

# Immunohistochemical detection of prolyl 4-hydroxylase (P4Hyd) in an experimental model of hydronephrosis

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## ABSTRACT

**Background:** Several studies have shown the importance of interstitial fibrosis in chronic kidney disease and its relationship with the progressive loss of organ function. Fibrosis is a histopathological condition which causes a vast set of changes in the extracellular matrix as a consequence of the imbalance between the synthesis and the degradation of extracellular matrix proteins, particularly collagen. Prolyl 4-hydroxylase (P4Hyd) is a key cellular enzyme, essential for the biosynthesis and stabilisation of collagen, and this study's main aim is to identify its new-expression in obstructive nephropathy.

**Methods:** To achieve this aim, the authors developed a polyclonal anti-prolyl 4-hydroxylase antibody, to be used in the immunoperoxidase staining method. The anti-P4Hyd was further applied to a 4 and 8 day experimental model of unilateral ureteric obstruction (UUO) in Wistar rats. The next step was to analyse the qualitative and semi-quantitative new-expression of the enzyme in the renal tissue of both kidneys (obstructed and contralateral).

**Results:** After the development of the antibody, the authors observed the significant expression of P4Hyd enzyme in the interstitial and tubular cells on the 4<sup>th</sup> day after-obstruction, with no expression in

glomerular cells. On the 8<sup>th</sup> day a significant increase was observed in the tubular cells with a decline in the interstitial cells. There was also new-expression of P4Hyd in some glomerular cells (epithelial and mesangial), particularly in fibrotic sections of the kidney.

**Conclusions:** These observations illustrate the role of prolyl 4-hydroxylase in kidney fibrosis and reveal the tubular and the interstitial cells' potential to synthesise collagen at the different periods of obstruction. They also reinforce the conclusions of other authors as to the concept of epithelial-mesenchymal transition of the epithelial tubular cells. The new-expression in the glomerular cells suggests the progression of the pathogenic mechanisms, from the tubulointerstitium to the glomerula.

### Key-Words:

Fibrosis; collagen; prolyl 4-hydroxylase; unilateral ureteric obstruction (UUO).

## INTRODUCTION

The progression of chronic kidney disease is associated with tubular and interstitial changes, characterised by an increase in the extracellular matrix, in which collagen plays a major role. Since the sixties, pioneer studies have revealed a close relationship between the

decline of kidney function and interstitial fibrosis, which impacts strongly on the disease's prognosis<sup>1-3</sup>. The pathogenesis of renal fibrosis is, in essence, a monotonous process characterised by an excessive accumulation and deposit of extracellular matrix.

As in chronic tubulointerstitial disease, the changes of the mesangial matrix in chronic glomerulopathy are also characterised by excess collagen, and this has stimulated research into the main cells responsible for collagen's abnormal synthesis. Several studies have shown the collagen synthesis potential of tubular, mononuclear cells (monocytes and macrophages) and fibroblasts in the interstitium<sup>4</sup> and of mesangial cells in the glomerula<sup>5</sup>. The pathogenic aggression causes a dynamic inflammatory response with cellular mobilisation, proliferation, and new-expression of phenotypes able to synthesise multiple cytokines and proteins of the extracellular matrix, including collagen. Myofibroblastic activation of mesangial cells and fibroblasts is an early fibrogenic response after injury, whereas tubular epithelial-mesenchymal transition often occurs at a later stage<sup>6</sup>. The hallmarks of mesangial and fibroblast activation and tubular epithelial-mesenchymal transition (EMT) are *de novo* expression of  $\alpha$ -smooth muscle actin, a contractile protein normally restricted to perivascular smooth muscle cells *in vivo*, and overproduction of the interstitial matrix components such as type I and III collagen and fibronectin<sup>7</sup>.

The prolyl-4-hydroxylase enzyme (P4Hyd) plays an important role in collagen biosynthesis by promoting hydroxylation of the proline, which provides the stability for the collagen molecule<sup>8</sup>. In 1973 and 1974 Al-Adnani *et al.*<sup>9,10</sup> presented studies describing the identification of proline hydroxylase as a marker for the fibroblast function in tissues, but despite this, the role of P4Hyd in the progression of kidney disease remains to be defined<sup>5</sup>.

