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ORIGINAL PAPER

A. Schmiedl · P.O. Schwille · E. Bonucci · T. Seitz
R.M. Schwille · M. Manoharan

Renal cortical calcification in syngeneic intact rats and those receiving an infrarenal thoracic aortic graft: possible etiological roles of endothelin, nitrate and minerals, and different preventive effects of long-term oral treatment with magnesium, citrate and alkali-containing preparations

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Abstract Renal cortical nephrocalcinosis (C-NC) is a rare disorder of uncertain etiology. Using highly inbred (syngeneic) male Lewis rats, we describe the spontaneous occurrence of histologically detectable C-NC in sham operated control rats (Sham; $n=12$), its aggravation following grafting of the ascending thoracic aorta from a donor rat to the infrarenal aorta of a recipient (ATx; $n=12$), and differences in C-NC inhibition after 12 weeks of oral administration of magnesium (Mg), citrate and alkali. C-NC is characterized by Kossa-positive areas located in cells of the proximal tubule close to blood vessels and also, to a lesser extent, within glomeruli. After ATx there was vascular overproduction of endothelin (ET-1) but decreased production of nitrate; in renal cortical tissue there was an excess of calcium over Mg and phosphorus and oxalate over citrate. In plasma there was an increase in calcium and creatinine within the normal range. Calcification of tubular cells was eliminated by a preparation containing potassium, sodium and bases (from citrate degradation and bicarbonate) in addition to Mg. Less effective than the latter was Mg-potassium citrate and least effective, Mg citrate. The former treatment also normalized calcemia and urinary nitrate, but only incompletely suppressed ET-1 and had no significant effect on glomerular calcification or tissue and urinary oxalate. Urinary ET-1 excess appeared directly related to the cortical tissue calcium/Mg ratio, and urinary excretion of Mg, citrate and total protein appeared to be inversely related to the severity of C-NC. It was concluded that (1) the highly inbred rat is prone to precipitation of calcium phosphate in the renal cortex; (2) this type of C-NC occurs in close proximity to and within renal vascular tissue and is associated with an imbalance of vasoconstrictors and vasodilators of endothelial origin; (3) effective inhibition of C-NC can be achieved by an alkalinizing combination of Mg, potassium, sodium and citrate, underscoring its utility in the prophylaxis of pathological calcium phosphate deposition. The significance of these findings for the etiology and treatment of clinical disorders with renal and vascular calcification is uncertain and requires further investigation.

Keywords Syngeneic rat · Aortal graft · Renal-cortical calcification · Renal-cortical minerals · Oxalate · Citrate · Endothelin and nitrate · Prophylaxis by magnesium

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A. Schmiedl · P.O. Schwille (✉) · T. Seitz · M. Manoharan
Mineral Metabolism and Endocrine Research Laboratory,
University of Erlangen, Maximiliansplatz, 91054 Erlangen,
Germany

R.M. Schwille
Department of Surgery,
University of Erlangen, Germany

E. Bonucci
Department of Experimental Pathology,
University of Rome, Italy

Introduction

The deposition of calcium phosphate in soft tissue is frequently seen in the kidney, as nephrocalcinosis (NC), and in blood vessels as a component of atherosclerosis. At present it is not known whether calcification in these organs is etiologically linked. NC has a predilection for the functionally most highly developed medullary region, and affects tubular cells and interstitial tissue [2]. It frequently occurs in association with systemic mineral metabolic abnormalities such as hyperparathyroidism and vitamin D excess but also with hypomagnesemia and dyslipidemia of dietary origin [2, 29, 30]. Vascular calcification is typically located in the subendothelial

region and the media of arteries, but it is not clear whether calcification occurs in renal arterioles and capillaries. If yes, then it would be of interest to know whether there is a link between NC and renal vascular calcification, and whether both can be ascribed to vascular or to local factors such as altered tissue minerals, or whether both factors are involved. The current literature contains no information on this.

Current research on atherosclerosis is focused on genetic and environmental risk factors. In addition, vascular injury resulting, for example, from hypertension, dyslipidemia or surgery is under discussion as possibly initiating a cascade of events ultimately leading to vascular calcification [27, 39]. In the case of vascular injury caused by surgery, transplantation of the juxtacardial artery ascending to the infrarenal aorta in the rat is followed by calcification of the graft endothelium and subendothelial media [22]. Working with a highly inbred rat strain, we observed that aortic graft calcification could be inhibited by magnesium (Mg) administered in the drinking water [34]. Further investigations revealed alterations of renal mineral concentrations, mainly in the cortical region, containing glomerular arterioles and post-glomerular capillaries. It was therefore hypothesized that the presence of calcifications in the kidney might be another characteristic of this tissue transplantation model [22, 34].

In the present work we illustrate our findings in intact and graft-bearing rats in more detail, in particular with regard to the renal cortical mineral content, to the accompanying histological changes of the kidney, and to two indicators of vascular endothelial and one of vascular connective tissue metabolic activity. In addition, the anti-calcification effects of several Mg preparations were evaluated. The combined data permit the assumption that in genetically uniform (syngeneic), intact rats, and especially in graft-bearing specimens, renal tubular and glomerular structures are prone to calcify, with simultaneous alteration of tissue minerals and vascular effectors, and that calcifications can be prevented by treatment with alkalinizing Mg preparations.

Materials and methods

Animals

The investigations described were approved by an ethics committee, as currently required by the German law for the protection of animals. Male rats of the syngeneic Lewis strain (Wiga, Sulzfeld, Germany), weighing 220–240 g, were housed individually in conventional cages under a 12-h light/12-h dark cycle. During a 1-week acclimatization period prior to the study they had free excess to tap water. Before surgery food, but not water, was withdrawn for 12 h.

Surgery

The animals were anesthetized by ether, then tracheotomized, intubated and treated with analgesics in a standardized manner (air–oxygen mixture, isofluorane). The donor operation, lasting 2–3 min, comprised a laparo-thoracotomy followed by the clamping and explantation of an 8- to 10-mm-long segment of the ascending thoracic aorta. The segment was then irrigated with 0.15 M sodium chloride at 2–4°C. The graft was then implanted in the recipient animal. The recipient operation lasted about 30 min and comprised laparotomy, dissection of tissue surrounding the infrarenal aorta, clamping between two clips, resection, and insertion of the graft within an ischemic period of 8–10 min. The sham operation included a laparotomy and identical clamping of the aorta. The abdomen was closed after 30 min to mimic the unspecific stress of the recipient operation. A total of 108 rats were used.

