Paricalcitol counteracts the increased contrast induced nephropathy caused by renin-angiotensin-aldosterone system blockade therapy in a rat model

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Abstract. – OBJECTIVE: The effect of vitamin D and renin-angiotensin-aldosterone system blockade medications in pathophysiology of contrast induced nephropathy (CIN) is controversial. The effects of paricalcitol (active vitamin D analogue) and losartan treatments in an experimental model of CIN were investigated in this study.

MATERIALS AND METHODS: Thirty-six male Wistar albino rats were examined in five treatment groups. Placebo group (Group A; n = 4) received no active medication; control group (Group B; n = 8) received only contrast media (CM); Group C (n = 8) received paricalcitol; Group D (n = 8) received losartan and Group E (n = 8) received paricalcitol plus losartan. CIN was induced by NG-nitro-L-arginine methyl ester and indomethacin before iohexol injection. Renal histopathological findings were categorized and renal immunohistochemical examinations by caspase-3 rabbit primary antibody were performed.

RESULTS: Creatinine and cystatin C levels significantly increased in the treatment groups, compared to Group A. However, creatinine levels were not significantly increased in Groups C, D and E compared to Group B. Compared to Group B, a significant increase of cystatin C levels was observed in Group D ($p < 0.01$). In Group E, when paricalcitol treatment was added to losartan treatment, cystatin C levels were similar to Group B ($p = 1.00$). In histopathological and immunohistochemical examination frequency of Grade 2/3 tubular necrosis and renal caspase 3 activity scores were significantly higher in the losartan treatment group compared to the other treatment groups. The histopathological effects related to losartan treatment were found to be reversed when paricalcitol treatment was combined.

CONCLUSIONS: Our findings suggest that paricalcitol treatment counteracts increased contrast induced nephropathy caused by losartan. These findings warrant further clinical studies to investigate the benefit of paricalcitol in CIN prophylaxis.

Key Words:
   Contrast induced nephropathy, Vitamin D, Losartan.

Introduction

With the increasing use of contrast media (CM) for diagnostic and interventional procedures, CM induced nephropathy has become the third leading cause of hospital-acquired acute renal failure accounting for 12% of the cases[1]. Patients experiencing contrast-induced nephropathy (CIN) following coronary angiography, have a worse outcome compared to patients without CIN[2]. The pathophysiology of radiocontrast nephropathy has not been completely understood. However, a combination of renal vasoconstriction with resultant renal medullary hypoxia and direct renal tubular toxicity of CM have been implicated in the pathogenesis[3,4]. A possible role of renin-angiotensin-aldosterone system (RAAS) modulating therapies in the pathophysiology of CIN development has also been reported[5,6]. The available data concerning the impact of RAAS blockade on frequency of CIN remains inhomogenous and there are controversial results[7-9]. Especially, the question whether such therapy should be interrupted periprocedurally, or not,

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has not been answered. Recent studies suggest vitamin D deficiency as a potential risk factor for renovascular disease[10-12]. Vitamin D is a potent negative regulator of the RAAS and inflammation[13]. Experimental studies indicate that vitamin D participates in the regulation of renin-angiotensin axis by directly suppressing the renin gene expression[14,15].

In this study, we evaluated the effects of paricalcitol (19-nor-1,25-$(OH)_2$-vitamin D_2; an analog of 1,25-dihydroxyergocalciferol, the active form of vitamin D_2 (ergocalciferol)), and losartan (angiotensin II receptor antagonist) on an experimental rat model of CIN.

Materials and Methods

Animals

All the animals (rats) in this study are treated according to the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. All the rats included in the study were born and bred in significant pathogen-free conditions in animal facilities of the Research & Development Unit of our institution. The experimental protocol was approved by the local Ethics Committee for Animal Experimentation (protocol no: 2012-12) and was conducted according to their guidelines. Thirty-six male Wistar albino rats weighing 200 ± 30 g at the age of 6 weeks were included. Each treatment group of rats was housed in separate, standard, stainless steel cages, which were maintained on a 12-hours-light/12-hours dark cycle at 22-25°C and were given access ad libitum to a standard rat diet and water.

