and international
381 standards for the humane care and use of laboratory animals (Animal Welfare Assurance Publication
382 A5427-01, Office for Protection from Research Risks, Division of Animal Welfare, NIH [National
383 Institutes of Health], Bethesda, MD, U.S.A.), as described previously [19]. Rats were randomly
384 divided into four groups: control (Ctrl, n=5), control+boldine (Ctrl+bold, n=5), 2K1C Goldblatt (2K1C,
385 n=5) and 2K1C Goldblatt+boldine (2K1C+bold, n=5). We used the 2K1C hypertensive model
386 previously described by the group of Guan et al., 1992. Rats were anaesthetized with
387 ketamine/xylazine (10:1 mg/kg of body weight, intraperitoneal); then, a retroabdominal incision in
388 the left flank was performed, the left kidney was exposed. Afterwards, the left renal artery of 2K1C
389 Goldblatt-groups animals was occluded by a silver clip (0.2 mm internal diameter of the vessel), while
390 the right kidney was not disturbed. Control-group rats were sham-operated by an incision made in
391 the flank [9]. After recovery, rats were maintained with food and water *ad libitum.* Before starting
392 treatment with boldine, Hypertension was confirmed by measuring systolic blood pressure (SBP)
393 weekly with a non-invasive plethysmography (NIBP machine, IITC Inc., Woodland Hills, CA, USA)
394 until SBP was ≥ 180 mmHg. Once 2K1C animals became hypertensive (pressure ≥ 180 mmHg), a
395 group of them (n=5) was selected at random to receive the oral treatment with boldine. Boldine
396 (50mg/Kg/day) or its vehicle (water), were given by *gavage* over six weeks. Administration was
397 stopped one day before the end of the experiments, to study boldine long-term effects without the
398 involvement of its acute administration effects. The last day of the experimental period animals were
399 placed on metabolic cages for 16 hours to collect urine in a container built into the cage. The next
400 morning, rats were anesthetized with ketamine/xylazine (10:1 mg/kg of body weight, ip). Blood
401 samples were obtained from the abdominal aorta, centrifuged and plasma was frozen for further
402 analysis. Urine was measured and aliquoted. Kidneys were processed for immunohistochemistry
403 and Western blotting. Animals were sacrificed by exsanguination under anesthesia.

404 *5.3. Blood pressure measurements*

405 SBP was determined once a week, in the morning, in conscious pre-warmed restrained rats by a non-
406 invasive plethysmography (NIBP machine, IITC Inc., Woodland Hills, CA, USA). At least four
407 determinations were made in every session and the mean of the four values was taken as the SBP
408 value.
409
410

5.4. Renal Function Measurements

Plasma and urinary creatinine levels were measured with the Jaffé alkaline picrate assay (VALTEK Diagnostica, Chile). Urinary protein concentration was determined by Bradford's method (Bio-Rad protein assay, Kidlington, OX, UK) [20]. Creatinine clearance over 24 hours was calculated according to the standard formula $C = (U \times \tilde{V})/P$, where C is creatinine clearance, U is creatinine urinary concentration, $\tilde{V}$ is the urine flow rate per minute, and P is creatinine plasmatic concentration [19].

5.5. Thiobarbituric Acid Reactive Substances (TBARS) Measurement

The amount of TBARS was estimated using the method described by Ramanathan and collaborators with slight modifications. Culture medium was mixed with SDS (8% *w*/*v*), thiobarbituric acid (0.8% TBA *w*/*v*), and acetic acid (20% *v*/*v*), followed by heating for 60 min at 90 °C. Precipitated material was removed by centrifugation, and the absorbance of the supernatant was evaluated at 532 nm. The amount of TBARS was calculated using a calibration curve obtained with malondialdehyde (MDA) as standard. MDA was obtained from Merck (Darmstadt, Germany).

5.6. Histological damage assessment

Tissue damage was evaluated by a semiquantitative morphometric analysis that was performed according to the degree of immunoreactivity observed in the kidney tissue sample for each of the antibodies used (OPN, ED-1, α-SMA, Col III). This was done from a score on a scale from negative to three (- to +++), defined according to the degree of immunoreactivity observed in the kidney tissue sample ([-] = 0-10%, [+] = 10-40%, [++] = 40-70% y [+++] =70-100% of the area observed). To assess the degree of fibrosis, staining of collagen fibrils by Sirius red F3BA was carried out as previously described [21]