Vertebrate prolyl 4-hydroxylases are  $\alpha_2\beta_2$  tetramers in which the  $\beta$ -subunit is identical to the enzyme and chaperone protein disulfide isomerase (PDI)<sup>11</sup>. Prolyl 4-hydroxylase had long been assumed to be of one type only, with no isoenzymes, until the  $\alpha$ (II)-sub-unit was cloned from mice<sup>12</sup> and humans<sup>13</sup>. Further reports indicated that the two prolyl 4-hydroxylase isoenzymes (I and II) have major differences in their expression patterns in many tissues. The type I enzyme was previously found to be the main form in many cells and

tissues, while the type II enzyme was the main form in cultured chondrocytes and chondrocytes present in foetal cartilage<sup>14</sup>. Present data suggest that type I prolyl 4-hydroxylase is expressed particularly by cells of mesenchymal origin, and it may often be present in less differentiated cells than the type II isoenzyme<sup>14</sup>. The type II isoenzyme is likewise expressed by many mesenchyme-derived cells, but especially by chondrocytes and endothelial cells<sup>14</sup>. Epithelial cells appear to express only small amounts of either prolyl 4-hydroxylase form, but the main or only enzyme in the epithelial cells in the kidney and bile ducts is type II<sup>11</sup>.

Experimental hydronephrosis, through unilateral ureteric obstruction (UUO), is a well-known model which may be of great use in studies into the progressive changes of the extracellular matrix and in evaluating the selective role of the main cells responsible for collagen biosynthesis.

## ■ AIMS

This study's aims are two-fold:

- 1 – Isolation of the prolyl-4-hydroxylase enzyme (P4Hyd) and synthesis of a polyclonal antibody for application in immunoperoxidase staining method.
- 2 – Immunolocalisation and semi-quantitative evaluation of the cellular expression of the prolyl-4-hydroxylase enzyme (P4Hyd) in the kidney with hydronephrosis.

## ■ MATERIAL AND METHODS

### ■ 1 – Isolation of the enzyme and production of the polyclonal anti-Phy antibody

Developing the polyclonal antibody was a four stage process: **1<sup>st</sup>** – Induction of fibrosis/cirrhosis in hepatic tissue; **2<sup>nd</sup>** – Isolation of the enzyme; **3<sup>rd</sup>** – Antibody production; **4<sup>th</sup>** – Application of antibody to the tissue embedded in paraffin and revelation by immunoperoxidase staining methods (regular controls – positive and negative).

### Induction of fibrosis/cirrhosis in hepatic tissue

As this enzyme is directly implicated in the biosynthesis of collagen, we presuppose its active expression in tissues with proven fibrotic degeneration, as in the example of cirrhosis of the liver. This led us to carry out the induction of hepatic fibrosis in fifty male and female adult Wistar rats (acquired from the Calouste Gulbenkian Foundation Biotery), twelve weeks old with a mean body weight of  $320 \pm 55$ g, in line with an established protocol<sup>15</sup>. We followed animal welfare criteria as set out by the European Union (Directive 86/609 EC) and in accordance with the Portuguese legislation in force (Decree Law no. 1005/92). The animals were kept at a constant temperature (37°C) during the procedure. At the end of this period the animals were humanely killed using excess anaesthetic. Their livers were collected and were weighed then embedded in a solution of Ringer lactate and frozen at -80°C.

The fibrosis of the hepatic tissue was macroscopically and histologically tested by means of the trichrome Masson and Van Gieson colourings, and 400g of liver was obtained for use in the following procedure.

### Isolation of the enzyme

The purification of the hydroxylase of the proline was carried out by adapting a protocol previously described by Kuuti *et al.*, 1975<sup>16</sup>.

The purification of the enzyme was as follows:

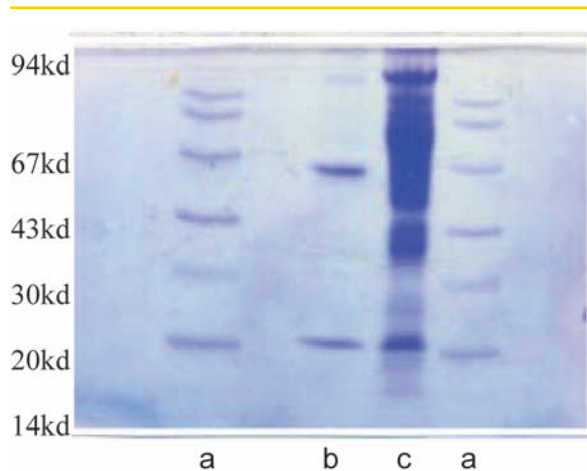
**1<sup>st</sup>** – The livers were subjected to -80°C and were then thawed, and each 100g was resuspended in three volumes of the designated solution, buffer A (0.1 M of Cl Na + 0.1 M of glycine + 0.01 M Tris HCl; pH=7.8) and 0.1% of "Triton X-100" and afterwards homogenized in a "potter"; **2<sup>nd</sup>** – We then proceeded to fragmentation with ammonium sulphate (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> and the precipitated material (30 - 60% p/v) was collected; **3<sup>rd</sup>** – This precipitated material was then resuspended in the homogenized buffer A; **4<sup>th</sup>** – The fraction obtained was applied to an affinity column containing poly-L-proline (M.M =30 Kd) balanced with the buffer A containing ditriitol (10 µM), and then eluted with 3 mg/ml of poly-L-proline

