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World J Urol. Author manuscript; available in PMC 2015 February 01.

Published in final edited form as:
World J Urol. 2014 February ; 32(1): 123–130. doi:10.1007/s00345-013-1050-2.

Calcium oxalate nephrolithiasis and expression of matrix GLA protein in the kidneys

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Abstract

Objectives—Polymorphism of the gene for matrix GLA protein (MGP), a calcification inhibitor, is associated with nephrolithiasis. However, experimental investigations of MGP role in stone pathogenesis are limited. We determined the effect of renal epithelial exposure to oxalate (Ox), calcium oxalate (CaOx) monohydrate (COM) or hydroxyapatite (HA) crystal on the expression of MGP.

Methods—MDCK cells in culture were exposed to 0.3, 0.5 or 1 mM Ox and 33, 66 or 133–150 $\mu g/cm^2$ of COM/HA for 3–72 h. MGP expression and production were determined by Western blotting and densitometric analysis. Enzyme-linked immunosorbent assay was performed to determine MGP release into the medium. Hyperoxaluria was induced in male Sprague–Dawley rats by feeding hydroxyl-L-proline. Immunohistochemistry was performed to detect renal MGP expression.

Results—Exposure to Ox and crystals led to time- and concentration-dependent increase in expression of MGP in MDCK cells. Cellular response was quicker to crystal exposure than to the Ox, expression being significantly higher after 3-h exposure to COM or HA crystals and more than 6 h of exposure to Ox. MGP expression was increased in kidneys of hyperoxaluric rats particularly in renal peritubular vessels.

Conclusion—We demonstrate increased expression of MGP in renal tubular epithelial cells exposed to Ox or CaOx crystals as well as the HA crystals. The most significant finding of this study is the increased staining seen in renal peritubular vessels of the hyperoxaluric rats, indicating involvement of renal endothelial cells in the synthesis of MGP.

Keywords

Urolithiasis; Matrix GLA protein; Calcium oxalate; Randall's plaque

Introduction

Nephrolithiasis, the formation of stones in the kidneys, is the third most common affliction of the urinary tract and is associated with a high rate of recurrence with an average rate of 6.78 years [1]. Stone formation is controlled by many factors, including urinary supersaturation, with respect to the crystallization of the stone-forming salts. Calcium oxalate (CaOx) is the most common type of kidney stone. CaOx supersaturation is controlled by the urinary calcium, oxalate, pH as well as the concentration of various mineralization modulators present in the urine. Pathogenesis involves not only

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crystallization but also crystal retention within the kidneys which is a highly regulated process requiring cellular changes and expression of a variety of macromolecules including the modulators of crystallization and inflammation such as osteopontin (OPN), Tamm–Horsfall protein (THP), bikunin (BK), hyaluronic acid, CD 44 and matrix GLA protein (MGP) [2, 3].

MGP is a vitamin K-dependent protein functioning primarily as an inhibitor of vascular calcification [4]. Although first isolated from bone [5], MGP mRNA expression is tenfold higher in lungs and heart and five times higher in kidneys [6]. MGP gene polymorphism is associated not only with vascular calcification [7] but also with kidney stone formation [8]. Results of studies show increased expression of MGP in renal tubules of rats fed ethylene glycol for 28 days [9] as well as in rat renal proximal tubular cell line, NRK-52E, after the exposure to Ox or CaOx monohydrate crystals [10].

In this study we investigated the expression of MGP in renal epithelial cells of distal tubular origin as represented by MDCK cells as well as in a rat model of CaOx nephrolithiasis induced by the administration of hydroxy-L-proline (HLP) [11]. In addition, we examined the response of cells to hydroxyapatite (HA) crystals, the most common crystal in the urine. Results show that MDCK cells do express MGP and its expression is increased when cells are exposed to Ox or crystals of CaOx monohydrate (COM) or HA. Increased MGP expression is seen in cells of the renal tubules, particularly those with CaOx crystal deposits. Most importantly, MGP staining is seen in the peritubular vessels of the renal medulla of hyperoxaluric rats.