Experimental groups

Animals were fed normal rat chow (Altromin, code 1000, Lage, Germany) with a calcium to phosphorus ratio of 1:1 and 0.9% Mg content (for other components of this standard diet [29, 30]) ad lib, and were given deionized water with or without additives to drink (see below). The observation period was 12 weeks ± 2 days. Five groups were used.

Group I

Sham operation (Sham), $n = 12$; these rats served as an intact-aorta control group.

Group II

Aortic graft (ATx), $n = 12$; animals received no further treatment.

Group III

ATx, $n = 12$; the animals received deionized water containing 13 g/l (29 mmol/l) water-soluble tri-magnesium di-citrate (MgC; Boehringer, Ingelheim, Germany), corresponding to 58 mmol citrate, 87 mmol Mg, 174 milliequivalent bases (from metabolic degradation of citrate to bicarbonate).

Group IV

ATx, $n = 12$; the animals received deionized water containing 4.3 g/l MgC and 12.0 g/l (39 mmol) neutral potassium citrate (PC; Fluka, Ulm, Germany), corresponding to 66 mmol citrate, 29 mmol Mg, 117 mmol potassium, 198 milliequivalent bases.

Group V

ATx, $n = 12$; the animals received deionized water containing 4.3 g/l MgC, 9.0 g/l PC, and 2.52 g/l sodium bicarbonate (SB; Fluka, Ulm, Germany), corresponding to 44 mmol citrate, 29 mmol Mg, 85 mmol potassium, 28 mmol sodium, 160 milliequivalent bases.

In the following groups I–V are alternatively named Sham, ATx, MgC, MgCPC, MgCPCSB, respectively. The intention to treat the latter three groups with magnesium, citrate and alkali was prompted by information obtained from previous work of our own in which an oral supply of Mg and bases proved to be an effective prophylaxis against cortico-medullary NC in rats [29]. This treatment prevented calcium oxalate crystallization in the postprandial urine of humans forming calcium-containing renal stones [31]. For the present experiments it was further assumed that, provided there was similar fluid intake in groups III–V, these rats would receive bases in amounts varying by a maximum of 20 per cent, while the supply of Mg, sodium and potassium would vary by several orders of magnitude. Differences in calcification would therefore be ascribable to differences in the amount of cations supplied, rather than to the amount of bases.

Procedures and collection of samples

Weekly arterial blood pressure measurement via the tail cuff method yielded mean values of between 120 and 140 mm Hg, but

these were not further evaluated because of inconsistency in individual rats (for heart weight, see below). During the last week of the experimental period, animals were relocated to a metabolism cage, and 24-h urine collected on 2 days, with the average of the analyses being taken for the results. After overnight fasting and subsequent anesthesia by i.p. injection of 48 mg/kg body weight (BW) pentobarbital-sodium (Abbot-Boehringer, Ingelheim, Germany), blood was aspirated from the abdominal aorta close to the iliac bifurcation and centrifuged in heparinized tubes. The heart was removed from exsanguinated animals, and its wet weight recorded. From a limited number of rats per group (see Table 2) the left kidney was removed, immediately shock-frozen in liquid nitrogen, and then stored at –80°C. Before analysis, this kidney was bisected,the upper half thawed and the cortical, medullary and papillary areas dissected. These were then dried overnight at 100°C, weighed and incinerated at 800°C, and the ash dissolved in 1 M hydrochloric acid. From the lower half, the same three anatomic regions were lyophilized, pulverized in a mortar, and dissolved in acid (see above). The right kidney was also removed and fixed in 4% buffered formalin. After embedding in paraffin, 5-μm sections were stained using the von Kossa method [14] and subjected to blinded histological examination using light microscopy.

Clinical chemistry

Routine methods were used, with few exceptions. The latter included high performance liquid chromatography (HPLC) for the determination of urinary and tissue oxalate and citrate [30] and urinary nitrate (see below). Since vascular endothelial production of endothelin-1 (ET-1) and nitric oxide is best reflected by their urinary excretion rate, ET-1 and nitrate (a stable end product of nitric oxide metabolism which accounts for about 90% of nitric oxide production [46]), were measured in daily urine, the former by enzyme-linked immunoassay (kit Immuno-Biological Laboratories, Hamburg, Germany), the latter by HPLC using a modification of the method described by Everett et al. [12]. For this procedure the eluent was sodium carbonate (2.7 mM) and sodium bicarbonate (0.3 mM), pumped at 1.5 ml/min. Urinary total protein (Lowry method), total plasma phospholipids (kit Bio-Mérrieux, Nürtingen, Germany), and total sialic acids [26] (derivatives of neuraminic acid, involved in the metabolism of vascular tissue glycoproteins and glycolipids), were measured colorimetrically.

Calculations and statistics

Renal Kossa-positive spots outside and inside glomeruli were evaluated by the pathologist, using the scores negative = 0, mild = 1, moderate = 3, and marked = 6. Results for plasma and urine are given as mean values (± SE). Differences between groups II (ATx) and I (Sham), and, in the case of renal tissue oxalate and citrate also between V (MgCPCSB) and I, were tested for significance ($P < 0.05$) using the *t*-test or Mann Whitney U-test as appropriate. Groups II–V were additionally subjected to one-way ANOVA followed by testing for least significant differences (LSD) using STATISTICA software (Statsoft, Tulsa/OK, USA).

Results

General state of sham rats, effects of ATx (groups I and II)

In the sham operated rats (group I) BW gain was unremarkable. A range of variables in plasma and urine (Table 1) were of the same order of magnitude as observed in outbred rat strains, such as Sprague-Dawley in our laboratory [29, 30] and Fischer-344 in the laboratory of others [11]. However, calciuria was low when compared with Wistar rats of similar BW [44]. Also unremarkable were plasma creatinine, which roughly reflects renal glomerular filtration rate, phospholipids which are thought to be mediators of calcification in bone [15] and renal medullary tissue [30] and urinary calcium and citrate, which are considered to be important promoters and inhibitors, respectively, of renal and urinary concretions containing calcium. Plasma levels of phospholipids were higher than those observed previously [30]. Sialic acid, increased levels of which are considered to signal the presence of a number of diseases including vascular disease [23], was almost twice as high as in healthy humans [26]. However in our rats this appeared to be unrelated to BW, heart weight, renal minerals, or calcification (see below). The mean urinary excretion of ET-1, a strong vasoconstrictor [45] also of kidney arterioles [19], and of nitrate, believed to be a nitric oxide-derived marker of vasodilation [20], amounted to approximately 20 pg (ET-1) and 6 μmol (nitrate) per day and 100 g BW (Fig. 1A, B).

