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BOLDINE TREATMENT PREVENTS KIDNEY DAMAGE IN RATS WITH 5/6 NEPHRECTOMY[1,2]

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Abstract

Chronic renal failure(CRF) results in the progressive loss of kidney function toward a terminal stage. It is manifested by the advancing decrease in glomerular filtration, due to morphological alterations such as tubular necrosis and glomerular sclerosis. Moreover, oxidative stress increases in patients with CRF. Therefore, the use of natural products, such as the antioxidant boldine, has been observed to be a promising focus of research. We propose that boldine treatment prevents kidney damage in 5/6 nephrectomized(5/6NX) rats.

Rats(n=5/group) were treated with boldine(50mg/kg/day, *gavage*) for 28 days after 5/6NX; kidney function was evaluated measuring urinary protein/creatinine ratio(Uprot/Ucrea); and oxidative stress measuring thiobarbituric acid reactive substances(TBARS). ED-1(marker of inflammation), Col III(marker of kidney damage) and Vimentin(recovery marker) were evaluated by Western blot and immunohistochemistry.

Uprot/Ucrea ratio(4.53±1.29AU in 5/6NX+Boldine; 13.30±4.14AU in 5/6NX) and TBARS(39.52±3.73nmol/g in 5/6NX+Boldine; 62.42±1.05nmol/g in 5/6NX) were lower in 5/6NX+boldine rats than in 5/6NX rats $p<0.05$. In 5/6NX +boldine rats, levels of ED-1 and Col III were lower compared to 5/6NX rats. Vimentin(mesenchymal marker) was increased in 5/6NX+boldine rats, compared with 5/6NX rats. These results suggest that boldine protects the kidney in rats with 5/6NX and that boldine could potentially be used as a nutraceutic.

Introduction

Chronic renal failure (CRF) is a condition that results in the progressive loss of kidney function until reaching a terminal stage *(1)*. It is manifested by an advancing decrease in glomerular filtration rate (GFR), resulting from a rise in damaged nephrons, in addition to tubular homeostasis and, finally, failure of the organ's hormonal functions *(1-2)*.

Unlike acute renal failure (ARF), where an abrupt but reversible decline in renal function is observed *(3)*, CRF involves the progressive recruitment of nephrons, which could have varying degrees of structural and functional damage *(4)*. CRF has different etiologies. Independent of the cause, however, the morphological characteristics, such as tubular necrosis and glomerular sclerosis, are similar *(5)*. The prevalence of this condition has increased worldwide and it has been estimated that over 10% of adults in developed countries suffer from some degree of CRF. For this reason, the search for a treatment for this disease is a major research topic in nephrology *(6)*.

CRF is characterized by atrophy and/or tubular dilation, interstitial leukocyte infiltration and increased deposition of the extracellular matrix, eventually developing into renal interstitial fibrosis *(7)*. Macrophage infiltration occurs at several sites of the inflamed kidney, and their presence in the interstitium and the glomerulus contributes to tissue

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damage and the progression of renal disease *(8)*. It has been postulated that the development of interstitial fibrosis causes irreversible renal dysfunction *(9)*. During kidney repair after damage, a sequence of events similar to the one observed during kidney development in embryonic stages can be observed. This is characterized by the proliferation of undifferentiated cells and the subsequent differentiation of daughter cells into specific tubular cells, thus opening the possibility that renal regeneration may recapitulate the genetic program activated during renal organogenesis *(10, 11)*.

When studying the basic pathophysiologic mechanisms of renal impairment, all factors that create a predisposition to oxidative stress are present *(12)*. Oxidative stress is defined as tissue damage caused by an imbalance between excessive generation of oxidant compounds and insufficient antioxidant mechanisms. 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. Major sources of these antioxidant molecules are plants. Therefore, the potential to prevent or delay the adverse effects associated with excessive production of reactive oxygen species (ROS) by using previously unexplored plant products *(13)* has proven to be an attractive target for investigation.

