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Co-targeting of endothelin-$_A$ and vitamin D receptors: a novel strategy to ameliorate cisplatin-induced nephrotoxicity

Short running title:

Potential cross-talk between vitamin D and endothelin receptors in cisplatin-induced nephrotoxicity

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Highlights

- A cross-talk has emerged between ET-1/ET_AR and VDR in cisplatin nephrotoxicity.
- BQ-123 attenuated cisplatin nephrotoxicity by reducing ET-1 and ET_AR expression.
- Co-targeting ET-1 and VDR provided greater renoprotection than targeting ET-1.
- Alfacalcidol augmented the renoprotective effect of BQ-123.
- Alfacalcidol and BQ-123 downregulated ET-1/ET_AR and upregulated ET_BR/VDR

Abstract

Background: Although modulation of the vitamin D receptor (VDR) and endothelin-$_A$ receptor (ET_AR) has previously been reported to offer renoprotection against cisplatin-induced nephrotoxicity, the possible interaction between the ET-1 and vitamin D pathways remains obscure. Therefore, the present study addressed the possible interaction between these signalling pathways using BQ-123 (a selective ET_AR blocker) and alfacalcidol (a vitamin D3 analogue) separately or in combination. **Methods:** Male Sprague–Dawley rats were divided into the following groups: control (DMSO orally), cisplatin (single dose of 6 mg/kg *ip*; nephrotoxicity model), cisplatin+BQ-123 (1 mg/kg

BQ-123 *ip* 1 hr before and 1 day after cisplatin), cisplatin+alfacalcidol (50 ng/kg alfacalcidol orally 5 days before and 14 days after cisplatin), and cisplatin+BQ-123+alfacalcidol. Nephrotoxicity was evaluated 96 hrs and 14 days following cisplatin administration. **Results:** Both BQ-123 and alfacalcidol counteracted cisplatin-induced nephrotoxic changes. Specifically, they reduced serum creatinine and urea levels; renal tumour necrosis factor-alpha (TNF-α), transforming growth factor-beta1 (TGF-β1), and phosphorylated nuclear factor-kappa B (*p*NF-κB) content; and caspase-3 activity. They downregulated ET-1 and ET_AR expression and ameliorated cisplatin-induced acute tubular necrosis. In addition, the treatments have increased VDR and endothelin-$_B$ receptor (ET_BR) expression; however, BQ-123 did not affect ET_BR. The effect of the combination regimen surpassed that of each drug alone. **Conclusion:** These findings highlight the potential cross-talk between vitamin D and ET-1 pathways and pave the way for future preclinical/clinical studies to explore further mechanisms involved in this cross-talk.

Abbreviations

AKI, acute kidney injury; DMSO, dimethylsulfoxide; ET-1, endothelin-1; ET_AR, endothelin-$_A$ receptor; ET_BR, endothelin-$_B$ receptor; IκB, inhibitory kappa B; MAPK, mitogen-activated protein kinase; *p*NF-κB, phosphorylated nuclear factor-kappa B; TGF-β1, transforming growth factor-beta1; TNF-α, tumour necrosis factor-alpha; VDR, vitamin D receptor.

Key words: Cisplatin**,** ET-1, nephrotoxicity, ET_AR, VDR

Introduction

Cisplatin is one of the most remarkably successful treatments in the 'war on cancer', especially against solid tumours [1]. However, nephrotoxicity, which is dose-, concentration-, and time-dependent, is the main dose-limiting adverse effect in 25% to 35% of patients treated with even a single dose of cisplatin [2]. Unfortunately, less nephrotoxic cisplatin analogues are less potent anticancer drugs; therefore, cisplatin cannot be completely replaced at the moment [3]. The pathophysiology of cisplatin-induced nephrotoxicity is very complex and multifactorial; it includes tubular injury, renal vasoconstriction, oxidative stress, apoptosis, and a robust inflammatory response that ultimately leads to the death of renal tubular cells [2]. Moreover, the results of conventional renoprotective measures are unsatisfactory because they provide only partial nephroprotective effects, suggesting the need for combination therapy [1].