(M.M=5.7Kd); **5<sup>th</sup>** – The product was then concentrated by precipitation with 70% p/v of (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> and applied to a chromatography column of molecular exclusion "P-100" (1.5x70cm) balanced with ((NH<sub>4</sub>)HCO<sub>3</sub> (50mM); pH:7.6) and then eluted with this buffer.

The affinity column was developed as follows:

**1<sup>st</sup>** – The gel obtained was resuspended in 1 mM (HCl) and washed in a porous glass funnel (200ml/g); **2<sup>nd</sup>** – The alloy was dissolved in a buffer (0.1M NaHCO<sub>3</sub>; pH=8.3) containing 0.5 NaCl (1-10mM). The gel/alloy mixture was placed in agitation by rotation for 1 hour at room temperature or for one night at 4°C; **3<sup>rd</sup>** – The excess alloy was washed using at least 5 volumes of the alloy buffer; **4<sup>th</sup>** – Incubation in Tris-HCl (0.1M; pH=8.0) or in ethanolamine (1M; pH=8.0) for two hours was carried out to block the remaining active groups; **5<sup>th</sup>** – Finally we proceeded with the washing with three cycles of solutions with alternate pH: acetate buffer (0.1 M; pH=4.0), ClNa (0.5M) and Tris HCl buffer (0.1 M, pH=8.0).

1mg of enzyme, whose specificity was confirmed by electrophoresis in polyacrilamide gel (SDS-PAGE) was obtained using this technique (Fig. 1).



**Figure 1**

SDS-Page gel of the applied and further eluted material from the affinity column (Sepharose 4B-poly-L-prolin); **a** – molecular patterns; **b** – eluted material from the affinity column with poly-L-prolin; **c** – material applied to the affinity column

All this process was also applied to normal liver, with a much lower output being observed at the end. This fact has proved that the production of the enzyme in normal tissue is much lower than the quantity obtained in liver with cirrhosis.

#### **Production of the polyclonal antibody anti-prolyl 4-hydroxylase (anti-P<sub>4</sub>Hyd)**

We carried out the inoculation of three three-week old female Balb/c rats acquired from the Calouste Gulbenkian Foundation Biotery. The inoculums consisted of 0.1ml of the 30 µg enzyme mixture dissolved in buffered saline solution (PBS) with an equal volume of complete Freund adjuvant (FCA), subcutaneously administered in the dorsal region and in a total volume of 0.2 ml. Three administrations with intervals of three weeks were carried out. At the end of that period, we verified the absence of the antibody production (indirect ELISA) in blood samples collected from the tail vein. This fact led to two more administrations of 40µg of the enzyme and incomplete Freund adjuvant, also with an interval of three weeks. At the end of three weeks, after the last inoculation, a blood sample was collected from the orbital sinus of the inoculated animals and we verified that in one animal the ELISA test presented a title of 1/3000 and the other two 1/2000.

#### **Application of the antibody to the tissue embedded in paraffin – immunoperoxidase staining method**

The application test consisted of antibody to the tissue being embedded in paraffin, using the method of the avidin-biotin-peroxidase complex, applying as a primary the polyclonal antiserum developed by us, in a 1/100 dilution and to which we attributed the denomination of anti-P<sub>4</sub>Hyd. As a secondary antibody we used an antibody from a biotinilated rabbit anti-mouse (DAKO-E354) and streptavidin-biotin – HRP (DAKO-P364) which was revealed by a solution of diaminobenzidín-peroxide of hydrogen. In order to obviate the eventual appearance of a crossed reaction with the intra-cellular biotin, we had previously incubated the tissue to be studied with free avidin, and then with free biotin as described above<sup>17</sup>. Haematoxylin was used to colour the nucleuses for a better contrast in the preparations. For a complete positive control hepatic both human and mouse tissue with fibrosis was selected.

The negative control was obtained by means of two techniques:

- 1<sup>st</sup> – complete negative control, obtained through the application of the antibody to a tissue confirmed as fibroblast and collagen free; the nervous tissue;
- 2<sup>nd</sup> – negative control, obtained by replacing the incubation of the cirrhotic tissue with the primary antibody, for an incubation with PBS; the rest of the technique remaining unchanged.

