

Halofuginone Reduces the Occurrence of Renal Fibrosis in 5/6 Nephrectomized Rats

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Abstract

Background: Halofuginone is a novel antifibrotic agent that can reverse the fibrotic process by specific inhibition of collagen type I synthesis.

Objectives: To evaluate the effect of Halo on the development of glomerulosclerosis and interstitial fibrosis in the 5/6 nephrectomy rat model

Methods: Male Wistar rats were assigned to undergo 5/6 NX or sham operation, and then divided into three groups: 5/6 NX rats (NX-Halo and NX-Control) and sham. Systolic blood pressure, proteinuria and body weight were determined every 2 weeks. At sacrifice (10 weeks) creatinine clearance was evaluated and remnant kidneys removed for histologic examination, sirius red staining and *in situ* hybridization

Results: Systolic blood pressure increased progressively in both 5/6 NX groups. Halo slowed the increase in proteinuria in 5/6 NX rats. As expected, creatinine clearance was lower in 5/6 NX groups when compared to sham rats. Creatinine clearance was significantly higher in the NX-Halo group at the end of the study period. Histologic examination by light microscopy showed significantly less severe interstitial fibrosis and glomerulosclerosis in Halo-treated rats. The increase in collagen $\alpha 1$ (I) gene expression and collagen staining after nephrectomy was almost completely abolished by Halo.

Conclusions: Halofuginone reduced proteinuria as well as the severity of interstitial fibrosis and glomerulosclerosis in 5/6 NX rats. The renal beneficial effect of Halo was also demonstrated by the blunted decrease in creatinine clearance observed in the treated animals

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Most forms of chronic renal disease are characterized by the accumulation of extracellular matrix in glomeruli and interstitium, leading to renal fibrosis and chronic renal failure [1]. The histologic changes observed in rats with subtotal nephrectomy include mononuclear infiltration, glomerulosclerosis and tubulointerstitial fibrosis. An increase in the amount of type I, III and IV collagen and expansion of mesangial ECM has also been shown to occur in human and experimental models of kidney fibrosis [2]. Very

Halo = halofuginone

5/6 NX = 5/6 nephrectomy

ECM = extracellular matrix

few drugs are able to halt the fibrotic process in experimental and human chronic renal diseases.

Halofuginone, an alkaloid originally isolated from the plant *Dichroa febrifuga*, has been found, in animals and humans, to be a specific and potent inhibitor of the synthesis of collagen type I at the transcriptional level. *In vitro*, halofuginone attenuated collagen $\alpha 1$ (I) gene expression by murine and avian skin fibroblasts and human skin fibroblasts derived from scleroderma and chronic graft-versus-host-disease patients [3]. In culture, halofuginone also inhibits rat mesangial cell proliferation and ECM production [4]. In animals, halofuginone prevented the increase in collagen synthesis in models of fibrosis involving skin, liver, urethra, heart, and surgical and traumatic adhesions [5]. Topical treatment using halofuginone in a patient with chronic graft-versus-host-disease caused a transient attenuation of collagen $\alpha 1$ (I) gene expression, demonstrating clinical efficacy in humans [6]. In all these models halofuginone inhibited collagen $\alpha 1$ (I) gene expression. Halofuginone affects collagen biosynthesis probably by blocking transforming growth factor- β -mediated smad3 activation [7]. In view of these findings, we assessed the effect of halofuginone on the development of glomerulosclerosis and tubulointerstitial fibrosis in rats following renal mass reduction.

Methods

All procedures were performed in accordance with institutional guidelines concerning animal experimentation. Male Wistar rats (weighing 300 ± 30 g at the start of the experiment) were used in this study. After acclimatization to their environment for 1 week, rats underwent either renal mass reduction by 5/6 nephrectomy or sham operation, under anesthesia with intraperitoneal injection of pentobarbital (35 mg/kg body weight). 5/6 NX was performed by ligation of two of three major branches of the left renal artery and right nephrectomy in the same session. Sham rats underwent exposition of the kidneys and removal of the perirenal fat. Twenty-four hours after the operation, rats were assigned to one of the following groups:

- NX-Halo (n=8): 5/6 NX rats receiving halofuginone by oral gavage (0.2 mg/kg/day)
- NX-Control (n=8): 5/6 NX rats receiving normal saline daily by oral gavage

- Sham (n=5): age-matched sham-operated rats serving as controls.

The optimal dose of halofuginone (0.2 mg/kg body weight) was established following pilot studies of dose efficacy in different animal models.