Accordingly, extensive strategies have been investigated to ameliorate the severity of cisplatin-induced nephrotoxicity without interfering with its anticancer activity [2, 4]. However, a problem arose when scientists realized that most of the signalling pathways implicated in cisplatin-induced nephrotoxicity are also those responsible for its anticancer actions. This fact necessitates further knocking down the possible interplay among these pathways to identify clinically applicable renoprotective strategies that do not diminish the anticancer efficacy of cisplatin [1, 5].

Among the involved pathways is the upregulation of the vasoactive peptide endothelin-1 (ET-1), which has emerged as an important player in the pathophysiology of cisplatin-induced renal damage. Cisplatin-induced nephrotoxic insult is linked to the binding of this peptide to its vasoconstriction-mediating receptor-$_A$ (ET_AR), rather than its vasodilation-mediating receptor-$_B$ (ET_BR) [6]. Consequently, inhibiting the function of ET-1 could reduce cisplatin-induced renal injury.

In the same context, the renoprotective role of vitamin D has led many researchers to explore the hidden mechanisms underlying the protection offered by vitamin D against different models of nephrotoxicity. Over the last few decades, it has become clear that vitamin D is more than a simple calcium hormone. This statement is further supported by the direct correlation between vitamin D deficiency and kidney disease [7, 8], the ability of the vitamin D analogue paricalcitol to ameliorate cisplatin-induced renal injury [9], and the presence of vitamin D receptors (VDRs) in renal tubular cells to regulate functions beyond calcium homoeostasis [10, 11]. Moreover, activation of VDR has been reported to have an important impact in downregulating endothelin receptors in cultured osteoblasts [12], which may help explain how vitamin D treatment attenuates endothelin-induced cardiac remodelling and left ventricular dysfunction [13].

Although the protective roles of vitamin D and ET receptor blockade on the severity of cisplatin-induced nephrotoxicity and other models of acute kidney injury (AKI) have been recognized in previous studies, the possible interaction between these two pathways remains obscure. Therefore, the current study investigated, for the first time, the possible interaction between the ET-1 and VDR pathways in cisplatin-induced

nephrotoxicity in male rats and explored whether this interaction might provide a renoprotective effect against cisplatin-induced nephrotoxicity.

Materials and Methods

Animals

The experiments were carried out on 128 male Sprague–Dawley rats (180–200 g). The rats were obtained from the Animal House of the Faculty of Pharmacy and Drug Manufacturing, Pharos University in Alexandria (Alexandria, Egypt). The rats were housed (4/cage) under normal environmental conditions of daylight and temperature for a minimum of one week prior to experiments for acclimatization and to ensure normal growth and behaviour. The animals had free access to water and standard nutritionally balanced chow. The experiments and animal handling were performed in strict accordance with the Guide for the Care and Use of Laboratory Animals protocol (NIH Publication No. 85-23, 1996), and the protocol was approved by the institutional Ethics Research Committee of the Faculty of Pharmacy, Cairo University (Cairo, Egypt), under the permit number PT 1109/June 2014. All efforts were made to minimize the suffering of the animals during the experimental period.

Drugs

Cisplatin (Cis) was used in a 1 mg/ml intravenous infusion (Cisplatine® Mylan 10 ml vial, Oncotec Pharma Produktion, Germany). Alfacalcidol (ALF; a vitamin D3

analogue) was prepared as a stock solution (0.2 mg/ml) by dissolving 10 mg of alfacalcidol powder (Selleckchem, Houston, USA) in 50 ml of 1% dimethylsulfoxide (DMSO; LOBA Chemie Pvt. Ltd., Mumbai, India). The stock solution was diluted by isotonic normal saline to a (0.00002 mg/ml) working solution. A BQ-123 solution (BQ; 1 mg/ml) was prepared according to the manufacturer's instructions by reconstituting a 5 mg BQ-123 sodium salt vial (Peptides International, Louisville, Kentucky, USA) in 5 ml of sterile water for injection. All other chemicals or reagents used were of high grade and purity.