The results obtained proved the antibody's reliability in identifying the enzyme prolyl 4-hydroxylase (P<sub>4</sub>Hyd) in renal sections, embedded in paraffin.

## **2 – Experimental Research**

As above mentioned, the aim of this phase was the identification of the prolyl-4-hydroxylase in the kidney tissue with fibrosis through the antibody previously produced (anti-P<sub>4</sub>Hyd). With this aim, an experimental model of hydronephrosis was developed by means of the unilateral ureteric ligation (UUO). Two groups of ten male Wistar rats (A and B), eight weeks old with a mean weight of 187.7±13.6g, were acquired from the Calouste Gulbenkian Foundation Biotery. Animal welfare criteria was followed in accordance with that established by the European Union (Directive no. 86/609 EC) and the Portuguese legislation in force (Decree Law nº 1005/92). Rats were anaesthetised with an intraperitoneal (i.p.) injection of 100mg/Kg of Ketamine, 12.5 mg/Kg of Xylazin and kept at a constant temperature (37°C) during the procedure. Under anaesthesia the left kidney and ureter were exposed via flank incision. The left ureter was ligated with 4-0 silk at the pielo-ureteric junction in both groups and the wound was closed in layers. Group A was sacrificed on the 4<sup>th</sup> day and group B on the 8<sup>th</sup> day by means of excess anaesthetic. Each group had a control (sham-operated), composed of five animals without any meaningful difference from the corresponding groups and which had undergone a similar procedure except for the ureter ligation. In the autopsy, the kidneys were removed, sagittally sectioned, fixed in buffered formaldehyde at 10% and embedded in paraffin. For the microscopic study, 5 µm sections were made for performing current optical microscopy techniques (Haematoxylin - Eosin, and

Masson's Trichromic). For the immunohistochemical study, we used the streptavidin-biotin complex method, using as primaries the polyclonal antibody anti-P4Hyd. As secondary antibody, we used an antibody from a biotinylated rabbit anti-mouse ("DAKO-E354"). We further used streptavidin-biotin-HRP ("DAKO-P364") which was revealed by a solution of diaminobenzidine-peroxide of hydrogen.

In order to obviate the eventual appearance of a crossed reaction with the intra-cellular biotin, we had previously incubated the tissue to be studied with free avidin and then with free biotin as described above<sup>17</sup>.

In the morphometric study, the frequency of tubular and interstitial cells, detected through the described immunoperoxidase staining method, was expressed as the number of tubular and interstitial cells in each area covered by a graded ocular grid measuring 1 cm<sup>2</sup> viewed under the x40 eyepiece of a Nikon microscope (0.0025 cm<sup>2</sup>). In each kidney 8-10 readings were made by section in the cortex-medulla direction.

The observations were always performed by the same observer and the mean results of group A, B and the control kidney were compared.

### ■ Statistical analysis

The morphometric data were plotted against the experimental durations and expressed as mean±SE. Statistical difference was assessed by analysis of variance. P<0.05 was considered to be significant.

## ■ RESULTS

### ■ Histology

Haematoxylin-Eosin and Masson's Trichromic

Compared to the control group (sham-operated) in group A (4 days), the left kidney (ligated) presented dilatation and atrophy of the tubular epithelia, interstitial oedema mainly in the medullar region, a moderate infiltration of the interstitium by mononuclear cells, and focal fibrosis (Fig. 2b). Approximate-

ly one fifth of the glomeruli presented atrophy. The vessels did not present relevant changes. In parallel, the contralateral kidney did not present any significant changes during this same period (Fig. 2a).

Compared to the control group (sham-operated) in group B (8 days), the left kidney revealed important changes in the tubulointerstitial architecture, with glomerular atrophy, increases in the cellular infiltration and interstitial fibrosis, which also involved some small and medium calibre vessels (Fig. 2c). In the right kidney (contralateral), there were no important detectable changes in the glomerula and tubulointerstitium.

### ■ Immunohistochemistry

Immunolocation of P4Hyd and morphometric analysis.