Materials and methods

Cell culture

MDCK cells were obtained from ATCC (Manassa, VA, USA) and maintained as sub-confluent monolayers at 37 °C in 5 % CO_2. The culture was grown in 75 cm^2 Falcon tissue culture flasks in a 1:1 mixture and modified Eagle's medium (DMEM) nutrient mixture and F-12 medium (DMEM/F-12) (Gibco BRL, Grand Island, NY, USA) containing 10 % fetal bovine serum (Gibco BRL, Grand Island, NY, USA), 2 % streptomycin/penicillin (Sigma Chemical Co., St. Louis, MO, USA) and pH 7.4. The medium was changed every 3–4 days.

The cells were seeded in 100-mm tissue culture dishes in the medium for Western blot; in 8-well chamber slide for TBE; in 24-well plates for SOD and in 96-well plates for LDH assays. Once they reached the confluence, the media were removed and cells were washed with phosphate-buffered saline (PBS). The cells were placed in serum- and sodium-pyruvate-free media supplemented with insulin/transferrin/ selenium mix (Gibco BRL, Grand Island, NY, USA), hydrocortisone (Sigma Chemical Co., St. Louis, MO, USA), tri-iodo-L-thyronine (Sigma Chemical Co., St. Louis, MO, USA) and prostaglandin E1 (Sigma Chemical Co., St. Louis, MO, USA) overnight to arrest the growth. The cells were then exposed to the serum- and sodium-pyruvate-free media supplemented with oxalate (Ox) (0.3, 0.5 and 1 mmol) or calcium oxalate monohydrate (COM)/hydroxyapatite (HA) crystals (33, 66 and 150 $\mu g/cm^2$) and incubated at 37 °C for 3, 6 or 24 h for MGP expression. Crystals were not detected in the medium at any concentration, but the presence of microscopic crystals cannot be completely ruled out. The culture media were collected in 2-ml microfuge tubes and stored at −20 °C until further analysis, and the proteins were isolated from the cells using TRIZOL Reagent (Life Technologies, Grand Island, NY, USA) according to the manufacturer's procedure and stored at −80 °C.

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Immunoblotting and ELISA

The proteins isolated from MDCK cells were subjected to sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and blotted with MGP mouse monoclonal antibody. The band cross-reacting with the antibody was analyzed by densitometry using Image J program (Version 1.33, NIH USA). After exposing the cell to Ox and COM/HA for the specified times, cells were dissociated from the wells by a cell scraper in cold phosphate-buffered saline (D-PBS) and washed two times in D-PBS with centrifuging at 4 °C, 430 g for 5 min. Cell pellets were resuspended in CellLytic^{TM} MT mammalian Tissue Lysis/Extraction reagent (Sigma Chemical Co., St. Louis, MO, USA) with protease inhibitors and homogenized on ice with cell homogenizer. The homogenate was centrifuged at 10,000×g for 10 min at 4 °C, and the supernatant was aliquoted and stored at −80 °C for further analysis. A Bio-Rad DC protein assay (Bio-Rad, Hercules, CA, USA) was performed to determine protein concentration according to the manufacturer's protocol, using BSA as a standard. Fifty microgram of protein samples was loaded per lane in a 4–20 polyacrylamide Tris–HCl Criterion gel, and it was run at 80 V for 90 min. After SDS–PAGE, the proteins were transferred from the gel to 0.45 μlm nitrocellulose membrane (Fisher, Suwanee, GA, USA) using a Bio-Rad immunoblotting apparatus at 80 V for 60 min. The membrane was blocked with 5 % nonfat milk in Tris-buffered saline/Tween solution (TBST) for 60 min. The blocking solution was decanted and the membrane incubated overnight at 4 °C in primary antibody (mouse monoclonal, MGP, Santa Cruz, CA, USA) at 1:1000 concentration in 5 % nonfat milk. The membrane was washed in TBST three times for 10 min each and then incubated in HRP-conjugated secondary antibody (1:1,000) in 5 % nonfat milk in TBST for 1 h at room temperature on a shaker. The membranes were again washed three times for 10 min with TBST. ECL Plus Western Blotting detection Reagents was used to visualize the bands on a KODAK X-ray.

The release of MGP into the medium was determined using Enzyme-Linked Immunosorbent assay (ELISA) kit (Life Sciences Advanced Technologies, Inc., St Petersburg, FL, USA) according to the manufacturer's instructions using standard procedures.