Experimental design

The animals were divided into 5 groups (n=16 each). The animals in the first group received an oral daily dose of vehicle (1% DMSO which has been diluted by isotonic normal saline the same way as in the alfacalcidol working solution) for 19 days, while those in the second group received a single dose of cisplatin (6 mg/kg, *ip*). In group 3, the rats received alfacalcidol at an oral dose of 50 ng/kg. Treatment started 5 days before the administration of cisplatin and continued for another 14 days afterwards. The rats in group 4 received 2 doses of BQ-123 (1 mg/kg, *ip*) one hour before and one day after the cisplatin dose. Finally, the rats in the last group received cisplatin, alfacalcidol, and BQ-123 in the same doses and routes of administration as in groups 2, 3 and 4. In each group, half of the rats were sacrificed 96 hrs after cisplatin administration, and the other half were sacrificed 14 days after cisplatin administration.

Notably, the selected doses and treatment regimens for both alfacalcidol and BQ-123 were obtained from our earlier pilot experiments that were carried out to evaluate the

dose-dependent renoprotective effects of both drugs with regards to improving the levels of two major kidney function markers, serum creatinine and serum urea. The rationale of the pretreatment strategy used is based on the notion of protecting the kidney before, during, and after the critical period of either drug- or procedure-induced nephrotoxicity; this strategy is now widely accepted in experimental settings and has been documented in previous published studies [14, 15].

The individual effects of alfacalcidol, BQ-123, and (cisplatin + DMSO) on the different parameters investigated in this study are provided as supplementary data.

Tissue preparation

Overnight-fasted rats were anaesthetized with thiopental sodium (35 mg/kg, *ip*), and blood samples were collected from the tail vein 96 hrs and 14 days after cisplatin injection using non-heparinized capillary tubes. Serum was recovered and stored at −70°C until analysis of the serum levels of creatinine and urea. After collecting the blood samples, the rats were euthanized, and the two kidneys were quickly removed. The right kidney was fixed in 10% formaldehyde solution in saline and embedded in paraffin blocks within 24 hrs for histopathological examination and immunohistochemical protein expression studies. The left kidney was washed with ice-cold saline, blotted dry, weighed, and homogenized (10%) in ice-cold saline. Extraction of both cytoplasmic and nuclear proteins from tissue samples was performed using a ReadyPrep™ Protein Extraction Kit (Cytoplasmic/Nuclear, Bio-Rad Laboratories, CA, USA; cat #163-2089) according to the manufacturer's instructions. Both the cytoplasmic and nuclear extracts

were divided into aliquots and stored at −70°C until used for the measurement of different renal parameters.

Biochemical analyses

Serum creatinine and serum urea levels were determined using BioSystems® assay kits (Biosystems S.A., Barcelona, Spain). Rat ELISA kits were used for determination of renal levels of ET-1 (Immuno-Biological Laboratories Co., Ltd. Gunma, Japan; cat #: 27165), tumour necrosis factor-α (TNF-α; Ray Biotech Inc., GA, USA; cat #: ELR-TNFalpha-001C), transforming growth factor-beta1 (TGF-β1; Kamiya Biomedical Company, WA, USA; cat #: KT-30309), caspase-3 activity (Caspase 3 Assay Kit Colorimetric; Abcam, MA, USA; cat #: ab39401), and phosphorylated serine 536 nuclear factor-kappa B (*p*(Ser536)NF-κB; RayBiotech, Inc., GA, USA; cat #: PEL-NFκBP65-S536). Kidney total protein levels were assessed using a Diamond Diagnostics® kit (Cairo, Egypt) according to the manufacturer's instructions. All parameters were determined in the cytoplasmic fractions except *p*S536NF-κBp65, which was determined in the nuclear fraction to enable assessment of the active nuclear translocated form.