ATx surgery had no effect on BW gain, and aortic grafts all remained patent. However, the relative heart weight (mg wet weight/g BW ×10) was slightly increased: 0.222%, 0.224%, 0.220% and 0.221% in the groups II–V, 0.215% in group I (Table 1), suggesting that in the former groups some degree of peripheral vascular resistance was present, and that to overcome this left ventricular hypertrophy developed. ATx elevated urinary ET-1 (Fig. 1A) as well as plasma calcium and creatinine within the normal range (Table 1), while urinary nitrate and protein were decreased (Fig. 1B; Table 1). Plasma phospholipids were high and urinary oxalate excretion was low (borderline significance) but plasma sialic acid and urinary citrate were unchanged (Table 1). ATx also increased renal cortical oxalate, left citrate unchanged (Fig. 2A, B), and preserved the cortico-papillary gradient of oxalate and citrate from low values in the cortex, higher in the medulla, to peak values in papilla (for minerals see below), which was also present in Sham rats (data not shown).

Renal tissue minerals and cortical calcification of Sham rats, effects of ATx (groups I and II)

Neither urinary tract stones nor any other particulate matter formed in either group. The cortico-papillary gradients of the three minerals were similar to those described earlier [29, 30]. However, Table 2 shows that in sham operated control rats (group I) the cortical mineral content (expressed as μmol/g dry tissue) was about 7 (calcium), 480 (phosphorus), and 22 (Mg), contrasting with the higher mean calcium and Mg, but lower phosphorus content found in the corresponding area of intact male Sprague-Dawley rats [30].

Histologically, cellular infiltrations were not found, thus excluding inflammatory processes. Kossa-positive areas, in general indicating phosphate deposited as calcium phosphate [13], were frequent and appeared as fine

Table 1 General data on animals, and variables in urine (*U*), serum or plasma (*P*). For the group symbols I-V see section on material and methods. Mean values (±SE). *n* number of observations. *$P \leq 0.05$ vs II

	I			II		III		IV		V		ANOVA (over II–V)	
	n = 12		*P*-value	*n* = 12		*n* = 12		*n* = 12[a]		*n* = 12		F	*P*
Final body weight (g)	404	(6)	0.24	399	(4)	404	(7)	391	(7)	406	(6)	1.2	0.32
Heart weight (mg)	871	(11)	0.24	885	(16)	903	(22)	899	(23)	896	(17)	1.4	0.34
P-Creatinine (μmol/l)	35	(2)	< 0.001	50	(2)	44	(4)	46	(4)	39*	(2)	5.0	0.002
P-Total calcium (mM/l)	2.31	(0.03)	0.05	2.35	(0.02)	2.32	(0.03)	1.99*	(0.11)	2.06*	(0.04)	6.4	0.002
P-Phosphorus (mg/dl)	6.0	(0.2)	0.45	6.0	(0.2)	5.7	(0.2)	6.1	(0.3)	6.2	(0.3)	0.66	0.58
P-Phospholipids (mg/ml)	0.90	(0.05)	0.11	1.02	(0.08)	0.88	(0.06)	0.91	(0.05)	0.76	(0.07)	2.6	0.09
P-Sialic acid (μg/ml)	1114	(17)	0.29	1092	(37)	1145*	(24)	1028	(32) [8]	1034	(33)	3.0	0.04
U-Protein (μg)[b]	276	(51)	0.04	174	(29)	216	(28)	612*	(69)	404*	(77)	12.9	< 0.001
U-Calcium (μmol)	4.2	(0.4)	0.22	4.5	(0.3)	6.2*	(0.4)	6.5*	(0.5)	8.3*	(0.6)	14.1	< 0.001
U-Magnesium (μmol)	68	(7)	0.18	79	(4)	120*	(5)	130*	(11)	168*	(8)	22.0	< 0.001
U-Phosphorus (μmol)	304	(19)	0.21	324	(15)	146*	(16)	203*	(29)	186*	(19)	14.2	< 0.001
U-Citrate (μmol)	62	(7)	0.43	63	(3)	86*	(4)	120*	(10)	142*	(5)	35.4	< 0.001
U-Oxalate (μmol)	5.6	(0.4)	0.08	5.0	(0.2)	6.0	(0.4)	7.5*	(0.7)	8.3*	(0.5)	9.2	< 0.001
U-pH	7.70	(0.13)	0.45	7.71	(0.06)	8.12*	(0.5)	8.18*	(0.18)	8.49*	(0.05)	9.7	< 0.001

[a] Except where indicated by [ ]
[b] Excretion rates are mean of 2 days and per 100 g BW