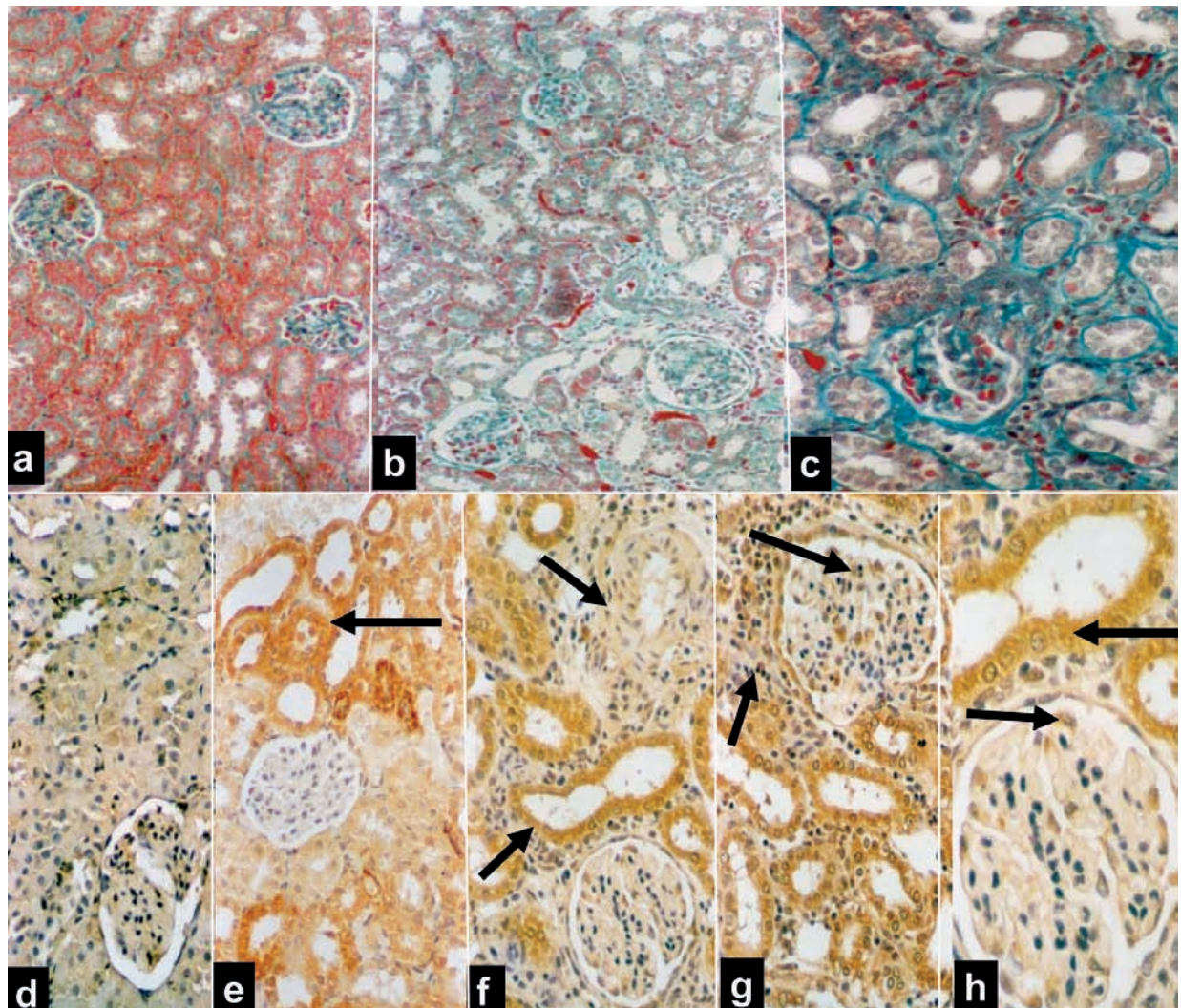
We found only a scant number of cells marked by this antibody in the kidneys of the control groups (sham-operated).

In the left kidneys, near the interstitium, the new expression of P4Hyd in mononuclear cells was observed on the 4<sup>th</sup> day, with a further decrease on the 8<sup>th</sup> day (14.1±2.7 % vs. 10.9±0.4 %; P<0.05) (Fig. 3). We also observed a progressive and meaningful increase in the expression of the enzyme phenotype in tubular cells detected by the antibody anti-P4Hyd from the 4<sup>th</sup> to the 8<sup>th</sup> day (39.7%±6.3% vs. 66.1±10.8 %; P < 0.05) (Fig. 4). This expression was clear near the proximal tubular epithelial cells, but also in distal cells, although less intensely (Fig. 2e).

We did not observe the marking of cells in the glomerula until the 4<sup>th</sup> day, but on the 8<sup>th</sup> day we observed the new expression of the enzyme in some glomerular cells (epithelial and mesangial) near fibrosis (Fig.2f-h).

On the vessels walls, near the fibrosis, some cells of the median layer were identified expressing the P4Hyd enzyme phenotype (Fig.2f).