Experimental Design

Model of Contrast Induced Nephropathy

We used a model of radiocontrast nephropathy combining injection of the radiocontrast iohexol (Omnipaque™, 350 mgI/ml solution; GE Healthcare Inc., Princeton, NJ, USA) with prior inhibition of prostanoids and nitric oxide synthesis. Rats were injected with a nitric oxide synthase inhibitor (NOSI, N^G-nitro-L-arginine methyl ester, 10 mg/kg; Sigma-Aldrich Co., St Louis, MO, USA) and an inhibitor of prostaglandin synthesis (IPS, indomethacin, 10 mg/kg; Sigma-Aldrich Co., St Louis, MO, USA) intraperitoneally (IP) before iohexol (350 mg iodine/ml, 1.5-3 g iodine/kg ip) injection. This model creates a repro-

ducible acute renal failure following radiocontrast injection as described before[16]. Sham mice received IP injections of saline simultaneously with the other treatment groups.

Study Groups

The rats were divided into 5 treatment groups (Table I). The placebo group (Group A, n = 4) received no active drugs and only IP injections of 0.9% isotonic saline serum were performed. The control group (Group B, n = 8) received only contrast media (1.5 g/kg, IP) following NOSI and IPS injections. Group C (n = 8) received three repeated IP injections of paricalcitol (0.4 μg/kg; Zemplar; Abbott Laboratories, Abbott Park, IL, USA) on the first, fourth and seventh days of treatment before NOSI, IPS and contrast media injections. Group D (n = 8) received two repeated IP injections of losartan (10 mg/kg; Sigma-Aldrich Co., St Louis, MO, USA) on the sixth and seventh days of treatment before NOSI, IPS and contrast media injections. Rats in Group E (n = 8) received three repeated IP injections paricalcitol (0.4 μg/kg) on the first, fourth and seventh days of treatment and two repeated IP injections of losartan (10 mg/kg) on the sixth and seventh days before NOSI, IPS and contrast media injections.

Experimental Protocol

Each group of rats was kept in separate standard stainless steel cages, which were maintained on a 12-hours-light/12-hours dark cycle at 22-25°C and were given access ad libitum to a standard rat diet and water. Drug administrations, collection of the blood samples and assessment of weight each day were performed between 9:00 and 10:00 a.m. to minimize circadian variation. On the first day, under general anaesthesia, blood was collected from each of the rats for determination of basal creatinine and cystatin C levels. Serum samples were kept at −70°C until analyzed. All the collection of blood for analysis from the tail vein and final sacrifications of the rats were performed under general anesthesia induced by intramuscular (i.m.) injection ketamine (75 mg/kg) and xylazine (10 mg/kg).

On the seventh day, the rats were deprived of access to water for twenty-four hours until the eighth day. On the eighth day, NOSI and IPS i.p. injections were performed as described, 15 minutes before the IP injection of contrast media (iohexol, 350 mg iodine/ml, 1.5-3 g iodine/kg) in groups B, C, D and E. Group A received the

Table I. Comparison of renal function parameters on the 8th day of the experiment.

Study groups	Creatinine (mg/dl)	Cystatin C (mg/L)	p^a_{Cr}	p^a_{Cys}	p^b_{Cr}	p^b_{Cys}
Group A	0.21 ± 0.01	0.14 ± 0.01	–	–	0.01	0.03
Group B	0.29 ± 0.05	0.18 ± 0.01	0.01	0.03	–	–
Group C	0.28 ± 0.04	0.19 ± 0.02	0.02	0.01	0.99	0.95
Group D	0.33 ± 0.05	0.23 ± 0.04	< 0.01	< 0.01	0.31	< 0.01
Group E	0.31 ± 0.24	0.18 ± 0.01	< 0.01	0.03	0.89	1