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% *(14)*. A large set of pharmacological activity has been attributed to boldine, such as cell protection, as well as anti-inflammatory and antipyretic effects. In addition, Boldine has the ability to 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 *(13)*.

Therapies currently available for human CKD are only partially effective in preventing end-stage renal disease (ESRD) or the decline in GFR. Most studies have demonstrated a reduction in the risk of renal end points by only 15–30% and a delay in the progression to ESRD of only a few months *(4)*. The current available strategies focus primarily on controlling blood pressure and maximizing the blockade of the Renin-Angiotensin-Aldosterone system. However, the beneficial results in reducing the progression of CRF are only partial, which generates the need to develop new treatments *(4)*. Consequently, based on the abovementioned evidence, this study assessed the effect of boldine on the progression of chronic renal failure in rats with 5/6 nephrectomy (5/6NX).

Materials and methods

Animals

Adult male Sprague–Dawley rats (220–250 g) were maintained under a 12 h light/12 h dark cycle, with food (20.5% protein, 5% fiber, 4% fat; Champion, Chile) and water *ad libitum* at the University animal care facilities. All procedures were in accordance with institutional and international standards for the humane care and use of laboratory animals [Animal Welfare Assurance Publication A5427-01, Office for Protection from Research Risks, Division of Animal Welfare, NIH (National Institutes of Health), Bethesda, MD, U.S.A.], as described previously *(15)*.

CRF induced by 5/6 Nefrectomy and boldine treatment.

A model that mimics the structural and functional damage of CRF was used *(Salas et al., 2011)*. Rats were anaesthetized with ketamine/xylazine (10:1 mg/kg of body weight, intraperitoneal); then, a retroabdominal incision in the left flank was performed and the kidney mass was reduced by clamping two branches of the renal artery. After 1 week, rats were subjected to contralateral nephrectomy. This moment was considered to be the initiation of kidney damage, which was prolonged for 4 weeks. Animals were randomized into four groups: sham rats without boldine (Ctrl; $n = 3$); sham rats with boldine (Ctrl+bold; $n = 3$); 5/6NX rats without boldine (5/6NX; $n = 5$); and 5/6NX rats with boldine (5/6NX +bold; $n = 5$). Boldine (Härting, Chile) was administered by gavage, in doses of 50 mg/kg for 28 days after completion of the 5/6NX. On the last day of treatment (day 28) before sacrifice, rats were housed in

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metabolic cages (Nalgene Nunc. Int, Rochester, NY) for 16 hours in order to collect urine in a container built into the cage. The next morning, rats were anesthetized with ketamine/xylazine (10:1 mg/kg of body weight, ip). Blood samples were obtained from the abdominal aorta and centrifuged, and the plasma was frozen for further analysis. Urine was measured and aliquoted. Kidneys were processed for immunohistochemistry and Western blotting. Animals were sacrificed by exsanguination under anesthesia.

Biochemical 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) *(16)*. 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 *(10)*. Oxidative stress was assessed in plasma through a colorimetric method, by determining the peroxidation of lipids that react with thiobarbituric acid (Merck; Darmstadt, GE) (TBARS). These levels were determined spectrophotometrically at 532 nm after the sample was boiled and condensed with thiobarbituric acid. The results were expressed as micromoles per liter *(17)*.

Tissue Processing and Immunohistochemical Analysis.

Renal slices (3-mm thick), including the cortex, medulla, and papilla, from different groups, were fixed by immersion in Bouin's solution for 24 h at room temperature. The tissue was then dehydrated, embedded in Paraplast plus (Monoject Scientific, St. Louis, MO), serially sectioned at 5-mm thickness with a rotatory microtome, mounted on glass slides, and stored until immunostaining *(18)*. To assess the degree of fibrosis, staining of collagen fibrils by Sirius red F3BA was carried out as previously described *(19)*.

Immunolocalization studies were performed using an indirect immunoperoxidase technique *(18)*.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).