In right kidneys (contralateral) we observed a slight but increasing marking of the epithelial tubular cells from the 4<sup>th</sup> to the 8<sup>th</sup> day (4.0±3.7 % vs. 6.5±3.7 %; P = ns), whereas interstitial cells evidenced a decreasing pattern (2.3±1.0 vs. 1.3±0.3 %; P <0.05) (Figs. 3, 4).



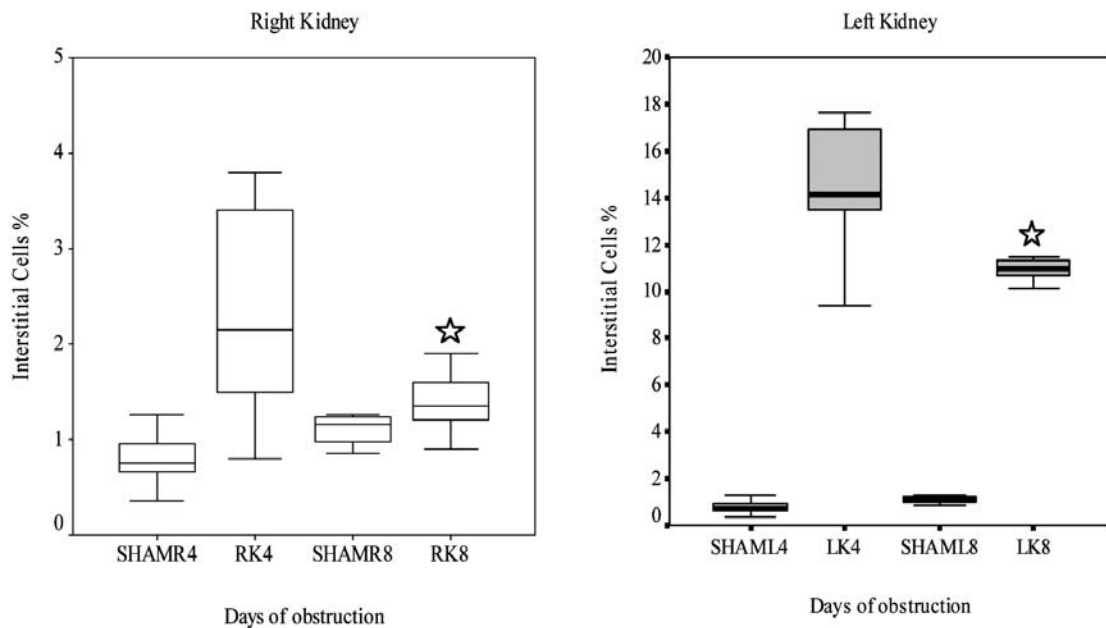
**Figure 2**

Kidney biopsy showing morphological changes in light microscopy and cellular new-expression of prolyl 4-hydroxylase (P4Hyd - immunoperoxidase staining method). **a** - right kidney (Masson's Trichromic x100); **b** - left kidney (group A - obstruction at 4 days; Masson's Trichromic x100); **c** - left kidney (group B - obstruction at 8 days; Masson's Trichromic x200); **d** - right kidney (anti-P4Hyd x200 - control); **e** - left kidney (group A - obstruction at 4 days; anti-P4Hyd in tubular cells x200 (black arrow)); **f-h** - left kidney (group B - obstruction at 8 days; anti-P4Hyd in tubular, glomerular (mesangial and epithelial), vascular (median layer) and interstitial cells x 400 (arrowhead)