Group A: placebo; Group B: only contrast media; Group C: paricalcitol treatment; Group D: losartan treatment; Group E: paricalcitol + losartan treatment. [a]p values in comparison to Group A: [b]p values in comparison to Group B.

same amounts of 0.9% isotonic saline injections. Twenty-four hours later, under general anaesthesia, rats were sacrificed by exsanguination from the abdominal aorta where blood samples were collected. After clamping the abdominal aorta proximal and distal to renal arteries, 10% formaldehyde solution was perfused through the renal arteries and kidneys were removed and stored in phosphate-buffered 10% formalin. Renal function was assessed by measuring changes in serum cystatin C and creatinine levels 24 h after radiocontrast injection.

Histopathologic Examination of the Renal Tissues

Histological slides of the formalin-maintained samples were prepared and then counterstained with hematoxylin, eosin (H&E) and periodic acid Schiff staining (PAS) using standard procedure. These steps were then followed by semi-quantitative analysis of the kidney sections by a pathologist functioning in a blind manner. Tubular necrosis and proteinaceous casts were graded according to a previous methodology[17] as follows: 0 = no damage; 1 = mild (unicellular, patchy isolated damage); 2 = moderate (damage < 25%); 3 = severe (damage between 25 and 50%) and 4 = very severe (> 50% damage).

Immunohistochemical Examinations

Paraffin sections (5 μm) were mounted on superfrost plus glass slides and deparaffinized in xylol and ethanol 96% and dried overnight at 37°C. After inactivation of endogenous peroxidases with 1.2% H_2O_2 in methanol and hydration, sections were pretreated with blocking buffer consisting of 0.1 M TRIS, 3% bovine serum albumin (BSA) and 20% normal calf serum for 30 minutes at room temperature. After rinsing with phosphate buffer solution, specimens were incubated for 30 minutes with anti-active caspase-3 rabbit primary antibody (caspase 3 (CPP32) Ab-4 rabbit polyclonal anti-

body, Thermo Scientific, Lab Vision Corp, Fremont, CA, USA). Sections were incubated with 1:50 anti-BrdU followed by biotinylated antimouse IgG for bromodeoxyuridine (BrdU) detection. Signals were amplified with streptavidin-biotinylated horseradish peroxidase complex, developed with 3-amino-9-ethylcarbazole (AEC), counterstained with haematoxylin and analysed under a light microscope.

Scoring was performed at a magnification of 200X. At the time of scoring, the investigators were blinded for clinical and histological data. The intensity of staining in tubular cells was scored semi quantitatively using a 4-point scale; 0: negative; 1+: weak intensity; 2+: intermediate intensity; 3+: strong intensity. This scoring system was performed on proximal as well as on distal tubules, which were recognized on their immuno-histochemical or morphological characteristics.

Statistical Analysis

All data are presented as a means ± SD for parametric variables and as percentages for categorical variables. Continuous variables were normally distributed according to Kolmogorov-Smirnov statistics. Differences between the groups were evaluated using the ANOVA with the Tukey's post-hoc test. The histopathological and immunohistochemical findings were reported as categorical variables and were tested by Pearson's χ^2 test and Fisher's Exact Test. All statistical studies were carried out using SPSS for Windows v. 11.5.0 (SPSS Inc., Chicago, IL, USA) software and a p value < 0.05 was considered statistically significant.

Results

All the animals in each group survived the corresponding treatments; no complications were

recorded throughout the experiment. In each study group, initial creatinine and cystatin C levels were not significantly different compared to Group A (placebo group) ($p > 0.05$ for each comparison). Changes in renal function parameters in the placebo, control and treatment groups are presented in Table I. After application of CM, creatinine and cystatin C levels significantly increased in the treatment groups, compared to Group A. However, creatinine levels were not significantly higher in Groups C, D and E compared to Group B ($p = 0.99$, $p = 0.31$, $p = 0.89$; respectively). Compared to Group B, a significant increase of cystatin C was observed in Group D (losartan treatment) ($p < 0.01$). In Group E, when paricalcitol treatment was added to losartan treatment, cystatin C levels were similar to Group B ($p = 1.00$). In addition, cystatin C levels were similar between Groups C and B.

