Antioxidant and renoprotective effects of paricalcitol on experimental contrast-induced nephropathy model

[1]E ARI, [2]A E KEDRAH, MD, [2]Y ALAHDAB, MD, [3]G BULUT, MD, [4]Z EREN, MD, [5]O BAYTEKIN, MD and [6]D ODABASI, MD

[1]*Department of Nephrology, Van Yuksek Ihtisas Hospital, Van, Turkey,* [2]*Department of Gastroenterology, Marmara University School of Medicine, Istanbul, Turkey,* [3]*Department of Pathology, Van Yuksek Ihtisas Hospital, Van, Turkey,* [4]*Department of Nephrology, Yeditepe University School of Medicine, Istanbul, Turkey,* [5]*Department of Biochemistry, Van Yuksek Ihtisas Hospital, Van, Turkey, and* [6]*Department of Cardiovascular Surgery, Yuzuncu Yil University School of Medicine, Van, Turkey*

Objectives: The aim of the study was to assess the effect of paricalcitol on the experimental contrast-induced nephropathy (CIN) model. We hypothesised that paricalcitol may prevent CIN.

Methods: 32 Wistar albino rats were divided into four groups ($n=8$ each): control group, paricalcitol group, CIN group and paricalcitol plus CIN group. Paricalcitol (0.4 μg kg^{-1} day^{-1}) was given intraperitoneally for 5 consecutive days prior to induction of CIN. CIN was induced at day 4 by intravenous injection of indometacin (10 mg kg^{-1}), Nω-nitro-L-arginine methyl ester (L-NAME, 10 mg kg^{-1}) and meglumine amidotrizoate (6 ml kg^{-1}). Renal function parameters, oxidative stress biomarkers, histopathological findings and vascular endothelial growth factor (VEGF) immunoexpression were evaluated.

Results: The paricalcitol plus CIN group had lower mean serum creatinine levels ($p=0.034$) as well as higher creatinine clearance ($p=0.042$) than the CIN group. Serum malondialdehyde and kidney thiobarbituric acid-reacting substances levels were significantly lower in the paricalcitol plus CIN group than in the CIN group ($p=0.024$ and $p=0.042$, respectively). The mean scores of tubular necrosis ($p=0.024$), proteinaceous casts ($p=0.038$), medullary congestion ($p=0.035$) and VEGF immunoexpression ($p=0.018$) in the paricalcitol plus CIN group were also significantly lower.

Conclusion: This study demonstrates the protective effect of paricalcitol in the prevention of CIN in an experimental model.

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Contrast-induced nephropathy (CIN) is a serious complication resulting from the use of iodinated contrast media [1]. It is the third leading cause of acute renal failure in a hospital setting, contributing to prolonged hospital stay and increased medical costs [1]. Whereas individuals with normal renal function are not considered to be at particular risk for CIN, patients with certain comorbid conditions, such as pre-existing renal insufficiency and diabetes with micro-vascular and macrovascular disease, are much more likely to experience acute renal failure after contrast administration [2].

The present evidence indicates that the pathogenesis of CIN is mainly related to renal medullary ischaemia due to intrarenal vasoconstriction, direct tubular toxicity and oxidative stress on renal tubular cells [1, 2]. Direct cytotoxic effects of contrast media, especially in association with the generation of oxygen free radicals, seem to represent the primary event in the pathogenesis of CIN [3].

Paricalcitol (19-nor-1,25-dihydroxyvitamin D_2) is an active, non-hypercalcemic vitamin D analogue that shows biological activity similar to vitamin D, but has fewer adverse effects [4]. In addition to its primary role in calcium metabolism and bone mineralisation, vitamin D and its non-hypercalcemic analogue paricalcitol have pleiotropic and antioxidant effects on cellular homeostasis [5]. Studies in experimental nephropathy models have focused on the effects of vitamin D and paricalcitol on glomerular damage and tubular toxicity [6–8]. Recently it has been shown that paricalcitol has antioxidant effects on the myocardium [9] and supresses the renin–angiotensin system (RAS) in the kidney [10].

Vascular endothelial growth factor (VEGF) is an endothelial-specific growth factor, the secretion of which is mainly stimulated by hypoxia [11] and plays an important role in the response to regional renal hypoxia [12–14].