Animal model of CaOx nephrolithiasis

CaOx nephrolithiasis was induced by the administration of hydroxyl-$_{L}$-proline (HLP) to male Sprague–Dawley rats as described earlier [11]. In brief, 8-week-old and weighing on average about 150 g rats were divided into two groups of six each: Group 1 rats were fed a normal rat chow and sterile water, while Group 2 rats received food supplemented with 5 % HLP (ICN Biochemicals, Aurora, OH). At day 28 posttreatment, the rats were killed and their kidneys removed and processed for light microscopic observations as previously described [11]. In this model rats quickly become hyperoxaluric with approximately 10–12-fold increase in urinary excretion of oxalate seen by day 14. Deparaffinized sections were incubated overnight at 4 °C with primary antibodies reactive to MGP. Isotype controls were performed using rabbit IgG. The staining was developed by the addition of diaminobenzidine (DAB) substrate (Vector Labs, Burlingame, CA) and counter-stained with hematoxylin. Images were taken using the Zeiss Axiovert 200 M microscope (Carl Zeiss Microimaging, Inc., Thornwood, NY, USA).

Statistical analysis

Statistical comparisons between the groups were made by means of one/two-way analysis of variance (ANOVA) with post hoc Dunnett's/Bonferroni posttest, using Graph Pad Prism5 and Graph Pad InStat3 (GraphPad Software, San Diego, CA, USA). The *p* value of less than 0.05 was regarded as significant.

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Results

MGP in cell culture

The extracts of control and treated MDCK cells rats (Ox 0.3, 0.5 and 1 mM; COM/HA 33, 66 and 150 $\mu g/cm^2$) were subjected to Western blotting and stained with MGP mouse monoclonal antibody (Fig. 1). Exposure to oxalate at any concentration had no effect on MGP expression for the first 6 h (Fig. 1a). However, 24-h exposures to various concentrations of Ox led to manyfold increases compared to the control, with no significant difference between exposures to 0.3 mmol or 0.5 mmol Ox. MGP expression was significantly higher on exposure to 1 mM Ox than when cells were exposed to other two lower concentrations.

Expression of MGP was significantly increased when MDCK cells were exposed to COM crystals. The response was both concentration and time dependent (Fig. 1b). The highest MGP expression was seen after exposures to higher concentrations of crystals. There was no significant difference in MGP expression between exposures to 33 and 66.7 $\mu g/cm^2$ crystal concentrations for various time periods. But a 24-h exposure to 150 $\mu g/cm^2$ COM crystals caused the highest increase in MGP expression.

The exposure to HA crystals also produced manyfold and significant increases in MGP expression. The increases in MGP expression were time and concentration dependent and somewhat similar to those seen after exposures to COM crystals (Fig. 1c). However, expression was manyfold higher when cells were exposed to the higher concentrations, 66.7 $\mu g/cm^2$ and 150 $\mu g/cm^2$, of HA crystals than to the lower concentration of only 33 $\mu g/cm^2$.

MGP release into the medium

The release of MGP into the medium was determined using an ELISA kit. Exposure to Ox, HA as well as COM crystals did not cause significant and consistent change in the secretion of MGP into the medium (Fig. 2) compared to their controls. However, the secretion of MGP into the medium increased significantly, compared to their controls, after the exposure to higher concentrations, 1 mmol of Ox or 133 $\mu g/cm^2$ of COM or HA for 48 h produced. The inconsistencies may be a result of MGP's tendency to bind to calcium-containing crystals.

Immunohistochemical analysis of the kidneys

Immunostaining was used to determine the production of MGP by renal cells in response to hyperoxaluria and CaOx crystal deposition. As described in our earlier publications, hyperoxaluria induced by administration of hydroxyl-L-proline to rats led to the formation of CaOx crystals and their deposition in the kidneys, mostly in the tubules of the renal cortex. MGP expression was almost nonexistent in kidneys of normal rats (Fig. 3). Occasional staining was, however, clearly evident in the glomeruli (Fig. 3b) as well as peritubular vessels (Fig. 3c). Kidneys of hyperoxaluric rats, on the other hand, showed heavy staining (Figs. 4, 5). Staining was associated with intratubular crystal deposits (Fig. 5). But most noticeable staining was seen in interstitial cells and peritubular blood vessels of the hyperoxaluric rat kidneys. Staining of the blood vessels was more pronounced in the renal medulla in complete absence of renal crystal deposits (Fig. 4a). Epithelial cells lining the renal medullary tubules showed no staining (Fig. 4b).