Histopathological studies

After fixation in formaldehyde saline, the right kidneys were embedded in paraffin wax and sectioned into 5 µm thick sections to be processed and stained with haematoxylin and eosin (H&E) stain. These sections were examined for histopathological changes under a light microscope, and the total histological damage in the kidney was blindly assessed semi-quantitatively as described in a previous study [15]. Briefly, 10

random sections from each kidney were examined (400×) and scored for tubular necrosis, inflammatory cell infiltration, and glomerular injury on a scale from 0 to 3 for each criterion, and the mean value of all 10 scores was computed for each kidney. For tubular necrosis, the tubular profile was assessed according to an arbitrary scale in which (0) denotes normal kidney histology; (1) denotes tubular cell swelling, brush border loss, and nuclear loss in up to one-third of the tubular profile; (2) denotes nuclear loss in one-third to two-thirds of the tubular profile; and (3 denotes nuclear loss in more than two-thirds of the tubular profile. Similarly, inflammatory cell infiltration and glomerular injury were assessed and each was scored as (0) absent, (1) mild, (2) moderate, or (3) severe. The total histological score for these three criteria ranged from 0 (normal) to 9 (severely damaged). Fig. 1 clearly demonstrates the criteria of the aforementioned scoring system.

Immunohistochemical study

The right kidney blocks were also used for immunohistochemical determination of the protein expression of the ET_A and ET_B receptors [16] as well as VDR [17]. Primary antibodies for ET_AR, ET_BR and VDR were purchased from GeneTex® Inc. (Irvine, CA, USA). Kidney sections were placed on positively charged adhesion microscope slides (Thermo Scientific®, Berlin, Germany), deparaffinized in xylene and rehydrated in a series of decreasing ethanol (100, 95 and 70%). The slides were rinsed gently with phosphate-buffered saline and drained. The antigenic determinants in the cells were unblocked by incubating the sections at 95–98°C for 20 min in citrate buffer (pH 6, Thermo Fisher Scientific, MA, USA) for heat-induced epitope retrieval. The slides were then rinsed with 1× TBST (50 mM Tris/HCl, pH 7.4, 150 mM NaCl, 0.1% Tween 20, (Thermo Scientific®). Endogenous peroxidases were blocked by adding 3% hydrogen

peroxide; the slides were then washed with 1× TBST, and a universal protein block was applied for 20 min. The appropriate primary monoclonal antibodies, *viz.*, rabbit anti-ET_AR, rabbit anti-ET_BR and rabbit anti-VDR, were diluted (1:200) as instructed by the manufacturer and applied to the slides for 45 min at 37°C. Negative controls were processed without applying the primary antibodies. The slides were then washed with 1× TBST, rinsed and incubated for 30 min with the secondary antibody (polyvalent horseradish peroxidase detection kit, Spring Bioscience®, Pleasanton, CA, USA). The chromogen 3,3′-diaminobenzidine (DAB) was prepared and applied as instructed by the manufacturer for protein visualization. Each slide was counterstained with haematoxylin and dipped in ascending concentrations of alcohol and then xylene. The immunohistochemical signals of ET_AR, ET_BR and VDR were quantified with ImageJ software (version 1.45s), and computer-assisted microscopy was used for this purpose with greyscale thresholding, as described previously [18].

Statistical analysis

Parametric data are presented as mean ± SD (n=8). Statistical comparisons between experimental groups were carried out using one-way analysis of variance (ANOVA) followed by Tukey's test. For the scoring data, the values are presented as median (min-max), and statistical analysis was performed using the Kruskal-Wallis test followed by Dunn's multiple comparisons test. Differences with values of $p < 0.05$ were considered statistically significant. Statistical analysis was performed and figures were drawn using the GraphPad Prism® software package (GraphPad Software, CA, USA, version 6).