Since the increase in oxidative stress may partially be responsible for the development of CIN, we hypothesised that administration of paricalcitol may prevent CIN through its antioxidant effects. To test our hypothesis we investigated the effects of paricalcitol on oxidative stress biomarkers, renal histopathology and VEGF expression in an experimental CIN model.

Address correspondence to: Dr Elif Ari, Department of Nephrology, Van Yuksek Ihtisas Hospital, 65170 Van, Turkey. E-mail: elifaribakir@gmail.com

Methods and materials

Animals

The experimental procedures were performed in accordance with the guide for the Care and Use of Laboratory Animals (National Institutes of Health) and were approved by the Institutional Animal Care Committee of Marmara University School of Medicine. The study included 32 male Wistar albino rats (7 weeks old), each weighing 230–250 g, which were born and bred in the Experimental Research Center of Marmara University. They were kept in stainless-steel cages, maintained on a 12 h light/12 h dark cycle at 22–25 °C, where they had unlimited access to standard rat chow and water. Individual metabolic cages were used for the collection of 24-h urine and for recording water intake.

Experimental design and drugs

The animals were randomly allocated into four groups.

Control group (n=8): control animals received intraperitoneal (ip) saline injections once daily, for 5 consecutive days (Days 1 to 5), and received three saline injections into tail veins at Day 4, at 15-min intervals.

Paricalcitol group (n=8): rats received paricalcitol (Zemplar®; Abbott Laboratories, Abbott Park, IL) ip at a dose of 0.4 μg kg^{-1} per day once daily for 5 consecutive days.

CIN group (n=8): CIN protocol, consisting of indometacin (10 mg kg^{-1}; intravenous, iv), followed after 15 min by Nω-nitro-L-arginine methyl ester (L-NAME, 10 mg kg^{-1}, iv) and after a further 15 min by 6 ml kg^{-1} high-osmolar radiological contrast agent 60% meglumine amidotrizoate (Urovist-Angiographin; Schering AG, Berlin, Germany), injected into the tail vein of rats under ether anaesthesia as described by Agmon et al [15].

Paricalcitol plus CIN group (n=8): in this group rats received intraperitoneal paricalcitol at a dose of 0.4 μg kg^{-1} per day once daily for 5 consecutive days. CIN protocol was applied to this group at Day 4 of paricalcitol treatment as described for CIN group.

Rats were allowed to recover in the metabolic cages for an additional 24-h period, at the end of which 24-h urine samples were collected for creatinine clearance (CCr) and fractional Na clearance (FENa%) measurement. Blood samples were withdrawn from the abdominal aorta and the right kidneys were excised under general anaesthesia, achieved by ip injections of ketamine (Ketalar®; Pfizer, Istanbul, Turkey) at 50 mg kg^{-1}. Serum samples were kept at −80 °C until analysis, and were used for measurements of renal functional parameters and serum malondialdehyde (MDA) levels.

Renal function parameters

Serum and urinary creatinine measurements were performed by spectrophotometric method (Modular P; Roche Diagnostics, Mannheim, Germany). Serum and urinary sodium were measured by the ion selective electrode method (Modular ISE; Roche Diagnostics). CCr was calculated as U × V/P, where U is urine creatinine (mg dl^{-1}), V is urine volume (ml min^{-1} 100 g^{-1}) and P is serum creatinine (mg dl^{-1}), and was expressed as ml min^{-1} 100 g^{-1} body weight. Fractional excretion of sodium (FENa) was calculated as (urine sodium/serum sodium) × (serum creatinine/urine creatinine) × 100.

Oxidative stress biomarkers

Kidney thiobarbituric acid-reacting substances (TBARSs) and serum MDA levels were studied. TBARS levels were measured by the method reported by Sozmen et al [16]. Tissue samples were incubated with thiobarbituric acid working solution for 30 min at 95 °C and were calculated using a calibration curve constructed from 1,1,3,3-tetraethoxypropan. MDA levels were assayed using a spectrophotometric method reported by Ohkawa et al [17]. Sample absorbances were measured at 532 nm and calculated using the absorbance of the standard.