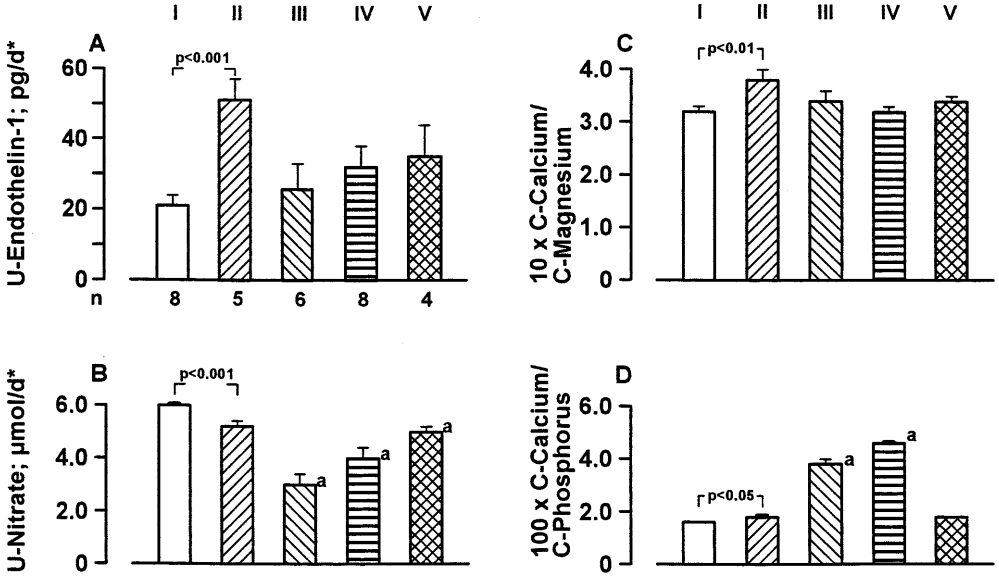

Fig. 1 Urinary excretion (U) of ET-1 and nitrate (**A** and **B**), and the renal cortical tissue molar ratios calcium/magnesium and calcium/phosphorus (**C** and **D**) in the syngeneic rat, as influenced by ATx surgery (group II), and three Mg-containing preparations (groups III–V). Bars are mean values (+/-SE). *I* Sham, *II* ATx, *III* MgC, *IV* MgCPC, *V* MgCPCSB. For abbreviations and further information see the section on material and methods. The number of observations (*n*) per group is 12, except where indicated. *: per day and 100 g body weight; [a] $P < 0.05$ vs. II

granules located exclusively in the renal cortex, but never in the medulla (Fig. 3A). Glomeruli were mostly free of calcification. Calcification was predominately found in the middle and, exceptionally, in the inner portion of the cortex, regions known to contain mainly proximal straight tubules, and the thick ascending limb of the nephron loop. Kossa-positive spots appeared to be intracellular, inhomogeneously distributed, and restricted to areas containing blood vessels, presumably post-glomerular capillaries. Importantly, when extraglomerular calcification was extreme, the glomeruli appeared almost normal (Fig. 3A, B). The semi-quantitative score for cortical calcification is given separately for glomeruli, the extraglomerular region, and as a total (Table 2).

ATx surgery increased urinary ET-1 and nitric oxide (Fig. 1A, B), and tissue oxalate but not citrate (Fig. 2A, B). It also reduced tissue Mg but not calcium (Table 2, group II), and consequently elevated the molar ratios of calcium/Mg and calcium/phosphorus (Fig. 1C, D). The mean extraglomerular calcification score and the summed score were moderately higher than in controls, but the mean glomerular score remained unchanged. The calcification, as described for the control group, (Fig. 3A) appeared to be more pronounced, as demonstrated in Fig. 3B. Unfortunately, for technical reasons, neither the calcium oxalate staining [13], nor electron microscopy and element analysis of calcified material could be carried out, thus preventing deeper insight into the nature, localization and morphology of the calcifications. Overall, in ATx rats there is excess of ET-1 over nitric oxide, tissue oxalate over citrate, and tissue calcium over Mg and phosphorus, combinations not previously described for renal calcification (for illustration of urinary ET-1/nitrate ratio vs. tissue calcium/Mg see Fig. 4A).

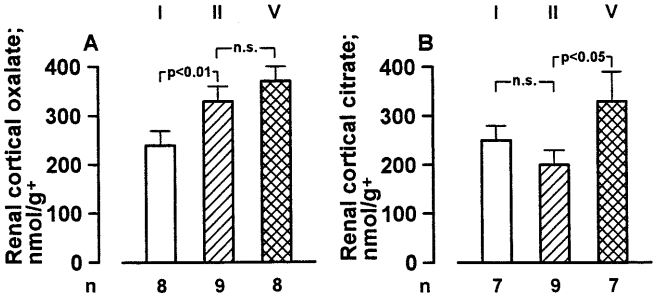

Fig. 2 Renal cortical oxalate (**A**) and citrate (**B**), as influenced by ATx surgery alone (group II), and by ATx plus long-term oral administration of the MgCPCSB preparation (group V). Bars are mean values (+/-SE). $^{+}$: dry tissue. For further information see legend to Fig. 1, and the section on material and methods

Response of ATx rats to treatment with Mg containing preparations

When the chemical variables responding to treatment regimens (groups III, IV and V) and the changes in renal calcification were compared with the corresponding data from untreated rats (group II), we found that treatment with MgCPCSB (group V) was generally most effective. This regimen normalized plasma calcium, phospholipids, and creatinine, but increased urinary pH and excretion of calcium, oxalate, Mg, citrate, and total protein (Table 1). It is worth noting that the high urinary pH indicates alkalization of metabolism, in turn stimulating citraturia [1]. In contrast, MgC treatment increased plasma sialic acid (Table 1) and resulted in minimum levels of both urinary ET-1 and nitrate, while MgCPC treatment led to intermediate levels of these two vascular effectors between MgC and MgCPCSB treatments (Fig. 1A, B; Fig. 4A).

MgCPCSB and MgCPC, but to a substantially lesser extent MgC, treatments kept renal cortical calcium within normal limits. The former preparation also prevented the high Mg found with MgC treatment as well as the low phosphorus seen with MgC and MgCPC (Table 2). MgCPCSB treatment failed to reduce renal cortical oxalate, but significantly increased citrate (Fig. 2A, B), and restored the calcium/phosphorus and calcium/Mg ratios to values almost identical to those of the non-grafted controls (group 1; Fig. 1C, D). Histological analysis showed that in MgCPCSB kidneys the cortical tubules appeared calcification-free, and the capillary basement membranes within the still calcified glomeruli appeared normally thick (see Fig. 3C). In contrast, after MgC treatment the kidneys showed some residual extra- and marked intraglomerular calcification, with a trend towards glomerular basement membrane thickening which was visible at higher magnifications (Fig. 3D). Overall, although MgCPCSB treatment failed to prevent glomerular calcification, it did prevent the extraglomerular calcification almost completely, as

Table 2 Minerals in renal cortex, calcifications in glomeruli, extra-glomerular-cortical region, and sum (Σ). For group symbols and further details see the section on material and methods. Mean values (±SE). *n* number of observations. $*P<0.05$, $**P<0.01$ vs II