Results

Effect of alfacalcidol, BQ-123, and their combination on kidney function parameters in cisplatin-induced nephrotoxicity in male rats

Fig. 2 shows a marked elevation in the serum levels of both (A) creatinine and (B) urea, which are markers of impaired renal function, 96 hrs and 14 days after cisplatin administration in the cisplatin group compared to the control group. Meanwhile, administration of alfacalcidol or BQ-123 significantly reduced both parameters following cisplatin administration at both time intervals. Furthermore, co-administration of both agents completely restored the creatinine levels at both time intervals, while urea levels were normalized only after 14 days. The individual effects of alfacalcidol, BQ-123, and (cisplatin + DMSO) on serum creatinine and urea levels are illustrated in supplementary table 2.

Effect of alfacalcidol, BQ-123, and their combination on the renal endothelin-1 (ET-1) pathway in cisplatin-induced nephrotoxicity in male rats

To determine whether the ET-1 pathway is involved in cisplatin nephrotoxicity, we measured ET-1 levels and the renal protein expression of ET_AR and ET_BR in rat kidney homogenates. Rats treated only with cisplatin exhibited sharp increases in the renal content of ET-1 (Fig. 3) and the protein expression of ET_AR (Fig. 4 A & B), but not ET_BR (Fig. 5 A & B), at both time intervals. These alterations were reversed by the use of either alfacalcidol or BQ-123 compared to cisplatin alone. However, the protein

expression of ET_BR was significantly induced only in the alfacalcidol and combination groups. Co-administration of alfacalcidol with BQ-123 further reduced ET-1 levels and ET_AR protein expression compared to administration of the individual agents.

Effect of alfacalcidol, BQ-123, and their combination on renal vitamin D receptor (VDR) levels in cisplatin-induced nephrotoxicity in male rats

Cisplatin treatment reduced the protein expression of VDR at both time intervals compared to vehicle treatment (Fig. 6 A & B). Pretreatment with alfacalcidol or BQ-123 significantly increased the protein expression of VDR at 96 hrs and 14 days following cisplatin administration; however, the effect of alfacalcidol on VDR was significantly greater than that of BQ-123. The level of VDR protein expression in rats treated with alfacalcidol and BQ-123 in combination was significantly higher than that in rats treated with either alfacalcidol or BQ-123 alone at 96 hrs after cisplatin treatment.

Effect of alfacalcidol, BQ-123, and their combination on the levels of renal phosphorylated nuclear factor kappa-B-p65 (p[Ser536]NF-κBp65), tumour necrosis factor-alpha (TNF-α), and transforming growth factor-beta1 (TGF-β1) and the activity of caspase-3 in cisplatin-induced nephrotoxicity in male rats

The cisplatin-induced ET-1/ET_AR activation affected downstream molecules, as depicted in Fig. 7. Cisplatin increased the levels of the transcription factor *p*[Ser536]NF-κBp65 (A) and its downstream cytokines TNF-α (B) and TGF-β1 (C) as well as the activity of the apoptotic marker caspase-3 (D). Pretreatment with alfacalcidol or BQ-123 hindered these alterations, with the blocker showing better anti-inflammatory and anti-apoptotic effects than the vitamin D3 analogue. However, pretreatment with both agents

further attenuated the changes in these biomarkers compared to pretreatment with the individual agents. This pattern was consistent at both time points, yet the effect was more pronounced 14 days after cisplatin treatment.

Effect of alfacalcidol, BQ-123, and their combination on renal histopathological changes in cisplatin-induced nephrotoxicity in male rats

The histopathological photomicrographs shown in Fig. 8 reveal that cisplatin (C & D) induced a typical acute tubular necrosis pattern with widespread tubular dilatation and sloughing of renal epithelial cells (red arrows). Moreover, condensed atrophic features of glomeruli (light blue arrows) with widening of Bowman's space (double-headed arrows↔), haemorrhage (yellow arrows) and infiltration of inflammatory cells (white arrows) were common in sections of this group compared to those of the normal control group (A & B). However, sections from the groups treated with alfacalcidol (E & F) and BQ-123 (G & H) showed limited injury to individual cells of the proximal tubules, whereas sections from combined pretreatment group (I & J) revealed marked reductions in the tubular necrosis induced by cisplatin at both time intervals.