Antibodies and chemicals.

The following primary antibodies were used: monoclonal antibodies against macrophages (clone ED-1), obtained from AbD Serotec (Kidlington, OX); antibodies against Collagen type III (Col III; SC1745), from Santa Cruz Biotechnology (Santa Cruz, MI); and antibodies against Vimentin (catalog #500513), from BD Pharmingen (San Diego, CA). Secondary antibodies and the corresponding PAP complexes were purchased from ICN Pharmaceuticals-Cappel (Aurora, OH). Triton X-100, 3,3'-diaminobenzidine, carrageenan, Tris-HCl, hydrogen peroxide, phosphate salts, and other chemicals were purchased from Sigma.

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). Protein concentrations were determined by using a detergent-compatible Bio-Rad protein assay kit (Bio-Rad, Richmond, CA). Fifty micrograms 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%

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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-ED-1 (1:1000), anti-Col III (1:1000) and anti-Vimentin (1:500) antibodies, and were then 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, Perkin-Elmer) and Kodak X-LS film. Densitometric analysis was performed using Scion Image software, as previously described *(18, 20)*.

Statistical Analysis.
The results were evaluated by ANOVA and the Bonferroni correction method was used to evaluate the difference between the two groups. Results are expressed as the average of the values of each independent experiment ± SEM and considered significantly different if $p<0.05$.

Results

Renal Function in rats. Effects of boldine.
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 *(21)*. The collection of urine for 24 hours (or around this time) is considered necessary to measure proteinuria. Nevertheless, this parameter has important limitations, that makes its single measurement not useful to assess renal damage in advanced stages. Therefore, we complemented the study of kidney damage by measuring creatinine *(21)*.
The functional damage induced by 5/6NX and the effect of boldine were evaluated by urinary protein/creatinine levels. The U Prot/U Crea ratio (Fig. 1) increased significantly in 5/6NX rats (15.68 ± 4.14 AU, * $P<0.05$), compared to control (0.37 ± 0.04 AU) and control+bold (0.39 ± 0.08 AU), but these levels were significantly lower in 5/6NX rats that were treated with boldine (5/6NX+bold; 4.53 ± 1.29 AU). This result allows us to suggest that boldine prevents the deterioration in renal function that occurs in rats with 5/6NX.

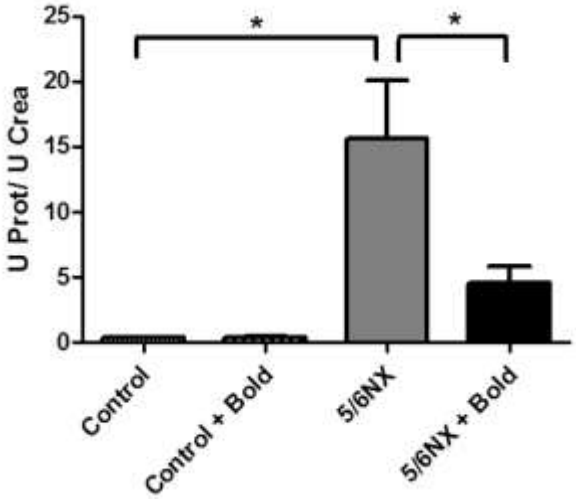

Figure 1. The U Prot/ U Crea ratio tends to normalize in animals with 5/6NX treated with boldine.
*Boldine was administered by gavage daily dose of 50 mg / kg for 28 days after 5/6NX. Protein and creatinine were measured in urine samples to assess renal function. Bars represent the mean ± SE. The differences were evaluated by analysis of variance followed by Bonferroni post test. *P<0.05 (n=5/group 5/6NX and 5/6NX+bold, n=3/group control and control + bold).*

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The data used to determine the U Prot/U Crea ratio is shown in Table 1. It can be observed that the major effect of boldine is on creatinine clearance, having much less effect on proteinuria.