Histopathological and immunohistochemical findings in the study groups are summarized in Table II and Figures 1, 2. In the placebo group, no form of renal damage was observed. Grade 4 tubular necrosis was not observed in any of the renal specimens. The distribution of tubular necrosis grades was similar for Group C and E when compared to Group B ($p = 0.99$; $p = 0.99$, respectively). Considering grades of proteinaceous casts, renal involvement was also similar in Group C and E compared to Group B ($p = 1.0$; $p = 0.66$, respectively). Whereas, frequency of Grade 3 (n=4) and Grade 2 (n=4) tubular necrosis was significantly higher in Group D compared to Group B ($p = 0.02$), Group C ($p = 0.01$) and Group E ($p = 0.01$). Likewise, frequency of Grade 1 (n = 6) and Grade 2 (n = 1) proteinaceous casts was higher in Group D compared to Group B, C and E but, the difference did not reach statistical significance ($p = 0.11$; $p = 0.11$; $p = 0.28$, respectively). In immunohistochemical examination, the renal caspase 3 activity scores were significantly higher in Group D (5 cases with Grade 3, 2 cases with Grade 2 and 1 case with Grade 1) compared to Group B ($p = 0.01$), Group C ($p = 0.01$) and Group E ($p = 0.03$). In histopathological and immunohistochemical examination, the severity of renal involvement was not significantly different in Group E (paricalcitol + losartan treatment) compared to Group B ($p > 0.05$ for each comparison).

Insult on renal function was evident both by serum markers and the histologic findings in the active treatment gorup compared to the placebo group (Group A), which show that our experimental model of CIN was appropriate.

Discussion

The main findings of this study are: (1) losartan treatment was associated with a more severe form of renal toxicity after CM exposure; (2) active vitamin D analogue (paricalcitol) treatment was not protective against renal toxicity; (3) concomitant use of paricalcitol with losartan treatment resulted in renal toxicity comparable to the

Table II. Histopathological and immunohistochemical findings in the study groups.

	Group A (n=4)	Group B (n=8)	Group C (n=8)	Group D (n=8)	Group E (n=8)
Tubular necrosis					
Grade 0	4	1	0	0	0
Grade 1	0	5	6	0	6
Grade 2	0	1	2	4	2
Grade 3	0	1	0	4	0
Presence of proteinaceous casts					
Grade 0	4	5	5	1	4
Grade 1	0	3	3	6	4
Grade 2	0	0	0	1	0
Caspase-3 activity					
Grade 0	4	1	0	0	0
Grade 1	0	6	7	1	5
Grade 2	0	1	1	2	3
Grade 3	0	0	0	5	0