Every 2 weeks, systolic blood pressure was measured by tail-cuff manometry and urine samples were collected individually in metabolic cages for determination of 24 hour total protein and creatinine excretion. Protein concentration in urine was determined by a colorimetric method using pyrogallol-red molybdate complex (Cobas integra 700™, Roche) [8]. At sacrifice (10 weeks after 5/6 NX), blood was withdrawn from abdominal aorta for determination of creatinine and halofuginone concentrations. Serum creatinine was measured with a Hitachi model 747 autoanalyzer using the kinetic Jaffe method. Blood level of halofuginone was determined by high performance liquid chromatography. Kidneys were removed for histologic evaluation.

Light microscopy studies

A pathologist blinded to the treatment groups assessed the presence and severity of renal fibrosis. Specimens were fixed in 10% buffered formaldehyde and embedded in paraffin. Histologic sections of 4–5 μm thickness were stained with hematoxylin-eosin, periodic acid-Schiff and Masson trichrome (light green). A semi-quantitative score was used to assess the degree of glomerulosclerosis and tubulointerstitial injury [9]. In brief, 30 glomeruli in each kidney were graded from 0 to 4 according to the severity of the glomerular sclerosis: 0 = normal; 1 = slight glomerular damage, the mesangial matrix and/or hyalinosis with focal adhesion, involving < 25% of the glomerulus; 2 = sclerosis of 25–50%; 3 = sclerosis of 50–75%; and 4 = sclerosis of > 75% of the glomerulus. Twenty fields of cortical tubulointerstitial tissue were examined and graded as follows: 0 = normal; 1 = the area of interstitial fibrosis, tubular atrophy, and dilation involving < 25% of the field; 2 = lesion area between 25 and 50% of the field; and 3 = lesions involving > 50% of the field. Indices of glomerular damage or tubulointerstitial lesion were calculated by averaging the grades assigned to all glomeruli and tubular fields.

Preparation of sections, *in situ* hybridization and sirius red staining

Samples from remnant kidneys were collected into phosphate-buffered saline and fixed overnight in 4% paraformaldehyde in PBS at 4°C. Serial 5 μm sections were prepared after the samples had been dehydrated in graded ethanol solutions, cleared in chloroform and embedded in Paraplast. Differential staining of collagenous and non-collagenous proteins was performed with 0.1% sirius red, with 0.1% fast green as a counterstain, in saturated picric acid. By this procedure collagen is stained red [10]. For hybridization, the sections were deparaffinized in xylene, rehydrated through a graded series of ethanol solutions, rinsed in distilled water (5 minutes), and incubated in 2 x saline/sodium

citrate at 70°C for 30 min. The sections were then rinsed in distilled water and treated with pronase (0.125 mg/ml in 50 mM Tris-HCl, 5 mM EDTA, pH 7.5) for 10 min. After digestion, the slides were rinsed with distilled water, post-fixed in 10% formalin in PBS, blocked in 0.2% glycine, rinsed in distilled water, rapidly dehydrated through graded ethanol solutions, and air-dried for several hours. Before hybridization, the 1600 bp rat collagen $\alpha 1(I)$ insert was cut out from the original plasmid (pUC18) and inserted into pSafyre. The sections were then hybridized with digoxigenin-labeled collagen $\alpha 1(I)$ probe [11].

Statistical analysis

All results were expressed as mean \pm SEM. Inter-group differences were analyzed by the Mann-Whitney U test within the SPSS system for Windows. A *P* value of less than 0.05 was considered statistically significant.

Results

After a small decrease during the first days following surgery in both nephrectomized groups, the increase in body weight was similar in NX-Halo and NX-Control groups, suggesting that food intake was similar in both groups throughout the course of the study (374 ± 11 vs 396 ± 28 g, respectively at 10 weeks). Plasma levels of halofuginone, as measured by HPLC, reached levels of around 1 ng/ml (levels that were found to be very potent in different successful animal models).

The influence of halofuginone on blood pressure, proteinuria and creatinine clearance

Systolic blood pressure increased progressively in both 5/6 NX groups. There was no significant difference between the two nephrectomized groups during the 10 weeks follow-up (179 ± 12 vs 177 ± 23 mmHg, respectively, NX-Halo vs. NX-Control). No significant variation from baseline level was noted in the sham-operated group [Figure 1A].

The halofuginone-treated group had lower levels of proteinuria than the control nephrectomized group. This difference was statistically significant, beginning from week 4 post-nephrectomy (335 ± 47 vs 530 ± 72 mg/day, *P* < 0.05, NX-Halo vs. NX-Control, at 10 weeks) [Figure 1B].