Histology

The excised kidneys were preserved in phosphate-buffered 10% formalin, embedded in paraffin wax and cut into 3-μm sections according to conventional techniques. The sections were stained with haematoxylin–eosin. Histopathological changes were analysed for tubular necrosis, proteinaceous casts and medullary congestion, as suggested by Solez et al [18]. Tubular necrosis and proteinaceous casts were graded as follows: 0, no damage; +1, mild (unicellular, patchy isolated damage); +2, moderate (damage less than 25%); +3, severe (damage between 25% and 50%); and +4, very severe (more than 50% damage) [18]. The degree of medullary congestion was defined as: 0, no congestion; +1, mild (vascular congestion with identification of erythrocytes by ×400 magnification); +2, moderate (vascular congestion with identification of erythrocytes by ×200 magnification); +3, severe (vascular congestion with identification of erythrocytes by ×100 magnification); and +4, very severe (vascular congestion with identification of erythrocytes by ×40 magnification) [18]. Evaluations were made by an experienced pathologist who was blinded to the data.

Immunohistochemistry for vascular endothelial growth factor

Immunohistochemistry for VEGF was performed on 3-μm-thick renal sections based on streptavidin–biotin–peroxidase complex formation, according to the manufacturer's instructions. In brief, paraffin-embedded sections were cleared in xylene, rehydrated in a series of ethanol washes. Endogenous peroxidase activity was inhibited with 3% H_2O_2. Antigen retrieval was performed by microwaving sections in citrate buffer (pH 6). Sections were blocked in phosphate-buffered saline (pH 7.4) for 20 min at room temperature and then protein blockage was performed for inhibition of non-specific staining. Sections were incubated with antiVEGF (Ab-1, RB-222-R7; Thermo Fisher Scientific, Fremont, CA) for 30 min, then incubated

Table 1. Renal functional parameters in the study groups

Parameter	Control group (n=8)	Paricalcitol group (n=8)	CIN group (n=8)	Paricalcitol + CIN group (n=8)
SCr (mg dl^{-1})	0.44 ± 0.12	0.48 ± 0.16	1.10 ± 0.44	0.51 ± 0.16[a]
CCr (ml min^{-1})	1.59 ± 0.58	1.57 ± 0.35	0.54 ± 0.37	0.98 ± 0.30[a]
FENa (%)	0.25 ± 0.10	0.27 ± 0.06	1.6 ± 2.18	0.23 ± 0.14[b]

CCr, creatinine clearance; CIN, contrast-induced nephropathy; FENa, fractional sodium excretion; SCr, serum creatinine.
Data are presented as mean ± standard deviation.
[a]$p<0.05$, *vs* CIN group.
[b]$p<0.001$, *vs* CIN group (one-way analysis of variation followed by Tukey's pairwise multiple-comparison test).

with biotinylated secondary antibody (UltraVision Detection System; TP-015-HD; Thermo Fisher Scientific) for 10 min. Finally, streptavidin peroxidase (UltraVision Detection System; TP-015-HD; Thermo Fisher Scientific) was added for 10 min and sections were washed in PBS (pH 7.4) before detection with DAB reagent. After briefly being counterstained with Mayer's haematoxylin, sections were dehydrated. Negative control sections were stained under identical conditions by omitting the primary antibody. Human colon cancer tissue was used as a positive control in staining.

To assess immunohistological staining, tubular VEGF immunostaining was assessed semi-quantitatively in the outer medulla of the kidney (nuclear staining of the tubular cells): 0, very weak or absent nuclear stain; 1, weak nuclear stain of <25% of tubular cells; 2, 25–50% of tubular cells were stained; 3, 50–75% of tubular cells were stained; and 4, >75% of tubular cells were stained intensely [19].

Statistical analysis

The statistical package SPSS v. 13.0 (SPSS Inc., Chicago, IL) was used for data analysis. One-way analysis of variance (ANOVA) followed by Tukey's pairwise multiple comparison test was performed to compare the characteristics between the four treatment groups. Student's *t*-test was performed for two group comparisons. Variables are presented as mean ± standard deviations, and values of $p<0.05$ were considered statistically significant.

Results

The rats tolerated the treatment well, and all survived until the end of the experiment. No statistically significant differences were observed for the baseline characteristics between the groups.