5.7. Tissue Processing and Immunohistochemical Analysis.

Renal slices (3 mm-thick), including the cortex, medulla, and papilla, from different groups, were fixed for 24 h by immersion in Bouin's solution at room temperature. The tissue was then dehydrated, embedded in Paraplast plus (Monoject Scientific, St. Louis, MO, USA), serially sectioned at 5-mm thickness with a rotatory microtome, mounted on glass slides, and stored until immunostaining. Immunolocalization studies were performed using an indirect immunoperoxidase technique [22]. Tissue sections were quickly dewaxed, rehydrated, rinsed in Immune Solution (IS) (0.11M Na_2HPO_4, KH_2PO_4 0.04 M, 1M NaCl, 0.32M Tris-HCl and 0.03M sodium azide) pH 7.6, and incubated with the primary antibody (1:100) overnight at 22°C. Afterward, sections were washed three times with IS for 5 min each, followed by 30 min incubation at 22°C with the corresponding secondary antibody (1:20) and with the peroxidase-antiperoxidase (PAP) complex (1:150). Immunoreactive sites were revealed using 0.1% (wt/vol) 3.3'-diaminobenzidine and 0.03% (vol/vol) hydrogen peroxide solution. The antisera and PAP complex was diluted in IS containing 0.25% (vol/vol) Triton X-100 and 0.7% (wt/vol) λ – carrageenan. The sections were rinsed with IS buffer between incubations, counterstained with hematoxylin, dehydrated and cleared with xylene, and coverslipped. Sections were counterstained

with hematoxylin and were observed and photographed using a Nikon Eclipse 600 microscope with a Nikon DXM1200 digital photographic system (Nikon Corporation. Tokyo, Japan).

5.8. Western blot.

Renal tissues were homogenized with an Ultra-Turrax homogenizer in buffer containing Tris-HCl 100 mM pH 7.4, EDTA 5 mM, SDS 1%, PMSF 1 μM and the protease inhibitor cocktail (Pierce, Rockford, IL, USA). Protein concentrations were determined by using a detergent-compatible Bio-Rad protein assay kit (Bio-Rad, Richmond, CA, USA). 50 μg of renal proteins were mixed with an equal volume of sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis sample buffer (100 mM Tris-HCl, pH 6.8, 200 mM dithiothreitol, 4% SDS, 0.2% bromophenol blue, 20% glycerol) and were boiled for 3 min. The proteins in renal samples were separated on 10% SDS-polyacrylamide gels and transferred to either nitrocellulose or polyvinylidene difluoride membranes. Nonspecific sites on the membranes were blocked by incubation in blocking solution (5% nonfat dry milk in Tris-buffered saline-Tween) for 30 min at room temperature. Membranes were blotted overnight at 4°C with monoclonal anti-ACE1 (1:500), then were stripped and reblotted with a polyclonal anti-ERK-1/2 (1:2000, Santa Cruz Biotechnology) antibody used as loading control. Immunoreactive bands were visualized by a chemiluminescent method (Western Lightning, Thermo Scientific, Pierce, Rockford, IL, USA) and Kodak X-LS film. The bands detected were digitized and subjected to densitometry analysis using the software Image J (Version 1.50i, NIH, Washington, DC, USA.

5.9. Enzyme-Linked Immunosorbent Assay

TGF- β ELISA assays was performed to determine the amount of TGF- β levels secreted in plasma under different conditions, following the manufacturer's protocol (TGF- β EIA kit, Enzo Life Science, Farmingdale, NY, USA). Results were presented in ng/ml.

5.10. Statistical Analysis.

Results were evaluated by ANOVA, and the Tuckey's post-test was used to evaluate the difference between two groups. Results are expressed as the average of values from each independent experiment ± SE and considered significantly different if $p < 0.05$. Analyses were performed with the GraphPad Prism 5 software for Windows (1992–2007, GraphPad Software, March 12, 2007, La Jolla, CA, USA).

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Autor Contributions

GIG and VV Designed research; GIG conducted research; GIG and VV analyzed data; GIG and VV wrote the paper. All authors read and approved the final manuscript.

Abbreviations

Renin-Angiotensin System	RAS
Chronic kidney disease	CDK
Glomerular filtration rate	GFR
Goldblatt two-kidney one clip	2K1C
Renovascular hypertension	RVH
transforming growth factor-β	TGF-β
Extracellular matrix	ECM
α-smooth muscle actin	α-SMA
Fibronectin	FN
Collagen type III	Col III
infiltration of macrophages	ED-1
Osteopontin	OPN
Reactive oxygen species	ROS
Oxidative stress	OS
Angiotensin converter enzyme	ACE
Systolic blood pressure	SBP
Enzyme-linked immunosorbent Assay	ELISA

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