## DISCUSSION

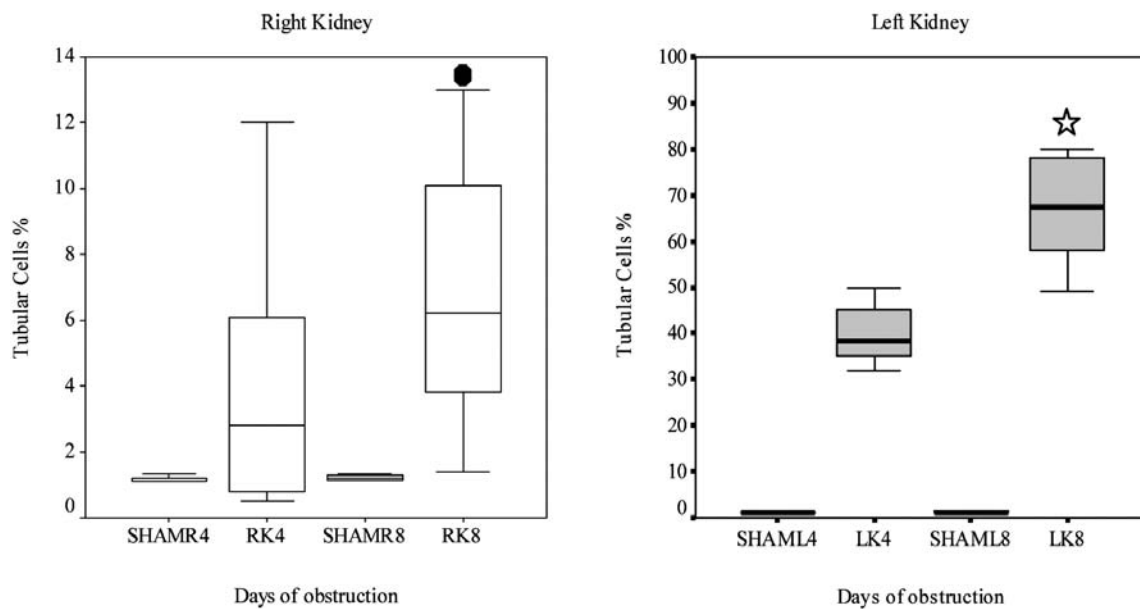
Studies into the mechanisms implicated in the pathogenesis of chronic kidney disease have already demonstrated the importance of the interstitial component of nephritis in the progression of renal disease. According to the literature, the relation between tubulointerstitial damage and the renal function is based on the combined interaction of several pathogenic mecha-

nisms, whose importance depends on the time of evolution and on the extension of the inflammatory process which they cause<sup>18</sup>. Fibrosis is characterised in general by the inadequate accumulation of extracellular matrix proteins which includes mainly types I, III and IV collagens, fibronectin and other proteoglycans<sup>5</sup>. This anomalous accumulation leads to the disorganisation of the normal structure of the organ with consequent loss of function. Other studies have demonstrated the impor-



**Figure 3**

Morphometric data for interstitial cells marked with anti prolyl 4-hydroxylase in kidneys with ureteral ligation (LK), contralateral (RK), and control (shamL/R) on the 4<sup>th</sup> and 8<sup>th</sup> day after obstruction (☆  $P < 0.05$ ; ●  $P = ns$ ).



**Figure 4**

Morphometric data for tubular cells marked with anti prolyl 4-hydroxylase in kidneys with ureteral ligation (LK), contralateral (RK), and control (shamL/R) on the 4<sup>th</sup> and 8<sup>th</sup> day after obstruction (☆  $P < 0.05$ ; ●  $P = ns$ ).

tance of the infiltrating cells of the renal interstitium, in particular of the monocytes, and their interaction with the resident glomerular and tubular cells of the kidney<sup>19,20</sup>. This interaction is mediated by means of multiple molecules, in which the autacoid substances, the cytokines, chemokines, and the growth factors are included, causing changes to the phenotype of the resident cells in the glomerula (mesangial and epithelial), the tubules, the interstitium and also in the vascular and perivascular wall<sup>21,22</sup>. According to some studies this new-expression of the cellular phenotype recapitulates at least in part, the phenotype expressed by the cell in early phases of the development of the foetal kidney<sup>23</sup>. The excessive matrix and extended production, surpassing the physiological capacity of resolution, is translated by the appearance of fibrosis, which basically results in an imbalance between the factors promoting the increase of the extracellular matrix and the factors responsible for its degradation<sup>24,25</sup>. In the chronic obstructive nephropathy model, and according to earlier studies, the progressive and heterogeneous increase in the interstitium is basically constituted of collagens of types I and III<sup>24</sup>.

Prolyl 4-hydroxylase resides within the endoplasmic reticulum, catalyses the formation of 4-hydroxyproline in collagens and more than 15 additional proteins with collagen-like sequences<sup>26</sup>. In our study, we searched for the cell elements implicated in the synthesis of collagen through the identification of the prolyl-4 hydroxylase enzyme phenotype, as an essential molecule to the synthesis and stability of the collagen triplice helix<sup>9</sup>. This stability may only succeed if the post-translational hydroxylation of proline occurs through the prolyl-4 hydroxylase enzyme. With the blocking of this enzyme, chains of collagen appear unable to be organised into triplice helical conformation, and therefore disintegrate<sup>26-28</sup>. Other studies on prolyl-4-hydroxylase have already been presented in the areas of hepathology<sup>29</sup>, ophthalmology<sup>30</sup>, cardiology<sup>31</sup> and nephrology<sup>32</sup>, mainly concerning the development of drugs to inhibit fibrosis.

In addition, cytoplasmic P4Hs play a critical role in the regulation of the hypoxia-inducible transcription factor HIF alpha. Collagen and HIF P4Hs constitute enzyme families as several isoenzymes have been identified<sup>33</sup>.