Groups	Weight (gr)	Proteinuria (mg/day)	Urine Creatinine (mg/day)	Plasma Creatinine (mg/ml)	Creatinina Clearance (ml/min/100gr)
Control (n=3)	376±10,2	13,86±3,0	38,56±9,1	0,80±0,2	0,89±0,08
Control + bol (n=3)	399±7,4	11,96±7,1	29,93±13,0	0,71±0,3	0,74±0,02
5/6NX (n=5)	307±25,7†	111,15±24,2*†	12,62±7,9*†	2,24±0,8*†	0,18±0,05*†
5/6NX + bol (n=5)	303±11,5	93,91±37,1	28,35±15,3‡	1,24±0,1‡	0,50±0,16‡

Table 1. Values for proteinuria, creatininuria, creatininemia and creatinine clearance in the 4 groups.
*Boldine was administered by gavage at doses of 50mg/kg during 28 days after 5/6NX. Samples of urine and plasma were obtained on the 28th day. Protein and creatinine were measured to assess renal function. Data are the mean ± SEM. The differences were evaluated by analysis of variance followed by Bonferroni post-hoc test. *P<0.05 vs. control, †P<0.05 vs. control+bold, ‡P<0.05 vs. 5/6NX (n=5/group 5/6NX and 5/6NX+bold, n=3/group control and control+bold).*

Boldine reduces oxidative stress and inflammation in rats with 5/6NX.
It has been suggested that oxidative stress (OS) is involved in the process of CRF *(22)*. In addition OS correlates inversely with the glomerular filtration rate (GRF) *(12)*. Moreover inflammation, which is also present in CRF, further increases the generation of oxidants *(23)*. Therefore we evaluated the levels of reactive oxygen species (ROS) by measuring TBARS and inflammatory markers, such as ED-1, in order to determine whether boldine decreases OS and inflammation.
The levels of malondialdehyde (MDA) (Fig. 2) in kidneys of rats with 5/6NX (12.48 ± 0.21 nmol/L, * P<0.05) significantly increased when compared with the control (8.79 ± 0.75 nmol/L) and ctrl+bold rats (8.32 ± 0.19 nmol/L), but these levels were significantly lower in kidneys of 5/6NX rats treated with boldine (7.9 ± 0.75nmol/L, *P <0.05).

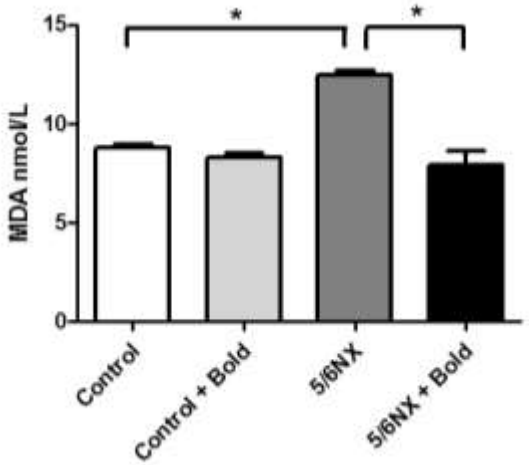

Figure 2. Boldine treatment normalizes levels of MDA in rats with 5/6NX.
*Plasma from each Rat of the four groups was used to measure reactive species of thiobarbituric acid (TBARS) as a measurement of ROS. Bars represent the mean ± SE. The differences were evaluated by analysis of variance followed by Bonferroni test. *P<0.05 (n=5/group 5/6NX and 5/6NX+bold, n=3/group control and control+bold).*

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The assessment of macrophages (ED-1) *(24)* showed an increase in the levels of this marker in 5/6NX rats. Nonetheless, fewer marker levels were observed in 5/6NX rat kidneys that were treated with boldine, being similar to that seen in control rats (Fig. 3A). Results obtained by immunohistochemistry were complemented with Western blot for ED-1 (Fig. 3B), where protein levels were significantly higher in 5/6NX rats (0.38 ± 0.02 AU, *P<0.05). However, it decreased in 5/6NX+Bold rats (0.20 ± 0.02 AU), similar to that observed in control rats (0.05 ± 0.02 AU) and the ctrl+bold group (0.06 ± 0.03 AU). Therefore, boldine prevented increases in OS and inflammation in kidneys from 5/6NX rats.