Group A: placebo; Group B: only contrast media; Group C: paricalcitol treatment; Group D: losartan treatment; Group E: paricalcitol + losartan treatment.
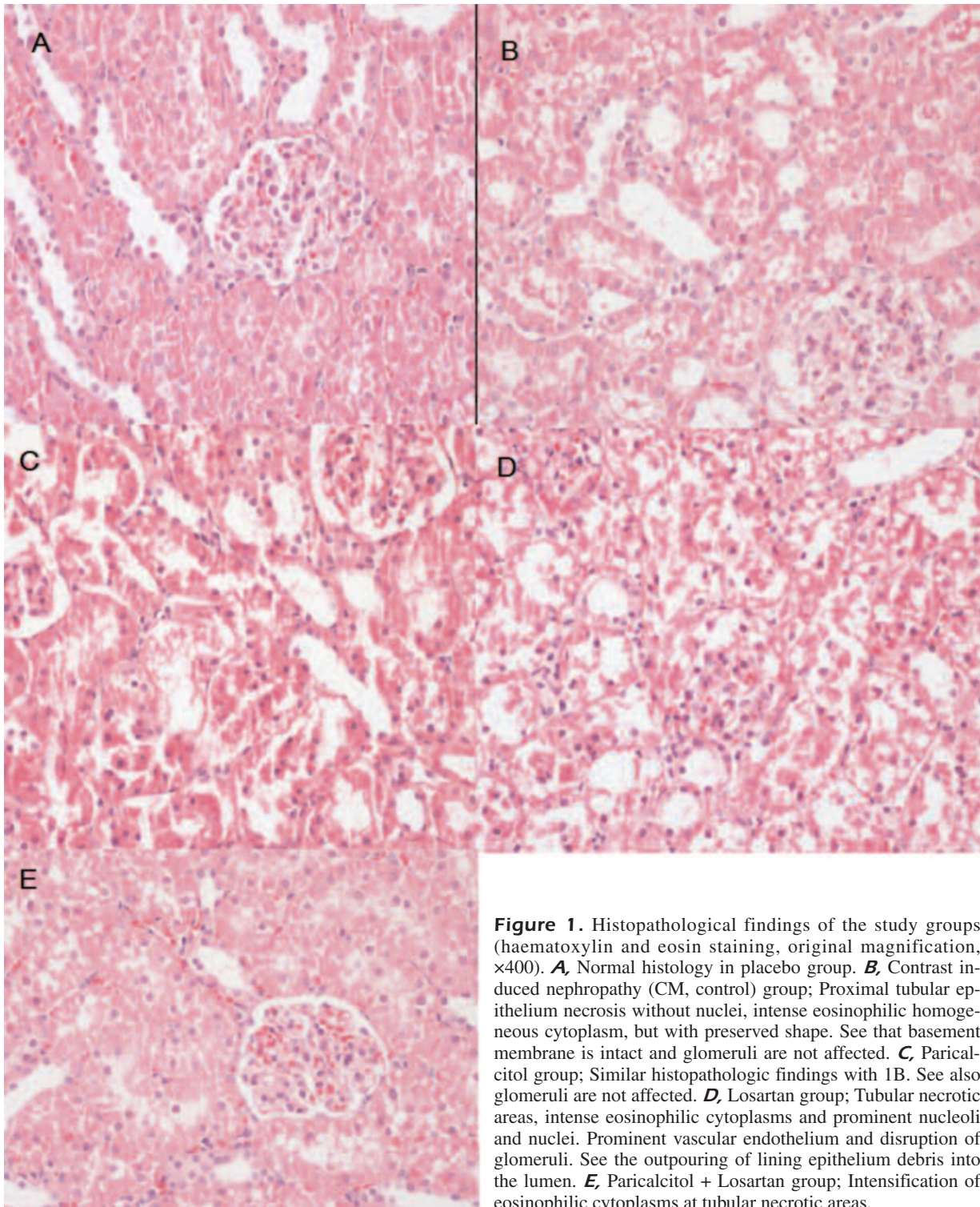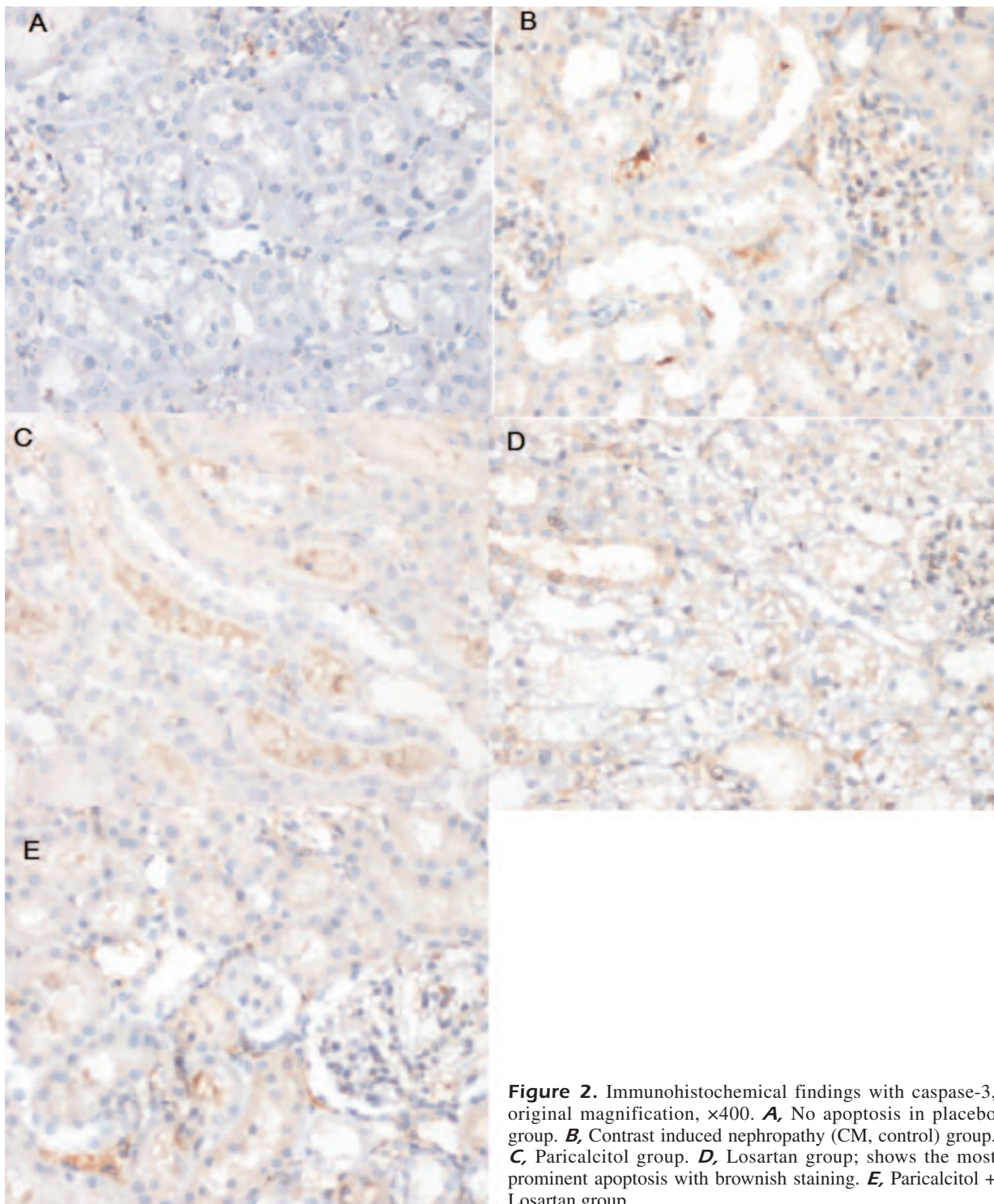
Figure 1. Histopathological findings of the study groups (haematoxylin and eosin staining, original magnification, ×400). *A,* Normal histology in placebo group. *B,* Contrast induced nephropathy (CM, control) group; Proximal tubular epithelium necrosis without nuclei, intense eosinophilic homogeneous cytoplasm, but with preserved shape. See that basement membrane is intact and glomeruli are not affected. *C,* Paricalcitol group; Similar histopathologic findings with 1B. See also glomeruli are not affected. *D,* Losartan group; Tubular necrotic areas, intense eosinophilic cytoplasms and prominent nucleoli and nuclei. Prominent vascular endothelium and disruption of glomeruli. See the outpouring of lining epithelium debris into the lumen. *E,* Paricalcitol + Losartan group; Intensification of eosinophilic cytoplasms at tubular necrotic areas.