As expected, creatinine clearance was lower in 5/6 NX groups when compared to sham rats. CCR was significantly higher in the NX-Halo group compared to the NX-Control group at the end of the study (0.44 ± 0.09 vs 0.35 ± 0.07 ml/min, *P* < 0.05, NX-Halo vs NX-Control) [Figure 1C].

Pathologic studies

Light microscopy. There was a significant decrease in tubulointerstitial fibrosis in the NX-Halo group when compared to the control group (NX-Control) (0.4 ± 0.2 vs 1.8 ± 0.2 , *P* < 0.01 at 10 weeks). A less accentuated trend was noted for the presence of glomerulosclerosis and mesangial proliferation, due to milder

PBS = phosphate-buffered saline

HPLC = high performance liquid chromatography

CCR = creatinine clearance

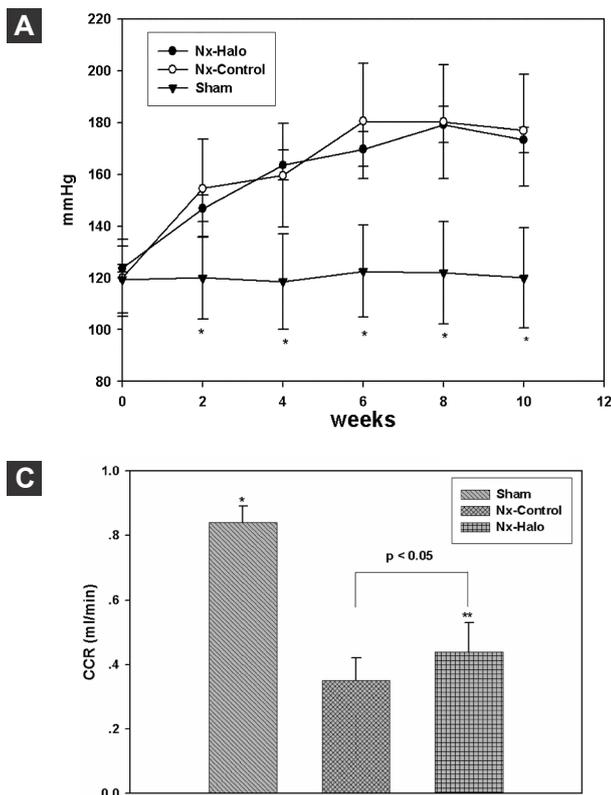


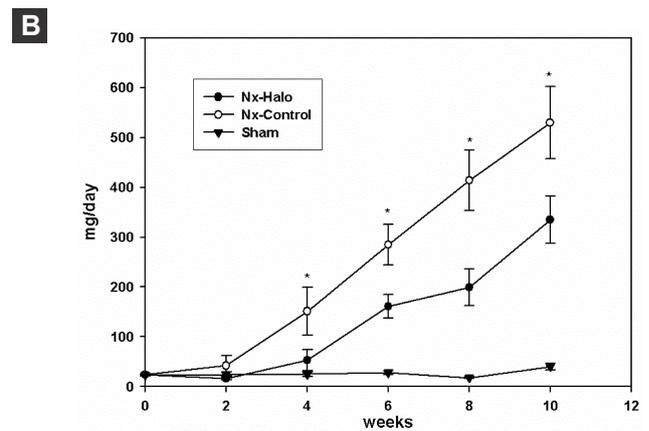
Figure 1. The effects of halofuginone on [A] systolic blood pressure (SBP), [B] proteinuria, and [C] creatinine clearance (CCR) in 5/6 nephrectomized rats.

- * SPB: significantly lower ($P < 0.05$) than both 5/6 NX groups
- Proteinuria: significantly higher ($P < 0.05$) than NX-Halo and sham groups
- CCR: significantly higher ($P < 0.05$) than both 5/6 NX groups
- ** significantly higher ($P < 0.05$) than NX-Control group)

sclerotic lesions in untreated nephrectomized rats (0.33 ± 0.5 vs 0.9 ± 0.1 , respectively for NX-Halo and NX-Control, $P < 0.01$). The sham-operated group showed no increase in either glomerulosclerosis or tubulointerstitial fibrosis at 10 weeks [Figure 2].



Figure 2. Light microscopic findings at week 10. Light microscopic findings at week 10 are shown by Masson trichrome stain (x100). Representative light microscopy findings of [A] sham-operated rats, [B] control nephrectomy (NX-Control), and [C] and halofuginone-treated (NX-Halo) rats.