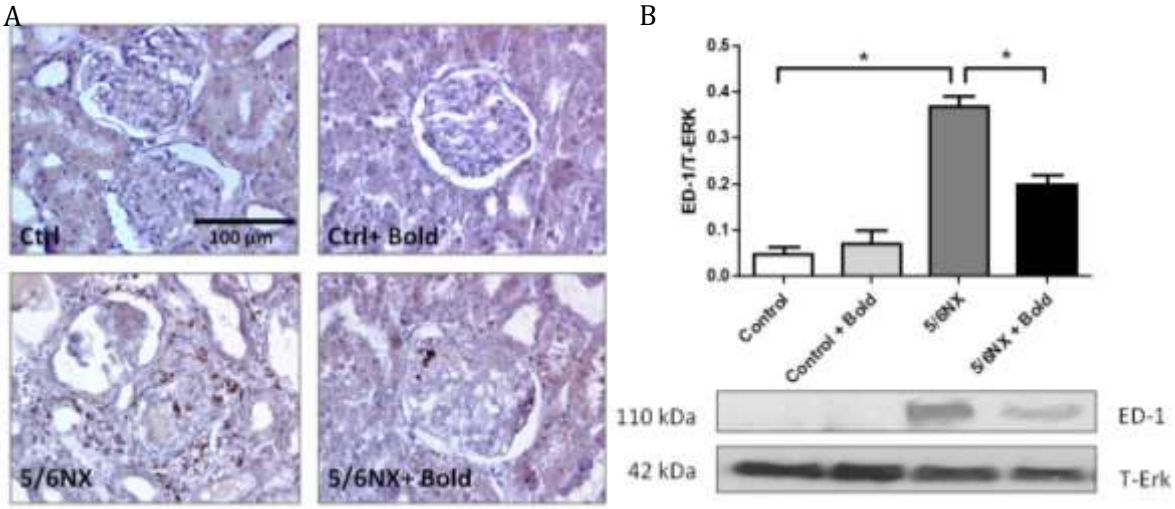

Figure 3. Boldine decreases levels of ED-1 in rats with 5/6NX.

*(**A**) ED-1 immunohistochemistry was performed in renal samples 28 days after 5/6NX. Representative pictures of at least 3 different kidneys are shown. Scale bar = 100µm. (**B**) Western blot for ED1. Bars represent the mean±SEM. The differences were evaluated by analysis of variance followed by Bonferroni test. *P<0.05 (n=5/group 5/6NX and 5/6NX+bold, n=3/group control and control+bold). Representative pictures of ED-1 positive bands and its loading control are shown under the graph.*

Boldine partially prevents damage to the renal tissue in 5/6NX rats.

Previously, we showed an improvement in renal function, OS and inflammation in 5/6NX+bold rats when compared to 5/6NX rats. We wanted to evaluate the effect of boldine on morphological markers of renal tissue damage. To evaluate kidney damage, we analyzed collagen 28 days after the 5/6NX *(25)*. First, the distribution of all collagens was evaluated with Sirius Red staining. We observed an increase in the levels of this marker in 5/6NX rats, however, a reduced distribution of collagen was seen in 5/6NX+bold rats, being similar to that observed in control rats (Fig.4A).

These results were complemented by Western blot, using an antibody specific for collagen III (Fig 4B), being its protein levels significantly higher in 5/6NX rats (0.64 ± 0.03 AU, *P<0.05), but lower in 5/6NX+bold rats (0.32 ± 0.03 AU), similar to what was observed in control (0.25 ± 0.03 AU) and ctrl+bold rats (0.33 ± 0.03 AU). With these results, we suggest that boldine is partially preventing renal injury by preventing renal fibrosis in rats with 5/6 nephrectomy.