	I			II		III		IV		V		ANOVA (over II–V)	
	n = 8		*P*-value	*n* = 8		*n* = 8		*n* = 7		*n* = 8		F	*P*
Minerals (μmol/g dry tissue)													
Calcium	7.1	(0.1)	0.38	7.0	(0.2)	8.6	(0.8)	7.2	(0.2)	7.3	(0.3)	2.6	0.06
Magnesium	22	(1)	0.002	19	(1)	25*	(2)	23	(1)	22	(1)	5.1	0.004
Phosphorus	480	(7)	0.002	423	(16)	235*	(15)	163*	(2)	440	(6)	140	< 0.001
Calcifications (score)													
Glomeruli	0.25	(0.16)	0.50	0.25	(0.16)	1.0*	(0.0)	0.29	(0.18)	0.5	(0.19)	9.2	< 0.001
Extraglomerular	3.4	(0.8)	0.30	4.0	(0.8)	1.4**	(0.5)	0.9**	(0.4)	0.4**	(0.2)	9.4	< 0.001
Σ	3.65	(0.7)	0.27	4.35	(0.7)	2.4*	(0.5)	1.19**	(0.5)	0.9**	(0.2)	5.1	< 0.005

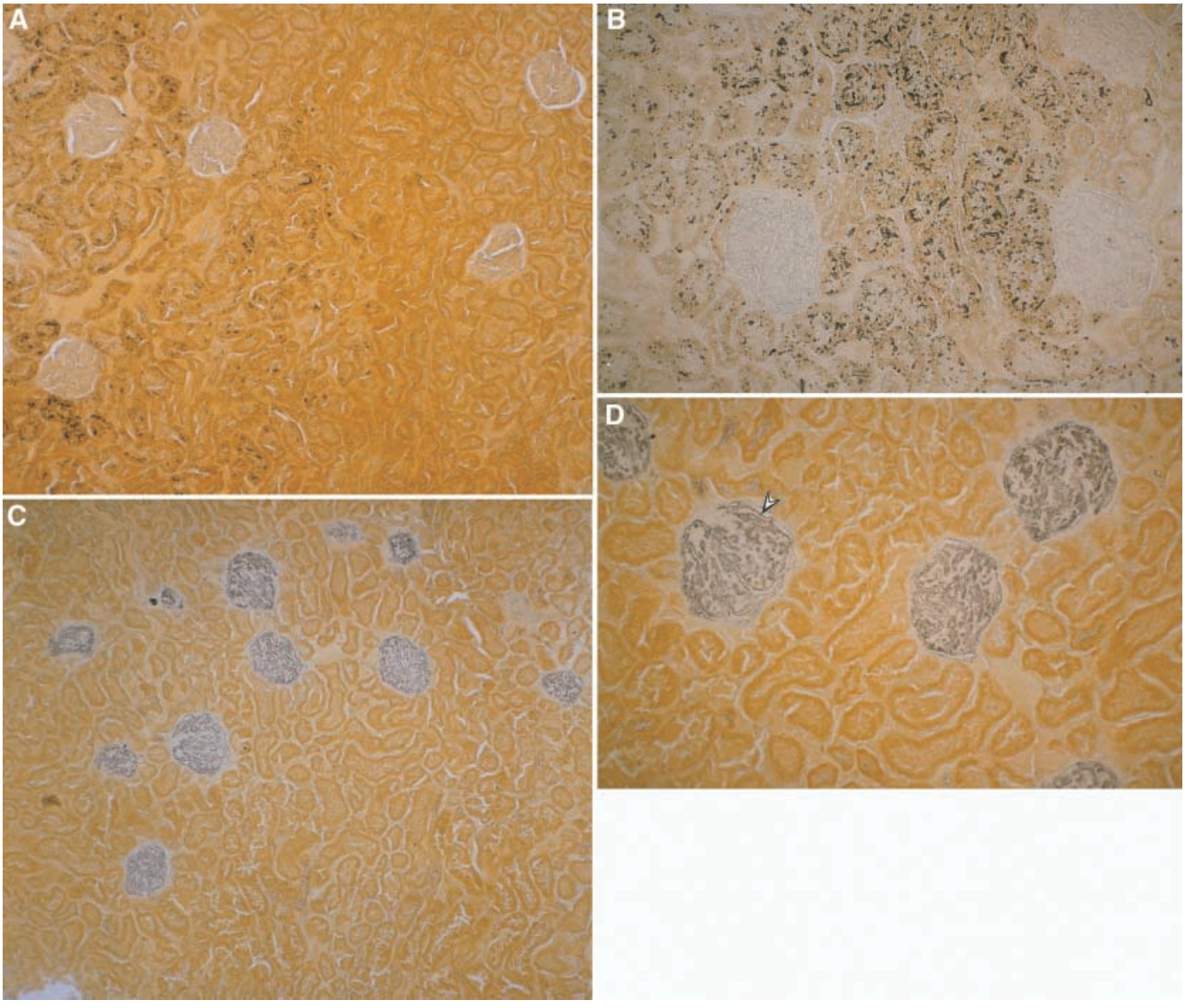

Fig. 3A–D Histology of the kidney of syngeneic rats, showing renal calcifications (dark spots) according to the von Kossa stain. **A** Calcified areas in tubules, mainly of the outer cortex. Note absence of calcification in the glomeruli. Magnification ×60. **B** Enlarged calcified areas of the cortex. Magnification ×100. Note absence of calcification in the glomeruli, and the absence of infiltration of inflammatory cells. **C** Moderately calcified glomeruli, but almost calcification-free tubules. Magnification ×60. **D** Intensive intra-glomerular, accompanied by moderate tubular, calcification. Arrowheads: thickened basement membranes. Magnification ×100. Note that picture **A** is characteristic for the kidney of rats after the sham operation, **B** for ATx, **C** for MgCPCSB, and **D** for MgC treatment. For further details see text

reflected in a roughly 80% reduction in the summed score (Table 2).

Relationships of variables (groups I–V)

The above observations led us to the question of the etiology of C-NC. However, considering the pilot character of the work and because correlations do not provide adequate information on cause –and effect relationships when data are limited, regression analyses were not carried out. Instead, an integrated view of all groups is given, covering the ET-1/nitrate ratio in urine vs. the cortical tissue calcium/Mg ratio, and urinary Mg, citrate and total protein excretion vs the summed cortical calcification score (Fig. 4). According to Fig. 4A, a rise in the tissue calcium/Mg ratio of approx. 14% requires an almost threefold increase in the ET-1/nitrate ratio (groups II vs. I), with the groups under treatment with Mg preparations taking intermediate positions. In contrast, the summed calcification score appears to be inversely related to Mg, citrate, and total protein (Fig. 4B–D). Most impressive is the superior anti-calcification effect of the alkalizing – because potassium, sodium and citrate-containing – Mg preparation (MgCPCSB), accompanied by high urinary Mg, citrate and total protein.