Renal function parameters

Table 1 shows the changes in renal function parameters induced by the experimental procedures in the four treatment groups. One-way ANOVA followed by Tukey's pairwise multiple-comparison test revealed that the mean serum creatinine value was significantly lower ($p=0.034$) and the mean CCr value was significantly higher ($p=0.042$) in the paricalcitol plus CIN group than in the CIN group. The mean FENa value was also significantly lower in the paricalcitol plus CIN group. There were no differences between the paricalcitol plus CIN group and either the paricalcitol group or the control group regarding renal function parameters.

Oxidative stress biomarkers

Table 2 shows the changes in oxidative stress biomarkers in the four treatment groups. Statistical analysis showed that the mean serum MDA levels were significantly lower in the paricalcitol plus CIN group than in the CIN group ($p=0.024$), but there were no differences between the paricalcitol plus CIN group and either the paricalcitol group or the control group. Similarly, mean kidney TBARS levels were significantly lower in the paricalcitol plus CIN group than in the CIN group ($p=0.042$), but there were no differences between the paricalcitol plus CIN group and either the paricalcitol group or the control group.

Histology

The histological findings in the four treatment groups are summarised in Table 3. The mean tubular necrosis score was significantly higher in CIN group than in the control group ($p<0.001$). Paricalcitol treatment significantly improved the mean tubular necrosis score as compared with the CIN group ($p=0.024$). Similar findings were evident for the mean scores of proteinaceous

Table 2. Oxidative stress biomarkers of the study groups

Biomarker	Control group (n=8)	Paricalcitol group (n=8)	CIN group (n=8)	Paricalcitol plus CIN group (n=8)
Serum MDA (μmol l^{-1})	2.88 ± 0.74	2.06 ± 0.45	7.48 ± 0.99	3.20 ± 0.38[a]
Kidney TBARS (nmol g^{-1})	26.20 ± 6.88	24.88 ± 5.95	38.56 ± 6.03	28.05 ± 4.24[a]

CIN, contrast-induced nephropathy; MDA, malondialdehyde; TBARS, thiobarbituric acid-reacting substances.
Data are presented as mean ± standard deviation.
[a]$p<0.05$, *vs* CIN group. Analyses were performed using one-way analysis of variation followed by Tukey's pairwise multiple-comparison test.

Table 3. Histopathological scores and tubular vascular endothelial growth factor expression scores of the study groups

Histopathology	Control group (n=8)	Paricalcitol group (n=8)	CIN group (n=8)	Paricalcitol + CIN group (n=8)
Tubular necrosis	0.26±0.44	0.28±0.40	2.48±0.51	1.27±0.51[a]
Proteinaceous cast	0.35±0.51	0.24±0.37	2.85±0.68	1.08±0.50[a]
Medullary congestion	0.76±0.58	0.85±0.36	3.46±0.52	1.36±0.43[a]
VEGF score	0.50±0.33	0.42±0.38	2.68±0.55	1.24±0.51[a]

CIN, contrast-induced nephropathy; VEGF, vascular endothelial growth factor.
Data are presented as mean ± standard deviation.
[a]$p<0.05$, *vs* CIN group (one-way analysis of variation followed by Tukey's pairwise multiple-comparison test).

casts and medullary congestion, which were significantly lower in the paricalcitol + CIN group than in the CIN group (p=0.038 and p=0.035, respectively). No significant differences were seen between the paricalcitol + CIN group and either the paricalcitol group or the control group. Histopathological findings of the study groups are shown in Figure 1.

Immunohistochemical findings

The results of immunohistochemical analysis of VEGF in the tubular cells in the four treatment groups are reported in Table 3. The mean VEGF score was significantly higher in the CIN group than in the control group ($p<0.001$). Paricalcitol treatment significantly improved VEGF expression as compared with the CIN group (p=0.018). No significant differences were seen between the paricalcitol plus CIN group and either the paricalcitol group or the control group. Immunohistochemical findings of the study groups are shown in Figure 2.

Discussion

The present experimental study demonstrated for the first time that paricalcitol treatment, initiated 4 days before the administration of contrast media, might confer protection against the development of CIN in rats. Paricalcitol attenuated the acute deterioration of renal function, decreased the systemic and renal oxidative stress due to contrast agent, reduced the tubular necrosis, medullary congestion and proteinaceous casts that occurred secondary to contrast media, and lowered VEGF immunoexpression that had presumably developed secondary to regional renal hypoxia.