Effect of alfacalcidol, BQ-123, and their combination on renal histopathological scores in cisplatin-induced nephrotoxicity in male rats

Confirming the histopathological changes, Fig. 9 shows the corresponding scores at (A) 96 hrs and (B) 14 days after cisplatin administration. The histopathological damage scores were significantly increased at both time intervals in the cisplatin group compared to the control group. However, pretreatment with alfacalcidol or BQ-123 significantly

reduced the histopathological damage score. In addition, pretreatment with both agents further reduced the score to control values at both time intervals.

Discussion

Although upregulation of ET-1 and ET_AR has been reported in several renal diseases, including cisplatin-induced nephrotoxicity [6], the exact mechanisms through which ET-1 mediates renal injury have not been completely elucidated. Likewise, vitamin D deficiency and downregulation of VDR have been reported in various models of renal injury [10, 19]. Nevertheless, the possible interaction between the ET-1 and vitamin D pathways remains obscure. Hence, the current study tested, for the first time, whether co-targeting these pathways would provide better renoprotection against cisplatin-induced nephrotoxicity than targeting each pathway alone.

The key findings reported in this study confirmed that (i) upregulation of the ET-1/ET_AR/NF-κB/TNF-α/TGF-β1 loop induced major downstream events associated with cisplatin-induced structural, biochemical, and functional renal injuries; (ii) downregulation of VDR played a role in cisplatin-induced renal injury; (iii) inhibition of ET_AR with the selective blocker BQ-123 downregulated ET_AR but restored VDR expression; (iv) only alfacalcidol, a vitamin D3 analogue, succeeded in increasing the expression of VDR and the beneficial receptor ET_BR, an effect that entailed the combination regimen, as well, although ET_BR was not altered by cisplatin; and finally, (v) co-administration of the ET_AR blocker and the vitamin D3 analogue markedly ameliorated cisplatin-induced renal injury and showed better results than treatment with

either agent alone by reducing renal ET-1 content and ET_AR protein expression while increasing VDR and ET_BR protein expression. These results hence verify an advantageous interaction between the ET-1 and VDR pathways in protecting against cisplatin-induced nephrotoxicity.

In the current study, pretreatment with BQ-123 alleviated the cisplatin-induced impairment of renal function, reducing both serum creatinine and urea, improving the renal histopathological structure and lowering the renal histopathological injury score. In a similar pattern, pretreatment with alfacalcidol showed almost the same effects. Thus, these findings confirm the previously reported renoprotective effects of BQ-123 [14] and vitamin D3 analogues [9]. However, the best effect on all these parameters was mediated by the combination regimen, showing that the effects of alfacalcidol augment those of the ET_AR blocker.

The nephrotoxic effect of cisplatin, as demonstrated by the two surrogate parameters of renal function, was more obvious 14 days after cisplatin than 96 hrs after cisplatin, consistent with the findings of previous studies [9, 14]. However, although the different treatment regimens lowered renal function parameters after both time intervals, their effects were most pronounced in the long-lasting insult after 14 days. The present findings support using renoprotective agents before, during and after the critical period of cisplatin-induced toxicity.

Elevated renal ET-1 levels and increased protein expression of ET_AR, but not ET_BR, in kidney tissue were observed in the cisplatin-treated groups in the current study, consistent with the findings of Lee and Ahn [6]. Increased ET-1, when bound to ET_AR,