Sirius red staining and in situ hybridization. The kidney sections from 5/6 nephrectomized rats exhibited high levels of the collagen $\alpha 1(I)$ gene expression compared with the sham rats, as demonstrated by *in situ* hybridization using the specific rat collagen $\alpha 1(I)$ probe [Figure 3A and B]. Oral administration of halofuginone (0.2 mg/kg/day) resulted in significant reduction in the collagen $\alpha 1(I)$ gene expression [Figure 3C]. The increase in collagen $\alpha 1(I)$ gene expression after 5/6 nephrectomy was accompanied by an increase in the collagen content in the tubulointerstitium as demonstrated by specific collagen staining [Figure 4B]. The amount of collagen in the kidney sections was decreased in rats treated with halofuginone [Figure 4C].

Discussion

Efforts to prevent the progression of renal diseases have relied largely on the effects of diet, antihypertensive medications (particularly angiotensin-converting enzyme inhibitors), angiotensin II blockers or endothelin receptor antagonists, and anticoagulant drugs [12]. The mediators involved in the progression of renal fibrosis have been studied in various models of renal diseases [13]. Hemodynamic influences of AgII are well established, but more recent data have suggested pro-inflammatory properties and a role in the regulation of matrix degradation for Ag II [14]. Modulation of glomerular capillary hydrostatic pressure by ACE

ACE = angiotensin-converting enzyme

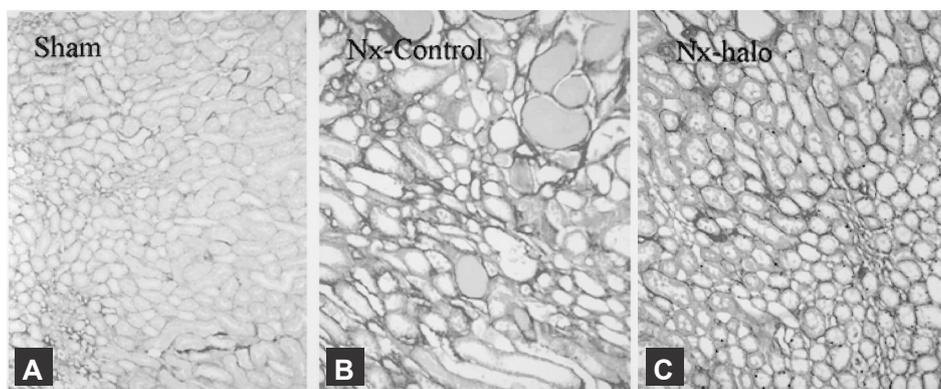


Figure 3. Effect of halofuginone on collagen $\alpha 1(I)$ expression. Samples of kidney biopsies were hybridized with rat collagen $\alpha 1(I)$ specific probe. Representative collagen $\alpha 1(I)$ expression of **[A]** sham-operated rats, **[B]** control nephrectomy (NX-Control), and **[C]** halofuginone-treated (Nx-Halo) rats. (Magnification x 100)

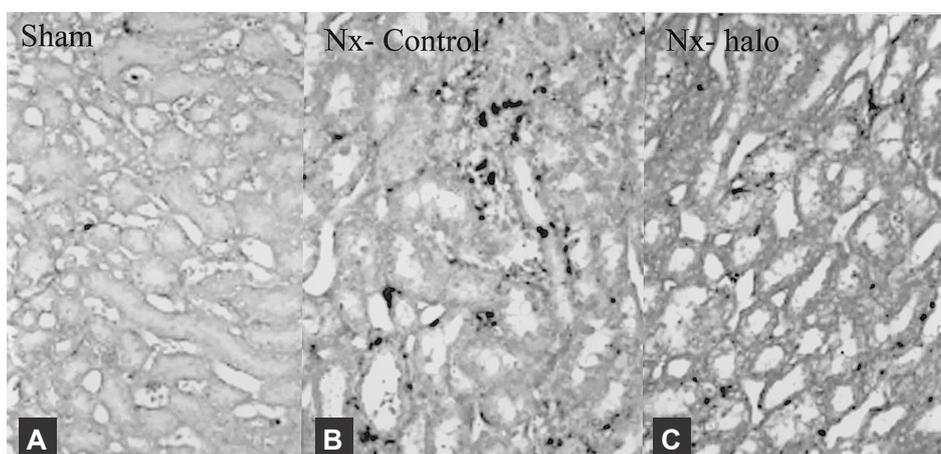


Figure 4. Effect of halofuginone on collagen content in 5/6 nephrectomized rats (10th week). **[B]** Note the increase in collagen staining after nephrectomy (NX-Control), **[C]** which was almost completely prevented by halofuginone (NX-Halo)(C). (Magnification x 100)

