Objective: This study evaluated the ability of heme oxygenase-1 to prevent or reverse renal fibrosis. Methods: Sprague-dawley male rats were submitted to unilateral ureteral obstruction and divided into groups: non-treated and hemin. Biochemical and histological analyses were performed. We also conducted RT-PCR to verify the expression of heme oxygenase-1, MCP-1, IL1-β, IL-6, TNF-α, COL-I, COL-III, PAI-1 and fibronectin mRNA. Results: heme oxygenase-1 expression significantly increased in treated animals. The non treated group showed significantly higher levels of proteinuria than the Hemin group. The protein/urinary creatinine ratio in obstructed pelvis was also higher in non treated group, which also showed greater albuminuria and higher percentage of fibrosis when compared to the Hemin group. The expression of pro-inflammatory and pro-fibrotic molecules was significantly higher in the non treated group. Conclusions: The treatment induced the expression of heme oxygenase-1, preventing the installation of fibrosis and even limiting its progression.

Keywords: Heme-oxygenase-1; Fibrosis/pathology; Kidney/pathology; Urologic surgical procedures/methods

INTRODUCTION

The past decades have witnessed a considerable incidence in progressive chronic nephropathies, which in turn lead to an increase in incidence and prevalence of end-stage renal diseases. In numerous countries this represents an important social and medical problem, as well as a financial burden. Based on the estimate that worldwide renal and urinary tract diseases are responsible for 830,000 deaths a year, chronic renal...
Cytoprotective role of heme oxygenase-1 upregulation in progressive renal disease

disease is considered a threat to public health throughout the world\(^1\).

Tubulointerstitial fibrosis is an important event in chronic renal disease and is also an important tool for estimating renal dysfunction. Fibrosis is more than just a response to tubular injury. It also plays an important role in the loss of nephrons – the functional units of the kidney. A number of renal diseases progress towards renal failure as a consequence of the functional adaptations that occur in the kidney, once the original disease has caused critical loss of nephrons\(^2\).

Heme-oxygenase-1 (HO-1) is an enzyme involved in the degradation of free heme, which results in formation of final products such as biliverdin (which biliverdin reductase rapidly converts into bilirubin), carbon monoxide and free iron. Cytoprotective action occurs due to the formation of byproducts that appear to have various important functions: 1) antioxidants – especially due to increase in biliverdin and bilirubin; 2) anti-inflammatory and antiapoptotic – due to production of CO; and 3) possibly, immune-modulator. Furthermore, the free iron is bound by ferritin, which also seems to have a cytoprotective effect. The HO-1 upregulation is associated with an increase in the expression of p21, a regulatory protein of the cell cycle, which is in part responsible for the enzyme antiproliferative effects\(^3\).

Chronic renal disease is a common problem in clinical practice. Despite a clear understanding of tubulointerstitial fibrosis, a characteristic of the disease, it is still poorly controlled. We believe that treatment with an HO-1 upregulator after full development of fibrosis in an experimental model of renal failure will limit or even reverse progressive renal disease.

**OBJECTIVE**

To evaluate whether the increased expression of HO-1 effectively improves renal dysfunction and reduces the gene expression of proinflammatory and profibrotic molecules.

**METHODS**

**Animals**

For the purpose of this study, we used male Sprague-Dawley rats, weighing more than 200 grams. Animals were provided by the Main Experimental Animal Facility of Universidade Federal de São Paulo (UNIFESP) and by the Experimental Animal Facility of Universidade Estadual de Campinas. Animals were kept at the Experimental Facility for Rats of the Discipline of Nephrology of UNIFESP. The animals were housed in collective boxes, no more than three animals per box, under an artificial 12-hour dark/light cycle. Food and water were available ad libitum and room temperature was kept constant at 22 °C. The project started only after approval by the Research Ethics Committee of UNIFESP (CEP n°. 029/2009).

**Surgery for unilateral ureteral obstruction (UUO) – experimental model for renal fibrosis**

Following anesthesia, animals were positioned on a dissection plate in dorsal recumbency. A midline incision was performed for exposure of abdominal organs, which were dislodged towards the thorax and protected using gauze moisturized with 0.9% physiologic saline solution (SF) (Sanobiol, Porto Alegre, Brazil). After exposure of the retroperitoneum, unilateral ligation (UUO) of the left ureter was performed using two sutures: one more distal and the other more proximal to the kidney. The portion of the ureter between these two sutures was cut off, causing interruption of urine flow. The contralateral (right) kidney was not manipulated. After this procedure the organs were placed back in the abdominal cavity and the abdominal wall and peritoneum were sutured. Until full recovery from anesthesia the animal was kept warm using indirect light exposure. On the day prior to surgery and two days after surgery the animals were housed in metabolic boxes to allow for 24-hour urine collection. The animals were humanely killed and the abdominal cavity was opened. We collected blood from the vena cava, urine from the obstructed renal pelvis, and the kidneys for histological analysis and real time PCR.