enhances a constellation of events and activates downstream molecules, including the transcription factor NF-κB; this activation occurs through phosphorylation and ubiquitination of inhibitory kappa B (IκB), which frees NF-κBp65 to be translocated into the nucleus [20]. NF-κB is an early responder to injurious cellular stimuli; therefore, its activation is most prominent at the peak of cisplatin injury [21]. This transcription factor activates several pro-inflammatory and apoptotic cytokines, including TNF-α [4] and TGF-β1 *via* p38 mitogen-activated protein kinase (p38MAPK) downstream of activated toll-like receptors [22] as well as the apoptotic marker caspase-3 [4]. These cytokines mediate the long-lasting deleterious effects of cisplatin on the kidney. These findings are echoed in the present work by the observed activation of *p*NF-κBp65 and caspase-3 and the increases in TNF-α and TGF-β1 levels in the cisplatin groups. In a vicious cycle, overexpression of TNF-α and TGF-β1 has been reported to increase the formation of ET-1 [23]. This dual relationship between ET-1 and cytokines in the kidney has been previously described to create a positive feedback loop augmenting renal injury and deterioration of renal function [24, 25].

Consistent with the findings of previous studies on different models of renal injury [19, 26], the current model of cisplatin-induced nephrotoxicity demonstrated a significant downregulation of VDR. The suppression of VDR was associated with activation of *p*NF-κBp65 and TNF-α, which are key players in cisplatin-induced inflammation and cell death. Interestingly, these observations suggest that VDR downregulation plays a role in the pathogenesis of cisplatin-induced nephrotoxicity by enhancing the inflammatory response and ensuing renal tissue damage. Renal VDR is

known to be involved in the modulation of inflammatory responses during AKI, and activation of VDR signalling intrinsically suppresses NF-κB [26, 27].

The previous findings prompted us to hypothesize that cisplatin-induced ET-1/ET_AR upregulation and the subsequent cytokine loop play a role in VDR downregulation. ET_AR and VDR could counterbalance each other, as evidenced by the restoration of VDR expression upon ET_AR blockade in BQ-123-treated rats. Thus, upregulation of VDR plays a role in the beneficial effects of BQ-123 against cisplatin-induced nephrotoxicity and is considered to augment the effects of ET_AR blockade, including the reduction in *p*NF-κBp65/TNF-α loop signalling, which in turn lowers renal ET-1. Previously, it was proven that inhibition of TNF-α reduces the recruitment and binding of NF-κB to the ET-1 gene promoter and ultimately reduces ET-1 levels [28]. Moreover, BQ-123 reduces the levels of TGF-β1, as previously reported by Sasser et al. [29], which further inhibits ET-1 release and consequently reduces ET_AR expression, as seen in the BQ-123 groups, since ET_AR expression is mainly induced by ET-1 [30].

The relationship between VDR and the altered endothelin system associated with cisplatin-induced renal damage was further investigated in this experiment. We investigated whether upregulation of VDR, as well as activation of VDR using the vitamin D3 analogue alfacalcidol, could offset the alterations in the endothelin system and attenuate the inflammatory response and subsequent renal damage. Interestingly, we report, for the first time, that alfacalcidol reduced renal ET-1 levels and ET_AR protein expression in a model of nephrotoxic AKI. Vitamin D has been reported previously to reduce ET-1 levels in a rat model of pre-eclampsia [31] and to reduce ET-1-mediated cardiac hypertrophy [13]. The alfacalcidol-mediated reduction in ET-1 could be

attributable, in part, to the reduction in TGF-β1, a potent ET-1 inducer, as observed in our study. This effect is in line with Tan et al. [32], who reported that a vitamin D analogue suppressed TGF-β expression in tubular cells. Furthermore, we demonstrated that alfacalcidol attenuated cisplatin-induced inflammation in the kidney, as manifested by reductions in *p*NF-κBp65 and TNF-α, which contribute to the production of ET-1, as mentioned previously. All the proposed mechanisms underlying alfacalcidol-mediated ET-1 reduction explain the downregulation of ET_AR protein expression in alfacalcidol groups, which was also previously reported by Milara et al. [30].