Discussion

The stone formation is a result of mineralization within the kidneys and involves crystal nucleation, growth, aggregation and their eventual retention within the kidneys. Since

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smooth movement of urine through the nephrons is essential for normal functioning of the kidneys, and crystals and stones interfere with this, the body produces a number of substances to inhibit the formation of crystals and stones. Some of these inhibitors are produced by the renal epithelial cells in response to urinary changes which may promote crystallization. Increased urinary excretion of oxalate and the formation of CaOx crystals are such harmful conditions. Animal model and tissue culture studies are performed to determine the renal cellular response to hyperoxaluria and CaOx crystal deposition to better understand the pathogenesis of CaOx nephrolithiasis, the most common type of kidney stone. Results of such studies have shown that renal epithelial cells respond by increased production of macromolecular inhibitors of crystallization such as OPN, bikunin and related proteins, prothrombin fragment-1, α-1-microglobulin, fibronectin, calgranulin and hyaluronic acid.

Matrix GLA protein is a vitamin K-dependent protein functioning primarily as vascular inhibitor of calcification [12, 13]. To become biologically active, MGP undergoes carboxylation of glutamic acid residues, which occurs at the blood vessel level and requires vitamin K as a cofactor. Mutations in the MGP gene lead to Keutel syndrome, a disorder associated with extensive soft tissue and vascular calcification [14]. The calcium deposition is primarily located within the media of the vessel wall. MGP knockout mice die within 2 months as a result of arterial calcification and blood vessel rupture [15], while restoration of MGP in these mice prevented arterial calcification [16]. Polymorphism of MGP may play a role in vascular calcification [17] and has shown an association with myocardial infarction [7]. Single nucleotide polymorphism of MGP gene has also been shown to be associated with CaOx kidney stone disease in both the Japanese [8] and Chinese populations [18].

A number of experimental studies have also shown an association between the formation of CaOx kidney stones and MGP. Renal epithelial cells express MGP mRNA which is regulated by growth factors and cell density [19]. The expression is increased in experimentally induced situations when renal cells come in contact with various types of crystals. MGP gene and protein expression was increased in time- and concentration-dependent manner in the rat renal epithelial cell line, NRK 52 E, when exposed in vitro to oxalate and CaOx crystals [10]. MGP mRNA expression was also increased in vivo in kidneys of rats made hyperoxaluric by the administration of ethylene glycol [9]. The renal expression of MGP was also increased in adenine phosphoribosyltransferase (APRT)-deficient mice with 2,8-dihydroxyadenine nephrolithiasis [20].

Interpretation of results obtained from animal model and tissue culture studies, with respect to their translation into understanding the pathophysiology of human diseases, is often challenging. Intuitively, cellular response to crystals and oxalate in tissue culture, within human kidneys, and kidneys of animals used in various studies, may well be different with respect to the concentrations required as well as consequences of cell exposures. Both mice and rats normally excrete manyfold higher oxalate in the urine than humans do, and still higher concentrations of oxalate are required for crystal deposition in the kidneys [21]. In fact, mice do not produce CaOx crystal deposition in their kidneys even in the presence of severe hyperoxaluria alone [22]. On the other hand, human data can only provide a snapshot of a long process and in case of stone disease, perhaps of the end when process of stone formation has already finished.