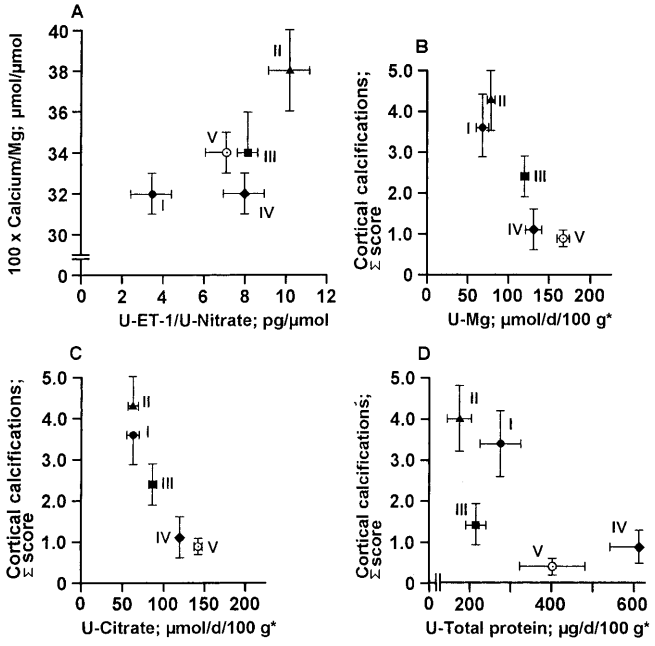

Fig. 4 Relationship of urinary (U) ET-1/nitrate ratio and renal cortical calcium/Mg ratio (**A**), excretion of Mg (**B**), citrate (**C**), total protein (**D**) and summed (Σ) score of renal cortical calcifications. *: BW. Symbols represent mean values (+/-SE) per variable in the groups I–V (for key see the section on material and methods)

Discussion

The animal model

The concept underlying tissue transplantation in syngeneic individuals is the post-transplant absence of immune reactions, rejection episodes, and signs of inflammation in the graft. The spontaneous occurrence of renal C-NC in such highly inbred rats is a new insight, and the associated data may give hints into its possible genesis. To definitively identify the factors, renal or extrarenal, which cause this type of NC, direct comparisons between inbred and outbred rats would be desirable. However, as already mentioned, in the NC-free intact outbred (Sprague-Dawley) rats, both cortical calcium and Mg are higher and cortical phosphorus lower than in the intact inbred (Lewis) rats used in our present work. Mg is an accepted inhibitor of calcium phosphate formation [6]. It follows that the higher cortical Mg of outbred rats may prevent them from developing C-NC, an assumption supported by the C-NC-attenuating effect of Mg administered prophylactically to inbred rats in our study (see below). That C-NC represents a nonspecific phenomenon, either due to the clamping of the aorta or to a false-positive Kossa stain, can be ruled out: for the latter, dissipation of such tissue areas by Mg treatment (groups III–V) is highly unlikely, while the inhibitory effect of Mg on formation and deposition of calcium phosphate is firmly documented [6, 29]. Clamping of the aorta was similar in duration in groups I and II but resulted in a threefold higher urinary ET-1/nitrate ratio in group II (Fig. 4A), compatible with the view that the tissue damage, if any, introduced by the initial (12 weeks ago) clamping, subsequent ischemia, and reperfusion was not solely responsible for the differences in cortical minerals and the degree of C-NC of the two groups. Additional factors may be responsible. Among these may be blood vessel transection, excess of urinary ET-1, tissue oxalate and calcium, a deficit of tissue Mg and citrate, and low proteinuria (Tables 1 and 2, Figs. 2 and 4). Although final proof is lacking, a loss of calcification inhibitors, inhibiting protein(s) included, and a gain of calcification promoters may have contributed to more serious C-NC in group II, and the triggering of calcification in inbred rats in general may be seen in this light.

C-NC diagnosis, pathophysiology

In humans, sporadic C-NC has been reported in adults in one series unrelated to other renal and urinary tract abnormalities [18], in a child with a similar type of calcification [38], and in a case of congenital oxalosis [24]. The latter, in particular, shows that advanced imaging technologies, emerging in vivo molecular imaging included, may be potent aids in detecting cortical NC in humans of all age classes, while kidney biopsies and other invasive methods are restricted for ethical reasons. With regard to oxalate, an etiological role of overproduction, which in humans is often due to well-defined inborn errors of metabolism, cannot be ruled out. In addition, oxalate binds to renal protein [36] and, can therefore accumulate in renal tissue [present work; 30] possibly predisposing it for the precipitation of calcium phosphate rather than calcium oxalate, an observation made in the rat [30]. Furthermore, in transplanted human kidneys, donated by more or less related individuals, cortical minerals increase due to the progressive alteration of renal hemodynamics, resulting in poor organ function and cell death [35]. In retrospect, this early report points, for the first time, towards the role of transection of blood vessels, the endocrine and morphological response to this kind of injury, subsequent alteration of tissue minerals, and possible interrelationships in calcification processes. In the renal cortex there is a close coupling of oxygen, calcium, and signalling substances mediating the cellular response to the alteration of oxygen levels [8]. Therefore, this area might be a key to understanding the basic features possibly shared by vascular and extravascular calcification [7] including those in the renal cortex.

ATx, ET-1 and nitrate

Reports in the literature suggest that common features of both ET-1 and nitric oxide production include their endothelial origin, and their modulatory effects on vascular tone. An imbalance of the two may manifest as

changes in blood pressure [25]. Currently, details of interactions between ET-1 and nitric oxide are the subject of intense research. In Sham and ATx rats the disparate patterns of ET-1 and nitrate, probably associated with slightly elevated blood pressure, indicate that the counter-regulatory actions of ET-1 and nitric oxide may be suboptimal. This is supported by the fact that Mg treatment regimens are followed by suppression of nitrate, but not ET-1 (Fig. 1A, B). Whether ET-1 excess (due to stimulation of secretion, diminishment of degradation or elimination) is the initial event, remains uncertain. ET-1 excess may possibly impair renal blood perfusion via ischemia and hypoxia [19], glomerular morphology, glomerular sieve function and proteinuria, transport processes of water, minerals and nonmineral substances in proximal tubules and other parts of the nephron [20, 25]. The association between nitrate deficit, an excess of ET-1, cortical oxalate and calcification after ATx suggests that ATX is a vascular injury that affects normally occurring events in such a way that the structure and function of cell membranes are altered. If ET-1 excess in fact induced tissue hypoxia, this could have led to intracellular acidosis [13] and enhanced citrate utilization [4], both plausible explanations for low tissue and urinary citrate (Figs. 2 and 4). Hypoxia and oxalate generate oxygen and nitrogen free radicals [3, 28] The resulting increase in oxidative burden of tissue can account for the disintegration of cell membranes via protein oxidation [5] and peroxidation of membrane phospholipids, a process resulting in apoptosis-like cell death [41]. In NC in general, higher –than normal plasma phospholipids seem to be common [this work; 29, 30], and one phospholipid subspecies has been shown to be involved in calcium phosphate deposition in the renal medulla [30]. Of note is also the fact that the combined occurrence of endothelial injury and apoptosis, mainly of renal glomeruli, has been ascribed to an immune process involving phospholipids [16, 40].