Research in the field of CIN pathophysiology suggests that this condition is most likely to be the result of renal ischaemia, oxidative injury and direct toxicity to tubular epithelial cells [1, 3]. After the administration of contrast media, reactive oxygen species enhance and cause lipid peroxidation and cytotoxic damage [3], suggesting that oxidative injury is a major factor in the pathogenesis of CIN. After the injection of contrast agent, free radicals react with nitric oxide to produce peroxynitrite, an oxidative and very reactive nitrosative species capable of reducing the bioavailability of nitric oxide, thereby increasing tissue damage [3]. This reactive species exerts its oxidative effects on the sulphydrylic groups and aromatic rings of proteins, cellular membrane lipids and nucleic acids [3]. MDA and TBARS are end products of lipid peroxidation of membrane polyunsaturated fatty acids by free radicals and are indicators of oxidative damage. Administration of contrast agent induces an

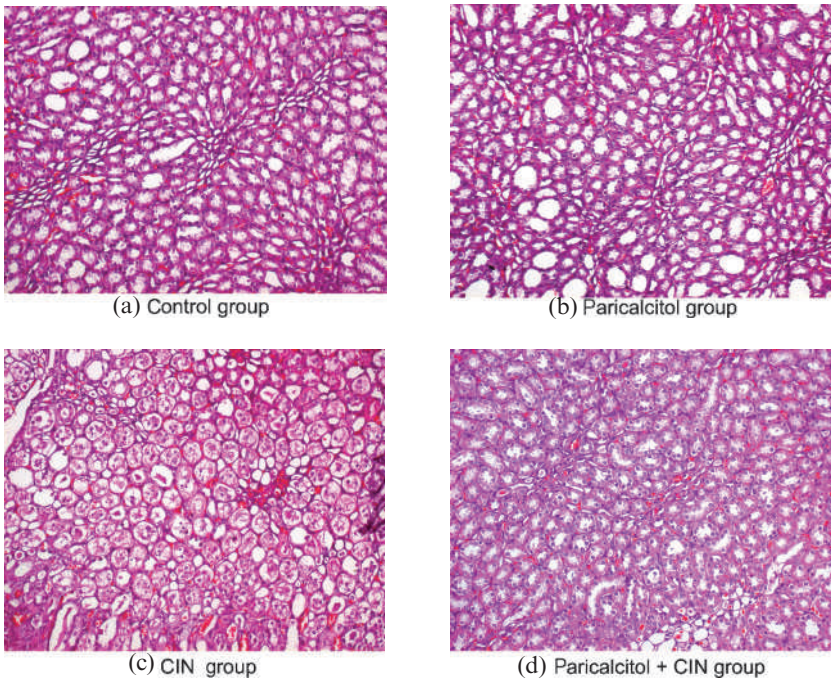

Figure 1. Histopathological findings of the study groups (haematoxylin and eosin staining, original magnification, ×200). Normal histology in (a) control and (b) paricalcitol groups. (c) Tubular necrosis areas in renal outer medulla of contrast-induced nephropathy (CIN) group. (d) Healthy tubular cells in renal outer medulla of paricalcitol plus CIN group.