**Experimental protocol**

To determine the ability of a heme-oxygenase-1 upregulator to prevent progression of chronic renal disease the animals were divided into groups as follows:

- Control Group (n = 5): normal animals that did not undergo surgical procedure or treatment;
- UUO Group (n = 5): non-treated animals that were humanely killed 7 and 14 days after UUO surgery;
- Hemin Group (n = 5): animals that were treated with Hemin 5 µM at 10 mg/kg on days -2 and -1 prior to surgery and that were humanely killed 7 and 14 days after UUO surgery.

In order to determine whether treatment can reverse fibrosis that had already developed, animals were divided into:

- Control Group (n = 5): normal animals that did not undergo surgical procedure or treatment;
- UUO Group (n = 5): animals that were not treated and were humanely killed 14 days after UUO surgery;
- Hemin Group (n = 5): animals treated with Hemin 5 µM at 10 mg/kg on days 6 and 7 after surgery and that were humanely killed 14 days after UUO surgery.

Renal function and histology analysis

Proteinuria was measured using the commercial kit Sensiprot (Labtest Diagnóstica, Sete Lagoas, Brazil). The modified Jaffé method (Roche, Mannheim, Germany) was used to determine urine creatinine levels. For the purpose of indirect urine albumin quantification we diluted the urine samples with equivalent amounts of sample buffer (distilled water; Tris – HCl 500mM, pH: 6.8; glycerol; SDS; sodium dodecyl sulfate 10%; β-mercaptoethanol; bromophenol blue at 0.05%), after this, samples were run on a 10% SDS-PAGE gel at a fixed amperage of 30 mA. For comparison, samples of bovine serum albumin (Sigma) were considered standard. The gel was stained using coomassie blue dye and then photographed in the G-Box equipment (Syngene, USA) using the GeneSnap program (Syngene, USA), and bands were quantified using the program GeneTools (Syngene, USA). Histology analysis was performed on paraffin embedded renal sections that had been fixed with 10% buffered formaldehyde solution and stained with Picrosirius dye. Images were captured using a video camera mounted on a polar light microscope and a 20x lens that sends images to the analyzer system (KS 300 Carl Zeiss) used for morphometric analysis. The images revealed the presence of areas of yellow-reddish birefringent collagen within the tubulointerstitial compartment. Said areas were marked by the observer and used to estimate the percentage of fibrosis in each field of the microscopic view.

Standard of gene expression

Kidney samples were frozen in liquid nitrogen. Total RNA was isolated from renal tissue according to the method of the TRIzol Reagent (Invitrogen, Carlsbad, California, USA) and quantification of extracted RNA was determined by spectrophotometer reading at 260 nm absorbance. Then, cDNAs were synthesized using reverse transcriptase MML-V (Promega, Madison, Wisconsin, USA). Real-time PCR was performed using the thermocycler ABI 7300 (Applied Biosystems, Foster City, USA). For the purpose of the Sybergreen assays the following primers were used: HPRT (sense) 5'-CTC ATG GAC TGA TTA TGG ACA GGA C-3' and (antisense) 5'-GCA GGT CAG CAA AGA ACT TAT AGC C-3'; MCP-1 (sense) 5'-AAG AGA AGA ATC ACC AGC AGG T-3' and (antisense) 5'-TTC TGG ACC CAT TCC TTA TTG G-3'; COL-I (sense) 5'-TGG CCA AGA AGA CAT CCC TGA AGT-3' and (antisense) 5'-AGA TCA GGT TTC CAC GTC TCA CCA-3'; COL-III (sense) 5'-ATG AGC TTT GTG CAA TGT GGG ACC-3' and (antisense) 5'-ACT GAC CAA GGT AGT TGC ATC CCA-3'; TGF-β (sense) 5'-TCA GTC GAG GT-3' and (antisense) 5'-GCC GAG TGT CAG TTC CGA CCT CCT TGA GC-3'. For the Taqman assays we used the following primers from Applied Biosystem (USA): HPRT (Rn01527838_g1), TNF-α (Rn99999017_m1), IL-1β (Rn00580432_m1), IL-6 (Rn00561420_m1), HO-1 (Rn00561387_m1), Fibronectin (Rn00569575_m1). Thermocycling was performed according to the following program: 10 minutes at 95 °C followed by forty-five 30-second-cycles at 95 °C, 30 seconds at 58 °C and 30 seconds at 72 °C. For the analysis of results the Sequence Detection Software 1.9 (SDS) program was used. Expression of mRNA was normalized by excess HPRT (control gene). Therefore, all results were expressed in relation to the expression of the respective control animals.