control group, which may indicate the protective effect of this active vitamin D analogue from deleterious effects of losartan on renal cells after CM exposure.

Radiocontrast nephropathy remains an important clinical problem despite the use of modern CM with lower osmolality. Especially, patients with pre-existing renal insufficiency, diabetes or

Figure 2. Immunohistochemical findings with caspase-3, original magnification, ×400. **A,** No apoptosis in placebo group. **B,** Contrast induced nephropathy (CM, control) group. **C,** Paricalcitol group. **D,** Losartan group; shows the most prominent apoptosis with brownish staining. **E,** Paricalcitol + Losartan group.

volume depletion are at high risk of developing radiocontrast nephropathy[18]. The pathogenesis of radiocontrast nephropathy appears to be multifactorial and includes a deleterious reduction of renal arteriolar blood flow and glomerular filtration rate as well as the direct renal tubular toxicity caused by the radiocontrast agents. Indeed, radiocontrast injection *in vivo* reduces renal blood flow, medullary PO_2 and glomerular filtration rate[4,19]. These effects could be due to modulation

of the intrarenal synthesis and release of vasoactive mediators after radiocontrast injection.

Endothelin-1, renin, and angiotensin II are some of the potential mediators leading to intrarenal vasoconstriction in experimental models of CIN and experimental data suggests that activation of renin-angiotensin-aldosterone system which increases endothelin-1 and reactive oxygen species (ROS) may play a role in the pathogenesis of CIN[6,20]. In recent studies, molecules with potent antioxidant properties (e.g. N-acetyl-cysteine, curcumin) have been shown to be protective against CIN[21,22].

1,25 hydroxy vitamin D also participates in the regulation of renin-angiotensin axis by directly suppressing renin gene expression[14,23]. Renin over-expression can be produced in wild-type mice by pharmacological inhibition of vitamin D synthesis[14]. In their experimental study, Kedrah et al[24] have shown that direct renin inhibitor aliskiren has a potential role in the prevention of experimental contrast-induced nephropathy in the rat. Also, Ari et al[25] have used paricalcitol for prevention of experimental contrast-induced nephropathy model and demonstrated the antioxidant and renoprotective effects of paricalcitol. In our study, we did not show significant difference regarding the severity of tubular necrosis, proteinaceous casts and caspase-3 activity staining in paracalcitol group compared to the control group. Thus, our findings do not support the protective effects of vitamin D therapy from CIN.

In our study, laboratory and histopathological examination revealed that losartan treatment was associated with the most severe form of renal toxicity after CM exposure. Previously, it has been hypothesized that administration of angiotensin converting enzyme (ACE) inhibitors and angiotensin receptor blockers may have a protective effect against CIN by attenuating afferent arteriolar vasoconstriction and reducing medullary ischemia[9,26], however recent studies did not yield confirmatory results about the beneficial effects of ACE inhibitors and angiotensin receptor blockers[5,27]. Moreover, some investigators believe that ACE inhibitor/angiotensin receptor blocker therapy should be discontinued prior to CM exposure[28]. A problem for the RAS inhibiting drugs is the appearance of compensatory renin increase, due to the disruption of the negative feedback inhibitory loop in renin production that eventually increases Angintensin II production and reduces the efficacy of RAS inhibition[29]. Renin increase can also activate the (pro) renin

receptor and cause renal and cardiovascular damage independent of Angiotensin II[30,31]. Our results comply with the recommendation to withhold RAAS blockade, 48-72 hours before contrast exposure, to improve renal outcome.

In this study, we observed lower levels of cystatin C in the paracalcitol + losartan group compared to the losartan group. Histopathological and immunohistochemical examinations also confirmed the extent of renal damage was less common in the combination therapy group similar to the control. However, the protective effects of paracalcitol were only seen when used in combination with losartan treatment. This protective effect of paracalcitol on renal cells may be a result of the renin inhibiting effects which antagonizes the increased renin activity secondary to angiotensin receptor blockade. Our findings may imply that vitamin D analogues may be beneficial in reducing the appearance of CIN in patients using ACE inhibitor/angiotensin receptor blockers.

Conclusions

In this study losartan treatment was found to be associated with more severe forms of renal toxicity after CM exposure. Vitamin D decreased this increased renal toxicity related to losartan. Further prospective clinical trials are warranted to investigate the clinical use of vitamin D in CIN.

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Conflict of Interest

The Authors declare that there are no conflicts of interest.

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