In this study, we investigated the expression of MGP in MDCK cells, which are generally recognized as of distal tubular epithelial origin. MDCK cells were selected because the distal tubular epithelial cells are most likely to come in contact with higher oxalate concentration as well as crystals, after the reabsorption processes in the proximal tubules and the loops of Henle, leading to supersaturation in the last parts of the nephrons. We

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investigated the time- and concentration-dependent effects of not only Ox ions and COM crystals but also HA crystals on the expression of MGP in the MDCK cells. HA crystals, as the constituent of Randall's plaques [23–25], play an essential role in the formation of CaOx kidney stones. MGP is expressed differently in response to Ox ions and COM or HA crystals. When the cells were treated with 0.3, 0.5 and 1 mM Ox for 3, 6 or 24 h, the MGP expression was seen only after 24-h incubation in a dose-dependent manner. On the other hand, exposure to COM or HA crystals leads to increased MGP expression rather quickly, indicating that MGP expression is more sensitive to crystals than to Ox ions, which has also been shown earlier for NRK 52E cells [10]. Oxalate ions and the crystals may be affecting different pathways, one perhaps more direct than the other.

The results of the animal model study show sporadic expression of MGP in both the renal cortex and medulla of the normal control rats. Staining was seen mostly in the glomeruli and the interstitium. Deposition of CaOx crystals in the kidneys led to a significant increase in MGP staining, which was seen in areas without crystal deposits, as well as in association with the CaOx crystals. In this model CaOx crystal deposition was mostly seen in the cortex. MGP staining was seen in the blood vessels and glomeruli as well as the organic material mixed with the CaOx crystals. MGP binds to calcific crystals [26] and has been found in the organic matrix of kidney stones [27]. In the renal medulla where crystals were rare, heavy MGP staining was evident in the peritubular vessels. Utilizing global transcriptome analyses, we have recently shown a highly significant increase in the expression of MGP gene in the kidneys of these hyperoxaluric rats [28]. Moreover, gene expression in the renal medulla was significantly higher than in the renal cortex. Heavy MGP staining of peritubular vessels in the kidney has not been previously reported in humans or rat models of CaOx nephrolithiasis but should not be a surprise since MGP is expressed in both the normal and calcified blood vessels in the humans [4, 5, 29, 30]. Moreover, endothelial cells play an essential role in extrahepatic posttranslational carboxylation of MGP necessary for its activation.

Both animal model and tissue culture studies presented here and elsewhere show that high Ox and CaOx and HA crystals can provoke renal epithelial cells to produce MGP. Our animal model studies also show an increase in MGP expression in the blood vessels, which may be a result of endothelial cell exposure to oxalate. Cellular exposure to oxalate leads to the production of reactive oxygen species and development of oxidative stress. Expression of MGP is enhanced in the endothelium by exposure to oxidative stress inducing atherogenic stimuli [31].

The molecular mechanism of calcification inhibition by MGP is not yet understood. However, it is suggested that MGP directly interferes with the crystal formation and their growth either by binding calcium and interacting with the apatite crystals [26, 32] or by binding bone morphogenetic proteins or other extracellular matrix components [33]. How does the MGP influence and inhibit stone formation? Recent studies [23, 24] have confirmed earlier observations [34] that CaOx kidney stones develop by deposition of CaOx crystals on apatitic plaques, called Randall's plaques, which are present on renal papillae. Such plaques are common in stone formers and have been suggested to start in the renal interstitium around loops of Henle [23] and/or blood vessels [35]. Increased expression of MGP in the blood vessels of the hyperoxaluric rat renal medulla points to the vessels as the site of MGP action in the kidneys during stone formation. It is also indicative of similarities between the development of Randall's plaques and vascular calcification.

Recent studies have provided evidence for communication between renal tubular epithelium and vascular endothelium, and such interactions are proposed to play significant role in the pathogenesis of renal diseases including hypertension [36]. As far as CaOx nephrolithiasis is

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concerned, there is evidence for collaboration between endothelial and epithelial cells in the presence of oxalate. Exposure to oxalate, of renal epithelial cells in co-culture with endothelial cells, resulted in increased apoptotic cellular death compared to when epithelial cells were exposed in individual cultures [37]. Interestingly, pretreatment with pyrrolidine dithiocarbamate, an antioxidant, reduced cell death in the co-culture, suggesting a role for reactive oxygen species.