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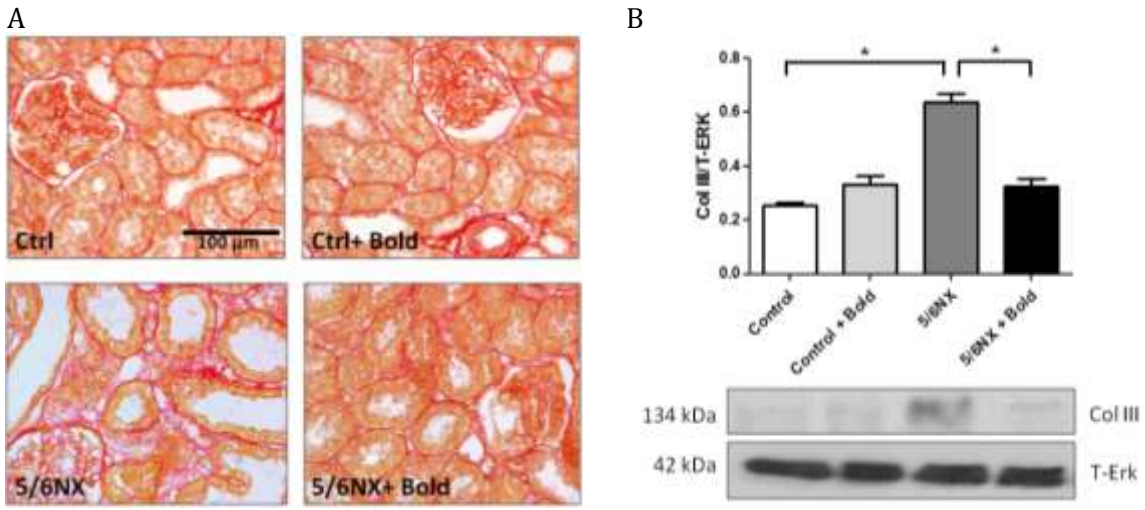

Figure 4. Boldine reduces renal tissue damage in 5/6NX rats.

*(**A**) Sirius Red staining was performed in renal samples 28 days after 5/6NX. Representative pictures of at least 3 different kidneys are shown. Scale bar = 100µm. (**B**) Western blot for Collagen type III. Bars represent the mean±SEM. The differences were evaluated by analysis of variance followed by Bonferroni test. *P<0.05 (n=5/group 5/6NX and 5/6NX+bold, n=3/group control and control+bold). Under the graph are pictures representing the score, with its load control. Representative pictures of Collagen III positive bands and its loading control are shown under the graph.*

Boldine treatment stimulates the regeneration of the kidney in rats with 5/6NX.

During kidney repair after damage, a sequence of events similar to the one observed during kidney development in embryonic stages can be observed *(Villanueva et al., 2006a)*. One of the most studied markers of this process is the mesenchymal factor Vimentin *(26)*. Based on the abovementioned evidence, we wanted to assess whether boldine could affect the expression of this marker in kidneys of rats with 5/6 nephrectomy.

An increase in immunostaining for Vimentin was observed in 5/6NX rats, relative to what was observed in control rats (Fig.5A). This marker was found in both internal and external renal medulla. Notably, in kidneys from 5/6NX+bold rats, Vimentin levels were still higher than the ones observed in 5/6NX rats. These results were complemented with Western blot (Fig.5B), where it was observed that Vimentin protein levels were significantly higher in 5/6NX rats (0.45 ± 0.02 AU, *P<0.05), and even higher in 5/6NX+bold rats (0.65 ± 0.04 AU), relative to that observed in control (0.23 ± 0.07 AU) and control+bold (0.18 ± 0.07 AU) rats.We hypothesize that boldine, by preventing renal damage, can stimulate kidney regeneration.