Statistical

Data was presented in graphs containing the mean and standard deviation. Data was compared using the Student t and the ANOVA tests. Differences were considered statistically significant when p < 0.05.

RESULTS

Firstly, we investigated the preventive effect of HO-1 upregulation in protecting the kidney from progressive fibrosis. To achieve this, animals were treated prior to their respective unilateral ureteral ligation surgery.

Hemin treatment induces gene expression of HO-1 and indicates improvement in renal function

To determine whether Hemin treatment induced gene expression of HO-1, we checked the gene expression of this molecule using RT-PCR on the kidney samples from the animals that underwent UUO with or without treatment. Seven days after ureter ligation, we noticed a significant increase in the amplification of this molecule in the treated group when compared to the non-treated group. Fourteen days after surgery, HO-1 expression decreased in both groups. Yet, even after this period, surprisingly HO-1 expression was higher in kidney samples from the treated group (Figure 1).
Since we determined that treatment induces positive upregulation of the HO-1 enzyme, we decided to evaluate its cytoprotective role. We noticed a marked protection in the proteinuria values of animals treated, which was evident from the start of treatment until the killing of the animals (Figure 2A). Once the ureter is ligated, urine accumulates in the obstructed pelvis. The urine samples were collected by puncture, and were used to determine if treatment protected the obstructed kidney, even in a situation of increased external pressure. In the treated group, seven days after surgery, we noticed a lower protein/creatinine ratio, which was significantly lower fourteen days after surgery (Figure 2B).

In order to determine whether the proteinuria observed after surgery was associated or not with the filtered albumin fraction, we obtained an indirect quantification of this protein using a 10% SDS-PAGE gel stained with coomassie blue dye. Albuminuria was much higher in the animals that underwent UUO surgery and did not receive any treatment. This indicates that protein loss in urine was higher in this group (Figure 3). Furthermore, we used Picrosirius staining to quantify the amount of interstitial fibrosis between the groups. The percentage of fibrosis was significantly higher in the non-treated group, when compared to the control and Hemin groups (Figure 4).
**HO-1 upregulation reduces the expression of pro-inflammatory and profibrotic molecules.**

In order to evaluate the mechanisms that protect renal function in the treated animals, we used RT-PCR to determine the expression of pro-inflammatory and fibroproliferative molecules. In treated animals we observed a reduction in the expression of the gene transcripts of TNF-α, IL-6, IL-1β, MCP-1, PAI-1, type I and III collagens and fibronectin. Yet, it is interesting that we did not observe any difference in the expression of TGF-β amplification between the groups (Figures 5 and 6).

We performed RT-PCR for HO-1 and noticed that the gene expression of this enzyme was significantly higher in the treated group (Figure 7). When dosing proteinuria from the bladder, we noticed that, at first, the protein loss profile was similar between the UUO and Hemin groups; yet, after treatment, we noticed that proteinuria progressed in non-treated animals, whereas treated animals maintained baseline levels of urine protein. The difference between groups was significant in the last points of dosing (Figure 8A). Analysis of urine samples drawn from the obstructed pelvis revealed a significantly lower protein/creatinine ratio in the treated group (Figure 8B).

**Late Hemin treatment reduces HO-1 and re-establishes renal function**

Since we observed that Hemin treatment prevents the development of interstitial fibrosis, we decided to study if HO-1 upregulation could also reverse tubulointerstitial disease. For this purpose we treated animals with Hemin after renal fibrosis had already developed.
When running the SDS-PAGE gel for the indirect determination of urine albumin, seven days after ureter ligation surgery, we noticed similar levels of urine albumin in the UUO and Hemin groups. After treatment, we noticed a progressive reduction in albumin levels in the treated group, while urine albumin levels progressively increased in the non-treated animals (Figure 9).

Histological analysis also revealed a higher percentage of fibrosis in the non-treated animals, when compared to control and treated animals (Figure 10).