inhibitors and AgII receptor blockers has been proven to be efficient in experimental and human studies [15]. Different approaches have been evaluated to prevent or reduce renal fibrosis [12,16]. Effective therapy might include inhibition of fibroblast activation, inhibition of matrix synthesis and/or stimulation of matrix degradation. Inhibition of plasma activator inhibitor-1 activity and synthesis for the prevention or the treatment of renal fibrosis *in vitro* has recently been reported [17]. In genetic PAI-1 deficiency mice renal interstitial fibrosis was significantly attenuated in a model of unilateral ureteral obstruction [18]. These studies establish an important role for PAI-1 in renal fibrosis.

Renal fibrosis is characterized by excessive synthesis of connective tissue, and collagen is a major component of fibrotic lesions. Increased amounts of type I, III and IV collagen accompany the process of renal fibrosis. Particularly the low levels of type I collagen expression in normal kidney increases significantly after renal injury. As halofuginone inhibits the expression of collagen type I gene in a broad range of cell types, its use in different models of fibrosis has been proposed [5]. In the present study, we tested the effect of halofuginone in 5/6 nephrectomized rats and observed

a significant reduction in interstitial fibrosis in the halofuginone-treated group. The effect on glomerulosclerosis was also significant, but we have to take into account the fact that glomerular sclerotic changes were not marked in this model. 5/6 nephrectomy was associated with increases in collagen $\alpha 1(I)$ expression and collagen content, which is in agreement with previous studies demonstrating specific increases in type I collagen in injured tissues [5]. The collagen $\alpha 1(I)$ expression and collagen content was almost completely abolished by administration of halofuginone. Northern Blot analysis of collagen I mRNA was not done in this study but was previously reported by our group in a different experimental model [19].

From the functional point of view, urinary protein excretion was lower and the decrease in creatinine clearance was partially blunted in halofuginone rats. As blood pressure values and body weight were similar in treated and untreated animals, the results demonstrate that the efficient action of halofuginone on the kidney was not mediated by changes in blood pressure or dietary protein intake.

Our results suggest that collagen I plays a significant role in the development of renal fibrosis, not only at the glomerular level but also, and perhaps especially, at the interstitial level. These results are similar to those obtained with pirfenidone, which did not significantly affect proteinuria but partially prevented the occurrence of renal fibrosis [20]. Pirfenidone reduces the expression of mRNA for type I and IV collagen in the kidney, as well as transforming growth factor-beta mRNA expression.

The improvement in renal function and histologic lesions (mainly in the tubulointerstitium) contrasts with the persistence of relatively high levels of proteinuria. This discordance between proteinuria and progression of renal disease has been reported in previous animal models and suggests that different mechanisms are responsible for the development of renal fibrosis and proteinuria. We believe that the amelioration of tubulointerstitial fibrosis induced by halofuginone plays a dominant role in the partial preservation of renal function in this model. The mechanism by which halofuginone reduces fibrosis was recently elucidated in a mouse model for scleroderma (dermal fibrosis), where a low dose of halo blocked TGF β -mediated SMAD3 activation in fibroblasts

PAI-1 = plasma activator inhibitor-1

TGF β = transforming growth factor-beta

[7]. Furthermore, halofuginone inhibited the collagen synthesis by hepatic stellate cells and prevented the fibrosis-induced alteration in insulin-like growth factor binding proteins by hepatic cells [21].

Other treatments have been proposed to depress the production of collagen, but most are non-specific, leading to a generalized reduction of several collagen molecules [16]. Interferon-gamma also delays the occurrence of fibrosis in 5/6 nephrectomized rats, however the lack of specificity of IFN γ interferes with the normal process of elastogenesis and bone formation, leading to severe side effects [22]. Recent *in vitro* studies have shown that halofuginone inhibits mesangial cell proliferation and ECM production, suggesting that halofuginone may also have a protective action in inflammatory glomerular disease characterized by mesangial cell proliferation and ECM expansion [4].

In conclusion, halofuginone, a collagen I inhibitor, has been shown to reduce renal fibrosis and proteinuria in the experimental model of 5/6 nephrectomy in rats. Such action was independent of blood pressure changes. Further studies are being performed in different models of renal diseases to confirm this beneficial and promising effect.

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