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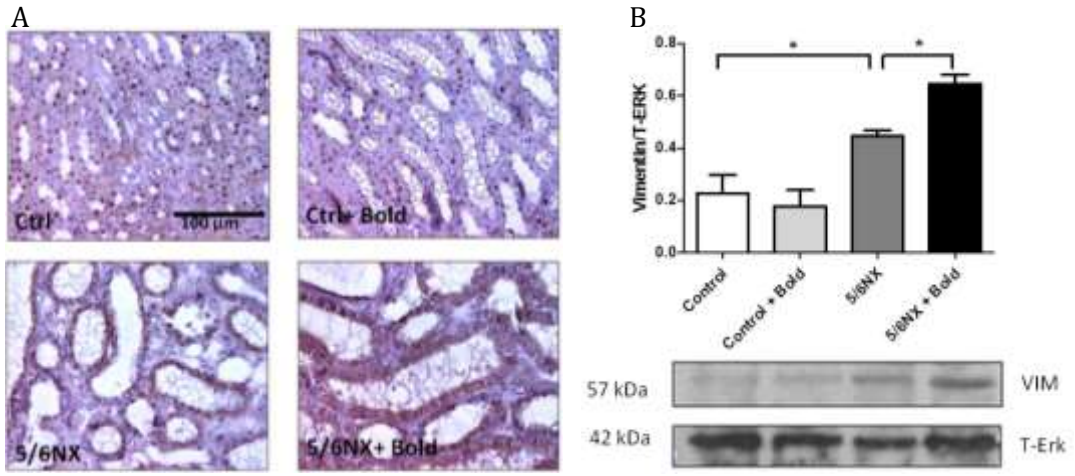

Figure 5. Boldine increases the expression of Vimentin, in kidneys from rats with 5/6NX.
*(A) Vimentin Immunohistochemistry was performed in renal samples 28 days after 5/6NX. Representative pictures of at least 3 different kidneys are shown. Scale bar = 100µm. (**B**) Western blot for Vimentin, bars represent the mean ± SEM. The differences were evaluated by analysis of variance followed by Bonferroni test. *P<0.05 (n=5/group 5/6NX and 5/6NX+bold, n=3/group control and control+bold). Representative pictures of Vimentin positive bands and its loading control are shown under the graph.*

Discussion

The incidence of CRF is increasing worldwide and the current available therapies cannot reduce this phenomenon. Current therapies focus on blood pressure control and optimizing blockade of the Renin-Angiotensin-Aldosterone System *(4)*. However, these treatments are partially effective in advanced and late stages of kidney disease. Furthermore, the development of new therapies must be directed specifically to reverse or prevent the pathological features of CRF, such as inflammation, fibrosis and tubular atrophy.

To evaluate the degree of renal damage, which is characteristic of patients with CRF, renal function was assessed by measuring the ratio of urinary protein/creatinine (Fig. 1), which strongly correlates with protein excretion in 24 hours *(27)*. Treating 5/6NX rats with boldine (50mg/kg/day) resulted in a significant prevention of the increase in this ratio. This allows us to suggest that boldine prevents loss of renal function, maintaining glomerular filtration and preventing kidney damage during CRF. These results are consistent with those observed in other studies, where the antioxidants, methyl-Bardoxolone and NZ-419 (5-hydroxy-1-methylimidazoline-2,4-dione) were used and improvements in GFR in rats and patients with CRF were observed *(28, 29)*. This confirms the participation of reactive oxygen species in this process, as has been suggested by other authors *(12)*.

Boldine has been demonstrated to be a potent antioxidant for its ability to scavenge $HO^{\cdot}$ radicals in several experimental models *(13)*. With this background, we evaluated ROS levels in rats with 5/6NX by measuring TBARS (Thiobarbituric acid reactive species) and observed a decrease in OS. The reduction in TBARS (Fig. 2) suggests that boldine is reducing $HO^{\cdot}$, and by doing that it is reducing the amount of species that can react with Thiobarbituric acid in the plasma of rats with 5/6NX.