**Hemin treatment reduces gene expression of pro-inflammatory and profibrotic molecules**

Similarly to the chronic renal disease limitation study, expression of mRNA from TNF-α, IL-6, MCP-1, PAI-1, type I and III collagen, and fibronectin decreased in treated animals. There was no difference in analysis of amplified IL-1β and TGF-β between the groups (Figures 11 and 12).
The HO-1 is a molecule with proven cytoprotective activity and is the last enzyme involved in the degradation of free heme, which results in the production of byproducts with cytoprotective, anti-inflammatory, antioxidant, antiproliferative and antiapoptotic effects\(^{(3)}\). Animal studies suggest that the upregulation of this enzyme has a protective effect in models of acute\(^{(5-6)}\) and chronic\(^{(7-8)}\) renal insults. In our study, HO-1 gene expression was significantly higher in the treated group, demonstrating that protection was associated with HO-1 upregulation.

Unilateral ligation of the ureter by itself increases HO-1 expression. This is also observed in our results and is associated with the fact that this enzyme is a stress response protein, and therefore is present as an attempt to reduce renal lesion\(^{(9)}\).

Kim et al. suggested that the higher expression of HO-1 induced by hemin treatment protects the kidney against the renal fibrosis caused by the UUO model, due to its antiapoptotic action\(^{(10)}\). Another study showed that renal fibrosis, inflammation and epithelial-mesenchymal transcription increased in HO-1 deficient mice that underwent UUO\(^{(11)}\).

All these data and the results of the present study suggest that HO-1 upregulation by affected cells represents an early response to the initial injury and treatment with a drug that potentiates the expression of this enzyme could be extremely beneficial to the development of progressive renal disease.

Regarding urine excretion of proteins, we noticed that after treatment proteinuria stabilized in the hemin group, whereas proteinuria progressively increased in the non-treated group. The progressive proteinuria profile was also observed in another experimental model for chronic renal disease, such as 5/6 nephrectomy\(^{(12)}\).

In patients with chronic renal disease, proteinuria comprises the different protein fractions, and albumin is the most important predictor of renal dysfunction. Despite its intermediate molecular weight, its negative charge causes reduced albumin permeability in the glomerular filtrate. The fraction of albumin that is actually filtered at first undergoes endocytosis by the binding proteins megalin and cubilin in the proximal tubular cells. When the reabsorption threshold is exceeded, albumin is excreted in urine, and is a valuable tool for estimating the extent of renal lesion\(^{(13-14)}\).

In our study, we noticed higher albumin levels in both UUO groups: prevention and reversion. This last analysis revealed that while urine excretion of albumin progressively increased in the non-treated group, albuminuria progressively decreased in the Hemin treated group. A study that evaluated the effect of TGF-\(\beta\) antagonist treatment in a diabetic nephropathy model showed a reduction in the levels of urine albumin.
in treated animals, when compared to progressively increasing albuminuria in non-treated animals\(^{(17)}\).

In few hours the experimental UUO model induces cell infiltration in the tubulointerstitial compartment. These infiltrating cells (especially macrophages) secrete growth factors and cytokines, which first cause inflammation and then disrupt the balance between apoptosis and tubular cell proliferation, and induce the activation and proliferation of fibroblasts\(^{(16)}\).

In our study, we noticed a lower expression of MCP-1, TNF-\(\alpha\), IL-6 and IL-1\(\beta\) in treated animals, which suggests this group has a lower inflammatory profile. A study demonstrated an increase in proinflammatory cytokines in patients undergoing hemodialysis and patients with chronic renal diseases, when compared to the control group\(^{(17)}\). MCP-1 is a molecule that shows potent chemical reaction towards monocytes and macrophages in inflamed areas\(^{(18)}\).

The lower expression of this chemokine in treated animals suggests that HO-1 upregulation can restrict its release, thus improving tissue repair. Macrophages produce ROS that directly injure resident renal cells, including tubular epithelial cells that develop tubulointerstitial fibrosis\(^{(19)}\). The antioxidative effects of HO-1 upregulation block these ROS and have a protective effect on the injured tissue.

The gene expression of TNF-\(\alpha\), the standard inflammation cytokine, was considerably reduced in all treated animals when compared to the UUO group. This indicates that cytokine TNF-\(\alpha\) is less involved in the progress of renal fibrosis. Meldrum et al. demonstrated that neutralization of TNF-\(\alpha\) with its soluble receptor could improve UUO induced fibrosis\(^{(20)}\). In regard to IL-6, Costa et al. conducted a clinical study with patients undergoing hemodialysis, who were resistant to treatment with recombinant human erythropoietin and noticed a significant increase in the levels of serum IL-6, C-reactive protein and soluble IL-2 receptor. A previous study performed by our group reported a strong upregulation in the gene expression of IL-6 after UUO. This suggests that this might be the most important proinflammatory cytokine of the process\(^{(21)}\).