NC prevention by Mg

The reason why Mg treatment is ineffective for glomerular calcification is unknown, and why the described three treatment regimens suppress differently extraglomerular C-NC is not immediately obvious from the data presented here. In Mg-deficient rats, mild hypercalcemia is frequent, and in some way related to parathyroid hormone [9]. In the present work, the trend towards hypertension, increased calcemia and calciuria (Table 1), and high renal tissue calcium over Mg and phosphorus after ATx (Fig. 1C, D) is therefore thought to signal a relative intracellular Mg deficit, high plasma ionized calcium, but low parathyroid hormone. In a satellite study in our laboratory such a constellation has indeed been confirmed (P.O. Schwille et al., manuscript in preparation) and is consistent with the anomalous transport of the two ions across cell membranes in the presence of mild hypertension [21]. Because complete correction of this cellular defect, normalization of plasma parathyroid hormone included, is seen only with the MgCPCSB but not the two other preparations [33; this work], a concerted action of Mg, potassium and sodium, and bases (from citrate plus bicarbonate) appears to be necessary. The high C-NC score in the presence of low citraturia, and vice versa (Fig. 4C), implies that, in the setting of incipient NC, there is a need for citrate, an important intracellular and urinary buffer [17]. Finally, the common syndrome of ''sick cell disease'' is characterized by Mg and potassium loss from inside cells, and intracellular accumulation of calcium, particularly in patients with cardiovascular disease [37, 42]. This syndrome is amenable to treatment with combined Mg and potassium, but not Mg alone or potassium alone [43].

Conclusions

In a previous study showing that medullary NC of outbred rats with Mg deficit or dyslipidemia is associated with an increase of uric acid precursor oxypurines in urine, an etiological role of hypoxemia at the level of kidney was discussed because this could have led to enhanced degradation of cell-energizing adenosine phosphonucleotides, subsequent pathological calcification, and cell death [32]. In the present work on syngeneic rats, the spontaneous occurrence of histologically detectable C-NC is demonstrated. Since vascular injury in the form of ATx enhances calcification and allows this phenomenon to be traced back, at least in part, to excess ET-1 and diminished nitrate, substances produced by the vascular endothelium appear to be involved in the control of blood pressure and, according to data, also cortical tissue minerals, oxalate, and citrate. Because prophylactic administration of the Mg-, potassium-, sodium- and base-containing preparation, but not alkali-free Mg citrate, shows superior anti-calcification effects and is accompanied by restoration of normocalcemia, malregulation of extra- and intracellular ions may play a fundamental role in the etiology of C-NC. To date, the clinicil significance of our findings is uncertain, and it remains to be shown whether these impact on the calcification in arteries, renal medulla and papilla, the latter two being discussed as a possible source of calcium-containing renal stones [10].

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References

1. Anaizi NH, Cohen JJ, Black AJ, Wertheim SJ (1986) Renal tissue citrate: independence from citrate utilization, reabsorption, and pH. Am J Physiol 251 (Renal Fluid Electrol Physiol 20):F547
2. Anderson HC (1983) Calcific diseases. A concept. Arch Pathol Lab Med 107:341