Inflammation, which is also present in CRF, further increases the process of oxidant generation (23). There is evidence that in a wide range of renal diseases macrophage infiltration is closely related to the up-regulation of the expression of tubular osteopontin, which is a potent chemo-attractant that is expressed in kidney damage and acts as an adhesion molecule for monocytes and macrophages *(30)*. We studied macrophage infiltration by using the ED-1 marker in rats with 5/6NX. Our results (Fig. 3) show that the increase in ED-1 was lower in 5/6NX rats treated with boldine than that observed in untreated 5/6NX rats. Accordingly, one can postulate that boldine would reduce the

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infiltration of macrophages and could be affecting the expression of osteopontin, the major chemo-attractant that is expressed during kidney damage *(30)*.

CRF is associated with the development of renal interstitial fibrosis. This is characterized by atrophy and/or tubular dilatation, macrophage infiltration and increased interstitial matrix deposition *(7)*. Moreover, kidney development in embryonic stages is characterized by the proliferation of undifferentiated cells and subsequent differentiation of daughter cells into specific renal cell. During kidney damage, a similar sequence of events to the ones that occur during fetal development can be observed, suggesting that renal regeneration recapitulates the genetic program that runs during kidney organogenesis *(11, 15)*. If this hypothesis is true, we could speculate that boldine, due to its antioxidant properties *(13)*, could prevent renal damage by inducing the regeneration of this damaged organ. For this reason, we evaluated the effect of boldine on the expression of a characteristic marker of kidney damage such as collagen III (Col III) *(25)* and the recovery marker Vimentin *(26)*.

Renal interstitial fibrosis is a common injury in chronic kidney disease. It is thought that the development of interstitial fibrosis causes the irreversibility of renal dysfunction. Different cells and cytokines have been postulated to be involved in fibrosis, but myofibroblasts are the main effector cells and type III collagen is one of the main markers of interstitial fibrosis (31). It was long thought that myofibroblasts originated from local fibroblasts, but lately it has been postulated that they may also arise from epithelial cells through epithelial to mesenchymal transition. This suggests that there might be many mechanisms involved, being the increased expression of TGF-β1 an important one *(32)*. It may therefore be pivotal to understand the activation mechanisms of myofibroblasts in matrix production, so as to design a therapeutic strategy for the effective treatment of renal interstitial fibrosis *(7)*. As a way to approach this problem, we evaluated the effect of boldine in the levels of type III collagen in 5/6NX rats and assessed whether it could prevent the development of renal interstitial fibrosis. As shown in Fig. 4, Boldine prevented the development of fibrosis in kidney damage, which could be due to the inhibition of the expression of TGF-β1 and the interference with the fibrotic process during kidney damage.

On the other hand, Vimentin, the mesenchymal cell marker, which is not found in renal tubules of healthy adults, is expressed during regeneration and tubular proliferation *(26)*. This description is consistent with our results (Fig. 5) that show elevated Vimentin protein levels in the kidneys of 5/6NX rats. Interestingly, Vimentin protein levels were even higher in 5/6NX rats treated with boldine. Thus, we could speculate that by reducing the tissue damage, cells can produce a higher level of Vimentin, which could help the cells to acquire a similar state of differentiation than the one observed during the early stages of development and promote a better regeneration after renal damage induced by 5/6NX. This result is similar to what was observed in the work of Villanueva et al., using basic fibroblast growth factor (bFGF) to induce regeneration in kidney tissue during acute kidney injury *(15)*.

Conclusion

In conclusion, these results suggest that Boldine partially protects the kidney in rats with CRF induced by 5/6NX. We postulate that due to its properties, boldine has a high potential to be used as a nutraceutical compound.

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V.V. designed research; G.I.G. conducted research; V.V and G.I.G analyzed data; V.V and G.I.G wrote the paper. V.V. had primary responsibility for final content. All authors read and approved the final manuscript

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