As mentioned earlier, single nucleotide polymorphisms of MGP genes have been found associated with CaOx kidney stone formation as well as arterial calcification and myocardial infarction. An association also exists between stones and arterial calcification and myocardial infarction [38]. Perhaps, a genetic variability of MGP plays a role in both conditions and is induced by oxidative stress. We propose that production of MGP is a normal response to lithogenic challenges be they crystals or high oxalate. Production of defective protein by the tubular epithelial or endothelial cells leads to crystallization in the tubular lumen or basement membrane of the peritubular vessels, respectively, latter leading to the development of Randall's plaque.

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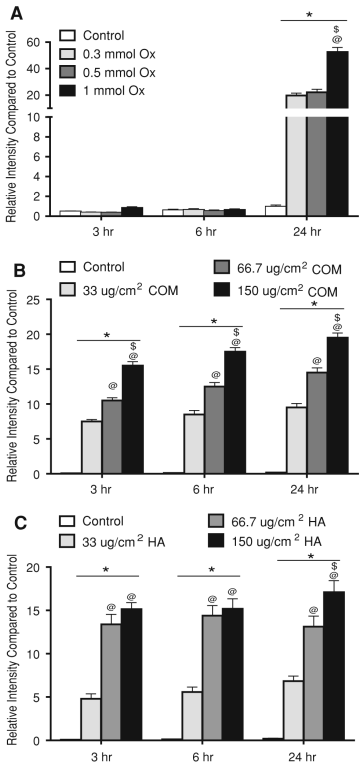

Fig. 1.

Relative densitometric expression of MGP by Western blotting in MDCK cells, exposed to **a** Ox 0.3, 0.5 and 1 mM, **b** COM and **c** HA 33, 66 and 150 μg/cm^2, for 3, 6 and 24 h. *p < 0.05, control versus all concentrations; $^@p$ < 0.05, 0.3 mmol/33 μg/cm^2 versus 0.5 and 1 mmol/66 and 150 μg/cm^2; $^\$p$ < 0.05, 0.5 mmol/66 μg/cm^2 versus 1 mmol/150 μg/cm^2

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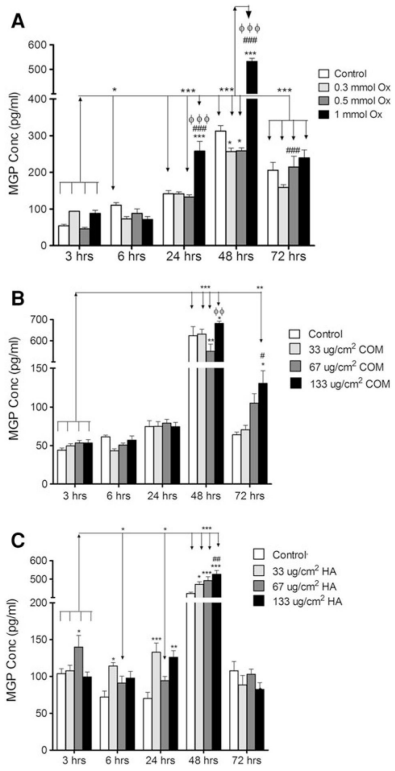

Fig. 2.

Changes in MGP excretion in media by MDCK cells exposed to **a** Ox 0.3, 0.5 and 1 mM; **b** COM and **c** HA, 33, 66 and 133 μg/cm^2 for 3, 6, 24, 48 and 72 h using enzyme-linked immunosorbent assay *(ELISA). Arrows* show significant difference across the groups, that is, 3 h versus 6, 24, 48 and 72 h. *$p < 0.05$, control versus all concentrations; $^{\#}p < 0.05$, 0.3 mmol/33 μg/cm^2 versus 0.5 and 1 mmol/66 and 133 μg/cm^2; $^{\varphi}p < 0.05$, 0.5 mmol/66 μg/cm^2 versus 1 mmol/150 μg/cm^2 within the time period