Regarding IL-1\(\beta\), a difference between groups was only noticed in the study that limited progressive renal disease. Chung et al. demonstrated a higher protein expression of IL-1\(\beta\) in animals that underwent UUO\(^{(22)}\). This cytokine is involved in the so-called inflammasome, a multimeric protein complex that connects the process of microbial product recognition and metabolic stress to the proteolytic processing of the active form of pro-IL-1\(\beta\). The outflow of potassium and ROS generation are the main non-pathogenic signals that trigger this complex\(^{(23)}\).

According to our model, the presence of ROS produced by the infiltrating cells activates the inflammasome and induces the production of the active form of IL-1\(\beta\). The antioxidative effects of HO-1 byproducts probably neutralize ROS, which would explain the lower expression of IL-1\(\beta\) in treated animals.

Renal fibrosis leads to formation of excess connective tissue and is observed in humans and in experimental models of renal disease. This excess connective tissue contributes to progressive decrease of glomerular filtration and tubular function\(^{(24)}\). In our study we observed a lower expression of the fibrogenic molecules PAI-1, types I and III collagen and fibronectin. It is worth noting that none of the studies showed a difference between groups in relation to the main profibrotic cytokine TGF-\(\beta\).

Matsuo et al. demonstrated that severity of fibrosis in genetically modified animals with over-expression of PAI-1 (PAI-1 tg) was worse than that observed in wild animals. Fibrosis was evidenced by positive Picrosirius staining of the interstitium and total renal collagen\(^{(25)}\). Collagen fibers, especially type I collagen, are proteins present in areas of extracellular matrix deposits. In our studies we noticed a higher expression of type I and type III collagen molecules in non-treated animals. Literature data suggests that collagen is involved in renal fibrosis, especially in the UUO model\(^{(26)}\). Iwai et al. demonstrated a reduction in type I and III collagen levels in animals that underwent UUO and were treated with a HO-1 inducer. This is consistent with the results presented in this study\(^{(27)}\). Literature reports support our results regarding the increase of fibronectin in the experimental UUO model\(^{(28-29)}\).

An interesting finding in our study is that there was no difference in the gene expression of TGF-\(\beta\) between treated and non-treated animals, in the prevention study and in the study of tubulointerstitial disease reversal. Since TGF-\(\beta\) is regarded as a key cytokine in fibrogenesis, we expected to find a reduction in TGF-\(\beta\) expression in treated animals. Literature reports suggest that genetic manipulation of animals aiming to attenuate fibrosis, and the production of knockout animals or even RNAsi technology reduced the gene and protein expression of TGF-\(\beta\)\(^{(22,30)}\). Probably, the reason why there was no difference between groups in our study is that we quantified the gene amplification of total TGF-\(\beta\), instead of its activated fraction. Furthermore, we verified the mRNA expression of this factor, and the protein expression of its active form might be the best method of comparison. Although upregulation of HO-1 may not interfere with the production of this cytokine, it may be involved in its activation, as fibrosis was significantly reduced in treated animals. Since TGF-\(\beta\) plays an important role in fibroproliferative disorders,
this process might be attenuated without interfering with the TGF-β expression or the TGF-β effector role.

CONCLUSIONS
Based on the results herein presented, we conclude that Hemin pre-treatment protects the kidney from developing tubular interstitial disease, and that treatment after full development of fibrosis reverses progressive renal disease, despite of continuous injury. This opens new perspectives for the future treatment of patients with chronic renal disease, regardless of its stage – initial or more advanced.

ACKNOWLEDGEMENTS
The authors thank Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP 07/07139-3) and Conselho Nacional de Desenvolvimento Científico e Tecnológico ( CNPq) for the support provided for this work.

REFERENCES

CONCLUSIONS
Based on the results herein presented, we conclude that Hemin pre-treatment protects the kidney from developing tubular interstitial disease, and that treatment after full development of fibrosis reverses progressive renal disease, despite of continuous injury. This opens new perspectives for the future treatment of patients with chronic renal disease, regardless of its stage – initial or more advanced.

ACKNOWLEDGEMENTS
The authors thank Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP 07/07139-3) and Conselho Nacional de Desenvolvimento Científico e Tecnológico ( CNPq) for the support provided for this work.

REFERENCES