3. Azevedo LCP, Pedro MDA, Souza LC, Souza HPD, Janiszewsky M, Luz PLD, Laurindo FRM (2000) Oxidative stress as a signalling mechanism of the vascular response to injury: The redox hypothesis of restenosis. Cardiovasc Res 47:436
4. Baruch SB, Burich RL, Eun CK, King VF (1975) Renal metabolism of citrate. Med Clin North Am 59:569
5. Berlett B, Stadtman ER (1997) Protein oxidation in aging, disease and oxidative stress. J Biol Chem 272:20313
6. Bigi A, Foresti E, Gregorini R, Ripamonti A, Roveri N, Shah JS (1992) The role of magnesium on the structure of biological apatites. Calcif Tissue Int 50:439
7. Demer LL (1995) A skeleton in the atherosclerosis closet. Circulation 92:2029
8. De Rubertis FR, Craven PA (1978) Alterations in rat renal cortical and medullary guanosine 3′5′–monophosphate accumulation by oxygen- and calcium dependent and –independent mechanisms: evidence for a calcium-independent action of oxygen in renal inner medulla. Metabolism 27:855
9. Dirks H (1983) The kidney and magnesium regulation. Kidney Int 23:771
10. Drach GW, Boyce WH (1972) Nephrocalcinosis as a source for renal stone nuclei. J Urol 107:897
11. Erben RG, Eberle J, Stahr K, Goldberg M (2000) Androgen deficiency induces high turnover osteopenia in aged male rats: a sequential histomorphometric study. J Bone Miner Res 15:1085
12. Everett SA, Dennis MF, Tozer GM, Prise VE, Wardman P, Stratford MRL (1995) Nitric oxide in biological fluids: analyses of nitrite and nitrate by high performance ion chromatography. J Chromatogr A 706:437
13. Galley HF, Webster NR (1999) Acidosis and tissue hypoxia in the critically ill: how to measure it and what does it mean. Crit Rev Clin Lab Sci 36:35
14. Gallyas F, Wolff JR (1985) Oxalate pretreatment and use of a physical developer render the Kossa method selective and sensitive for calcium. Histochemistry 83:423
15. Glimcher MJ (1992) The nature of the mineral component of bone and the mechanism of calcification. In: Coe FL, Murray MJ (eds) Disorders of bone and mineral metabolism. Raven Press, New York, p265
16. Griffiths MH, Papadaki L, Neild GH (2000) The renal pathology of primary antiphospholipid syndrome: a distinctive form of endothelial injury. Q J Med 93:457
17. Hamm LL, Simon EE (1987) Roles and mechanisms of urinary buffer excretion. Am J Physiol 253:F595
18. Hoffbrand BI, Oppenheimer SM, Sachs ML, Wrong OM (1987) Nodular cortical calcifications: a benign and hitherto undescribed form of renal calcification. Nephron 46:370
19. Kon V, Yoshioka T, Fogo A, Ichikawa I (1989) Endothelin may be the vasoconstrictor causing the persistent glomerular hypoperfusion in post-ischemic kidneys (abstract). Kidney Int 35:410
20. Kone BC (1997) Nitric oxide in health and disease. Am J Kidney Dis 30:311
21. Laurant P, Touyz RM (2000) Physiological and pathophysiological role of magnesium in the cardiovascular system: implications in hypertension. J Hypertens 18:1177
22. Levy RJ, Qu X, Underwood T, Trachy J, Schoen FJ (1995) Calcification of valved aortic allografts in rats: Effects of age, crosslinking, and inhibitors. J Biomed Mater Res 29:217
23. Lindberg G, Rastam L, Gullberg B, Eklund GA (1992) Serum sialic acid concentration predicts both coronary heart disease and stroke mortality: multivariate analysis including 54,385 men and women during 20.5 years follow-up. Int J Epidemiol 21:253
24. Luers PR, Lester PD, Siegler RL (1980) CT demonstration of cortical nephrocalcinosis in congenital oxalosis. Pediatr Radiol 10:116
25. Plato CF, Garvin JL (1999) Nitric oxide, endothelin, and nephron transport: potential interactions. Clin Exp Pharmacol Physiol 26:262
26. Pönniö M, Alho H, Nikkari ST, Olsson U, Rydberg U, Sillanaukee P (1999) Serum sialic acid in a random sample of the general population. Clin Chem 45:1842
27. Ross R (1993) The pathogenesis of atherosclerosis: a perspective for the 1990s. Nature 362 801
28. Scheid C, Koul H, Will WA (1996) Oxalate toxicity in LLC-PK_1 cells: role of free radicals. Kidney Int 49:413
29. Schmiedl A, Schwille PO, Bergé B, Markovic M, Dvorak O (1998) Reappraisal of the quantity and nature of renal calcifications, and mineral metabolism in the magnesium-deficient rat – effects of treatment with potassium citrate or the combination magnesium citrate and potassium citrate. Urol Int 61:76
30. Schmiedl A, Schwille PO, Bonucci E, Erben RG, Grayczyk A, Sharma V (2000) Nephrocalcinosis and hyperlipidemia in the rat fed a cholesterol- and fat-rich diet – association with hyperoxaluria, altered kidney and bone mineral, and renal tissue phospholipid-calcium interaction. Urol Res 28:404
31. Schwille PO, Schmiedl A, Herrmann U, Fan J, Gottlieb D, Manoharan M, Wipplinger J (1999) Magnesium, citrate, magnesium citrate and magnesium-alkali citrate as modulators of calcium oxalate crystallization in urine: observations in patients with recurrent idiopathic calcium urolithiasis. Urol Res 27:117
32. Schwille PO, Schmiedl A, Manoharan M, Wipplinger J (1999) Is there a role for uric acid in an animal model of calcium phosphate nephrocalcinosis and calcium phosphate crystallization in urine of patients with idiopathic calcium urolithiasis? An orientational study. J Endourol 13:637
33. Reference deleted in proof
34. Seitz T (2001) Transplantation eines Segments der herznahen in die infrarenale Aorta bei der Ratte – Untersuchungen zur Wirkung von oralem Magnesium auf den Mineralstoffwechsel und den Gehalt an Calcium, Magnesium und Phosphat der transplantierten Aorta. Dissertation, University of Erlangen
35. Sells RA, McLoughlin GA, Tyrell I (1974) Proceedings: renal cortical cation composition as an index of human kidney graft viability (abstract). Br J Surg 61:326
36. Selvam R, Devaraj S (1991) Induction of oxalate binding by lipid peroxidation in rat kidney mitochondria. Biochem Int 24:857
37. Shechter M, Kaplinsky E, Rabinowitz B (1992) The rationale of magnesium supplementation in acute myocardial infarction. Arch Int Med 152:2189
38. Smith LE, Adelman RD (1981) Early detection of renal cortical calcification in acute renal cortical necrosis in a child. Nephron 29:155
39. Sun P, Dwyer KM, Bairey Merz CN, Sun W, Johnson CA, Shircore AM, Dwyer JH (2000) Blood pressure, LDL-cholesterol, and intima-media thickness. A test of the "Response to Injury" hypothesis of atherosclerosis. Arterioscler Thromb Vasc Biol 20:2005
40. Trachtman H, Gauthier B, Frank R, Futterweit S, Goldstein A, Tomczak J (1996) Increased urinary nitrate excretion in children with minimal change nephrotic syndrome. J Pediatr 128:173
41. Tyurina YY, Shvedova AA, Kawai K, Tyurin VA, Kommineni C, Quinn PJ, Schor NF, Fabisiak JP, Kagan VE (2000) Phospholipid signaling in apoptosis: peroxidation and externalization of phosphatidylserine. Toxicology 148:93
42. Welt LG, Smith EKM, Dunn MJ (1967) Membrane transport defect; the sick cell. Trans Assoc Am Physicians 8:217
43. Whang R, Whang DD, Ryan MP (1992) Refractory potassium depletion. A consequence of magnesium deficiency. Arch Int Med 152:40
44. Whiting SJ, Draper HH (1980) The role of sulfate in the calciuria of high protein diets in adult rats. J Nutr 110:212
45. Yanagisawa M, Kurihara H, Kimura S, Tomobe Y, Kobayashi M, Mitsui Y, Yazuki Y, Goto K, Masaki T (1988) A novel potent vasoconstrictor peptide produced by vascular endothelium cells. Nature 332:411
46. Zeballos GA, Bernstein RD, Thompson CI, Forfia PR, Seyedi N, Shen W, Kaminski PM, Wolin MS, Hintze TH (1995) Pharmacodynamics of plasma nitrate/nitrite as indicators of nitric oxide formation in conscious dogs. Circulation 91:2982