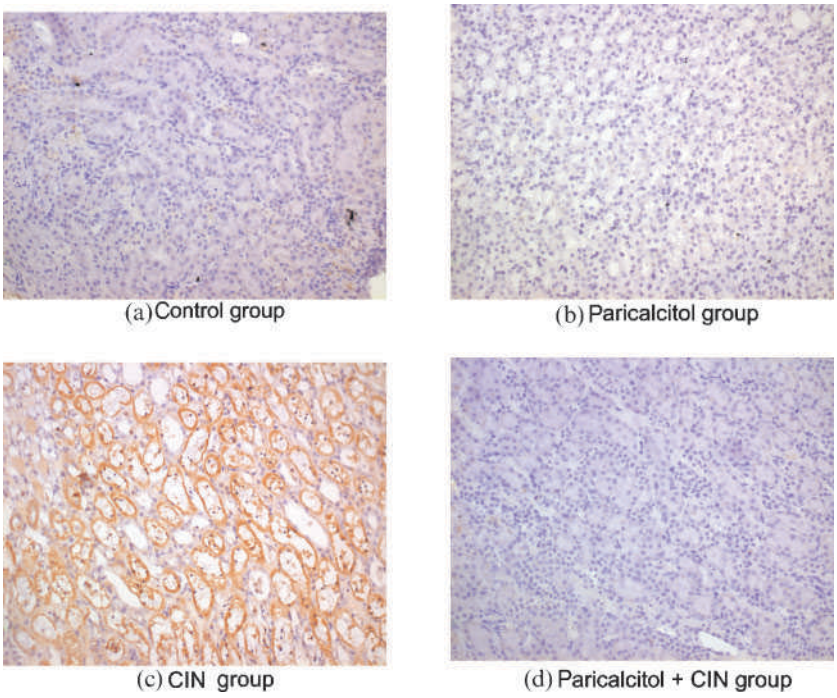

Figure 2. Immunoexpression of vascular endothelial growth factor (VEGF) in the study groups (original magnification, ×200). No immunoexpression of VEGF of tubular cells in the outer medulla of (a) the control, (b) paricalcitol and (d) paricalcitol + contrast-induced nephropathy (CIN) groups. (c) Severe immunoexpression of VEGF of tubular cells in the outer medulla of the CIN group.

increase of renal tissue and serum MDA levels and renal tissue TBARS levels on experimental CIN models [20–22]. To the best of our knowledge, this is the first study to examine the effects of paricalcitol on serum and renal tissue MDA, and TBARS levels after contrast exposure. We speculate that paricalcitol decreases contrast-induced explosion of serum MDA and renal TBARS levels, therefore ameliorating the renal histopathological findings (tubular necrosis, proteinaceous casts and medullary congestion) in the paricalcitol plus CIN group. Of note, paricalcitol has been shown to improve cardiac oxidative injury in uremic and aortic oxidative injury in atherosclerotic rats [9, 23].

Paricalcitol has been shown to attenuate ciclosporin A-induced nephropathy by suppression of inflammatory and apoptotic factors through inhibition of some protein kinase-signalling pathways [5]. De Zeeuw et al [24] demonstrated that paricalcitol lowers albuminuria in patients with diabetic nephropathy. Those findings that address the pleiotropic effects of paricalcitol and our own findings suggest that paricalcitol may well have benefits in the chronic as well as acute situations. Cetin et al demonstrated that ionic high-osmolar contrast medium administration, either alone or together with antecedent cisplatin treatment, leads to accelerated oxidative reactions in rat kidney tissues [20]. Their findings suggest that ascorbic acid supplementation might prevent contrast-induced explosion of renal MDA levels and protect the kidney against oxidant stress. Ascorbic acid has been shown to ameliorate renal damage in experimental models of ischaemic and toxic injury because of its antioxidant effects [25, 26]. It might be interesting to plan further studies to determine the possible beneficial effects of combination prophylaxis with ascorbic acid and paricalcitol on contrast-induced renal abnormalities.

The observed benefits of paricalcitol administration can further be associated with the inhibition of tubular expression of VEGF, even though the precise mechanisms by which paricalcitol ameliorates VEGF expression remain to be studied. Freundlich et al [10] reported that paricalcitol treatment reduces mRNA and protein expression of VEGF in the kidney on experimental chronic renal failure model, possibly through the inhibition of RAS [27]. Acute inhibition of VEGF overexpression by paricalcitol, observed in our study, might be related to the improvement of renal and systemic oxidative stress and subsequent amelioration of local renal hypoxia, and/or inhibition of RAS. Future studies are needed to shed more light on the potential pathophysiological effects of paricalcitol on tubular VEGF immunoexpression in this setting of acute kidney injury.

In conclusion, the active vitamin D analogue paricalcitol causes a reduction in the unfavourable histopathological findings of CIN, possibly through its antioxidant effects by inhibition of lipid peroxidation. Although further experimental and clinical studies are warranted, our findings provide evidence that paricalcitol has a significant potential as a therapeutic intervention for the prevention of CIN.

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