In this hydronephrosis model, the ischemia and chronic hypoxia are key pathogenic mechanisms in

the range of transformations which kidney tissue undergoes. The chronic hypoxia hypothesis proposed by Fine *et al*<sup>34</sup>, emphasises chronic ischemic damage in the tubulointerstitium as a final common pathway in end-stage kidney injury. The tubulointerstitial damage is associated with the loss of peritubular capillaries and interstitial fibrosis, which impairs oxygen diffusion and supply to tubular and interstitial cells, leading to cellular necrosis and/or apoptosis phenomena but also to cellular proliferation<sup>35,3</sup>.

Mammalian cells respond to changes in oxygen availability through a conserved pathway that is regulated by the hypoxia-inducible factor (HIF)<sup>37</sup>. Hypoxia inducible factor (HIF) is an alpha/beta heterodimeric transcriptional complex that plays a key role in directing cellular responses to hypoxia regulating the transcription of an array of genes, including those coding for glycolytic enzymes, erythropoietin, and VEGF<sup>37</sup>. Before the discovery of HIF regulation collagen was the only known hydroxyproline-containing protein. Recent studies have elucidated the mechanism of hypoxia-induced transcription, particularly that a prolyl hydroxylase (PHD) molecule regulates hypoxia-inducible factor (HIF PHD)<sup>38</sup>. Unlike the collagen prolyl 4-hydroxylase investigated in the present study, which acts on the tripeptide X-progly, those HIF PHDs require a much longer minimal HIF-derived peptide for optimal activity<sup>38</sup>. So, even though we have not probably studied the same isoenzymes, the PHDs are highly conserved and ubiquitously expressed, and it seems pertinent to establish a potential relationship between hypoxia, fibrosis, apoptosis and a coexistent intervention of the prolyl-hydroxylase (PHD) molecular family<sup>39,40</sup>.

As already mentioned, prolyl 4-hydroxylase isoenzymes have different expression patterns in several tissues<sup>41</sup>. While we did not characterise the specific isoenzyme in our model of obstructive nephropathy, we did detect a new and increasing expression of a P4Hyd molecule, related to the length of obstruction, and suggesting a close relationship between neo-expression of P4Hyd and progressive fibrosis. This new expression was patent in cells of the tubular epithelia, mainly proximal, which presented a growing expression of the P4Hyd, while an opposite relation was verified in the interstitial cells. These results still suggest that in a first stage after the obstruction (4<sup>th</sup> day), the interstitial and tubular cells actively participate in the inflammatory and ischemic

phenomena with a further and important contribution to the collagen synthesis. Later, on the 8<sup>th</sup> day, the enzyme expression in the interstitial cells suffered a decline, while an ongoing growth of the prolyl 4-hydroxylase expression by the tubular cells was observed, suggesting its dominant role in the collagen synthesis from this stage onwards.

This perspective strengthens the work of Strutz *et al*<sup>42,43</sup> and other authors<sup>6,44</sup>, on the potential of the tubular epithelial cells to differentiate into fibroblast, in a biological process designated as epithelial-mesenchymal transition. According to our observations, it seems that such phenotypic change must be followed by neo-expression of P4Hyd.

But our study also detected the neo-expression of prolyl 4-hydroxylase by cells in the median layer of the vessels wall, and also in the glomerular cells near fibrosis, on the 8th day of obstruction. This shows the participation of glomerular cells, namely epithelial and mesangial in glomerulosclerosis, as also smooth muscle cells in angiosclerosis, through their capacity to synthesise the collagen molecule. The detection of prolyl 4-hydroxylase neo-expression by glomerular cells in obstructive nephropathy must reflect the tubulo-glomerular extension of the fibrogenic mechanisms and reinforce the existence of a close physiopathological relationship between glomerular and tubulointerstitial disease.

Conversely, the incipient but curious expression of P4Hyd by some tubular and interstitial cells in the contralateral kidney suggests the role of haemodynamic and neuroendocrine adaptative mechanisms at the cellular and molecular level, with the potential to induce the transcription of collagen molecule.

In conclusion, our study emphasises the importance of collagen synthesis by tubular and interstitial cells, which may be a privileged target for the action of therapeutic agents, able to inhibit the prolyl 4-hydroxylase enzyme in different stages of the kidney disease and delay its progression.

Some interesting experimental models have shown promising results on this topic, but more research is needed, bearing in mind the words of James Black, Nobel Laureate in Chemistry for 1988: “The most fruitful basis of the discovery of a new drug is to start with an old drug”<sup>45</sup>.

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**Conflict of interest statement.** None declared.

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