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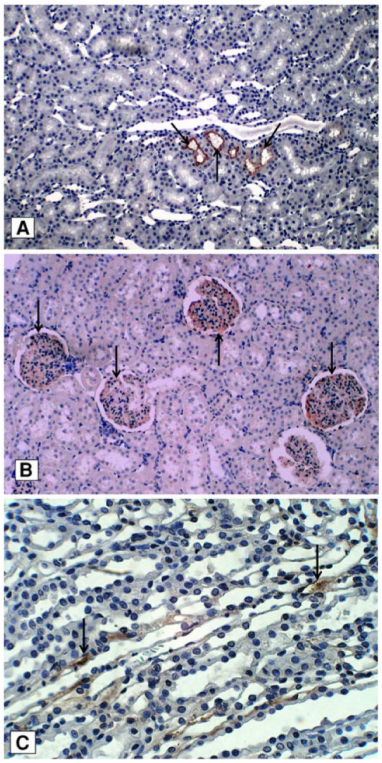

Fig. 3.
Representative slides of immunohistochemical staining for MGP expression from kidney of normal rat. **a** Normal control, MGP expression almost nonexistent. X10, *arrows* point to staining near the blood vessels. **b** Occasional expression in glomeruli (*arrows*). X10. **c** Sporadic staining of the peritubular vessels (**arrows**). X40

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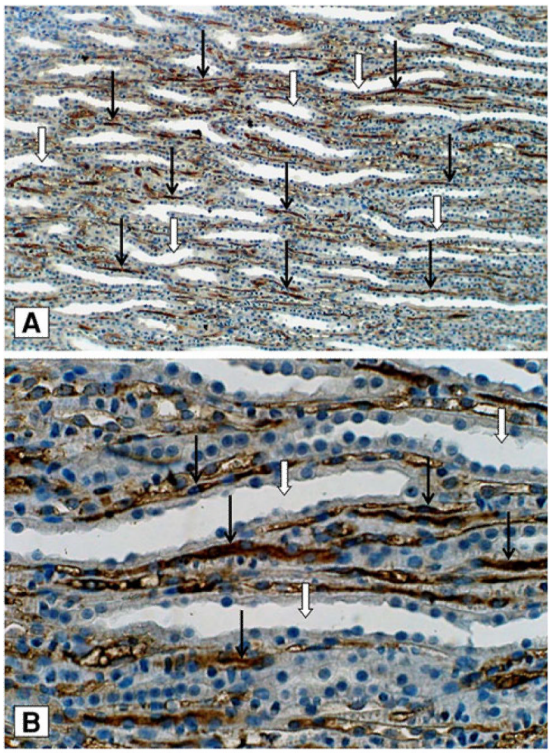

Fig. 4.

Representative slides of immunohistochemical staining for MGP expression in hyperoxaluric rat kidneys without noticeable crystal deposition. **a** Heavy staining in renal medulla blood vessels, while no staining in epithelial cell lining of the renal medullary tubules. **a** X10. **b** X40. *Dark arrows* point to staining in peritubular vessels. *White arrows* point to the renal tubules

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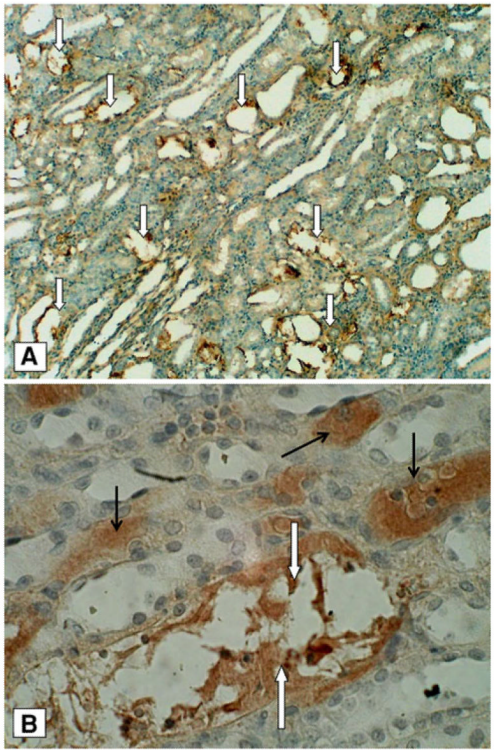

Fig. 5.

Representative slides of immunohistochemical staining for MGP expression in hyperoxaluric rat kidneys with heavy deposition of CaOx crystals. Intense staining associated with intratubular crystal deposits. **a** X10. **b** X40. *Dark arrows* point to peritubular staining. *White arrows* point to the renal tubules with crystal deposits. Crystals have fallen off during processing