Concerning the vasodilator ET_BR, we did not detect any change in ET_BR protein expression following treatment with cisplatin, consistent with the findings of Lee and Ahn [6]. Similarly, Bae et al. [33] reported that ET_BR expression was not affected by renal ischemic reperfusion injury despite enhanced ET-1 synthesis in their model. BQ-123 also had no effect on ET_BR protein expression compared to vehicle. Interestingly, unlike BQ-123, alfacalcidol increased ET_BR protein expression in the kidneys compared to cisplatin alone, an effect that extended to the combination group, indicating another mechanism for the renoprotective capacity of alfacalcidol. Upregulation of ET_BR has been reported to mediate vasodilation, improve renal blood flow, attenuate inflammatory cascades through the release of nitric oxide and prostacyclin [34], and enhance ET-1 clearance [35], representing an additional mechanism underlying the reduction in ET-1 and ET_AR expression in the alfacalcidol groups. These ET_BR-related events represent additional renoprotective mechanisms aside from the reductions in ET-1 and ET_AR expression in the alfacalcidol groups.

Conclusions

Our results clearly show that co-administration of alfacalcidol with BQ-123 offers a marked renoprotective effect against cisplatin-induced renal injury that surpasses the known renoprotective effect of either agent alone. Our study is the first to highlight the existence of an interaction between ET-1 and vitamin D signalling pathways in the pathogenesis of a model of drug-induced renal injury, specifically cisplatin-induced nephrotoxicity. This study revealed that the improved functional, biochemical, and structural effects rely in part on the ability of these interacting pathways to downregulate ET-1 and ET_AR and upregulate VDR and ET_BR expression. Taken together, these results indicating the existence of cross-talk between the endothelin system and vitamin D pathways pave the way for further preclinical and clinical studies to elucidate other functional and molecular mechanisms of this interaction and to document potential beneficial therapeutic outcomes in the context of renal injury.

Conflict of interest:

The authors declare that this research was conducted in the absence of any commercial or financial relationships that could be construed as potential conflicts of interest.

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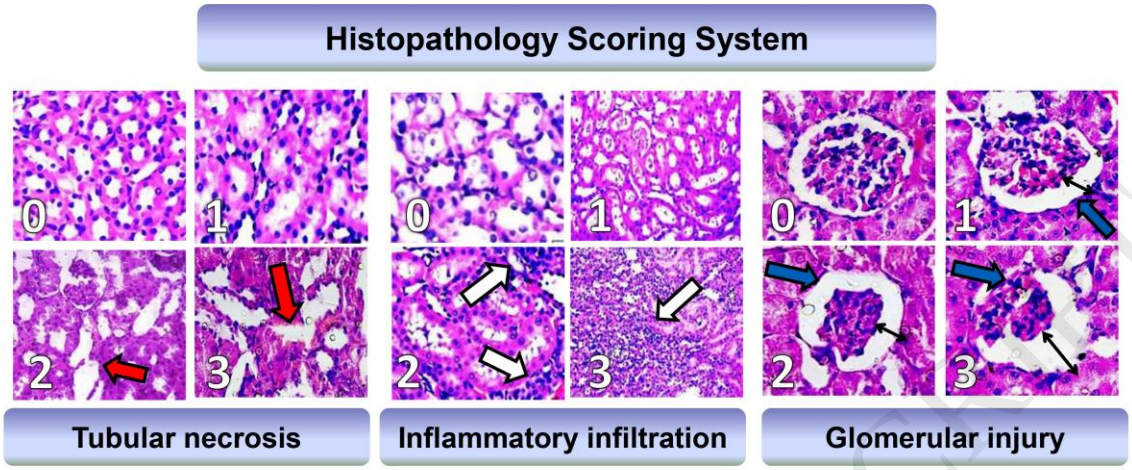


Fig. 1: Photomicrographs showing the progressive stages and scores for tubular necrosis, inflammatory cell infiltration and atrophic glomerular injury. The red arrows indicate tubular cell necrosis, tubular dilatation and sloughing of renal epithelial cells. The white arrows indicate inflammatory cell infiltration. The light blue arrows indicate atrophic features of renal glomeruli. The black double-headed arrow (↔) indicates the widening of Bowman's space (H&E, 400×).

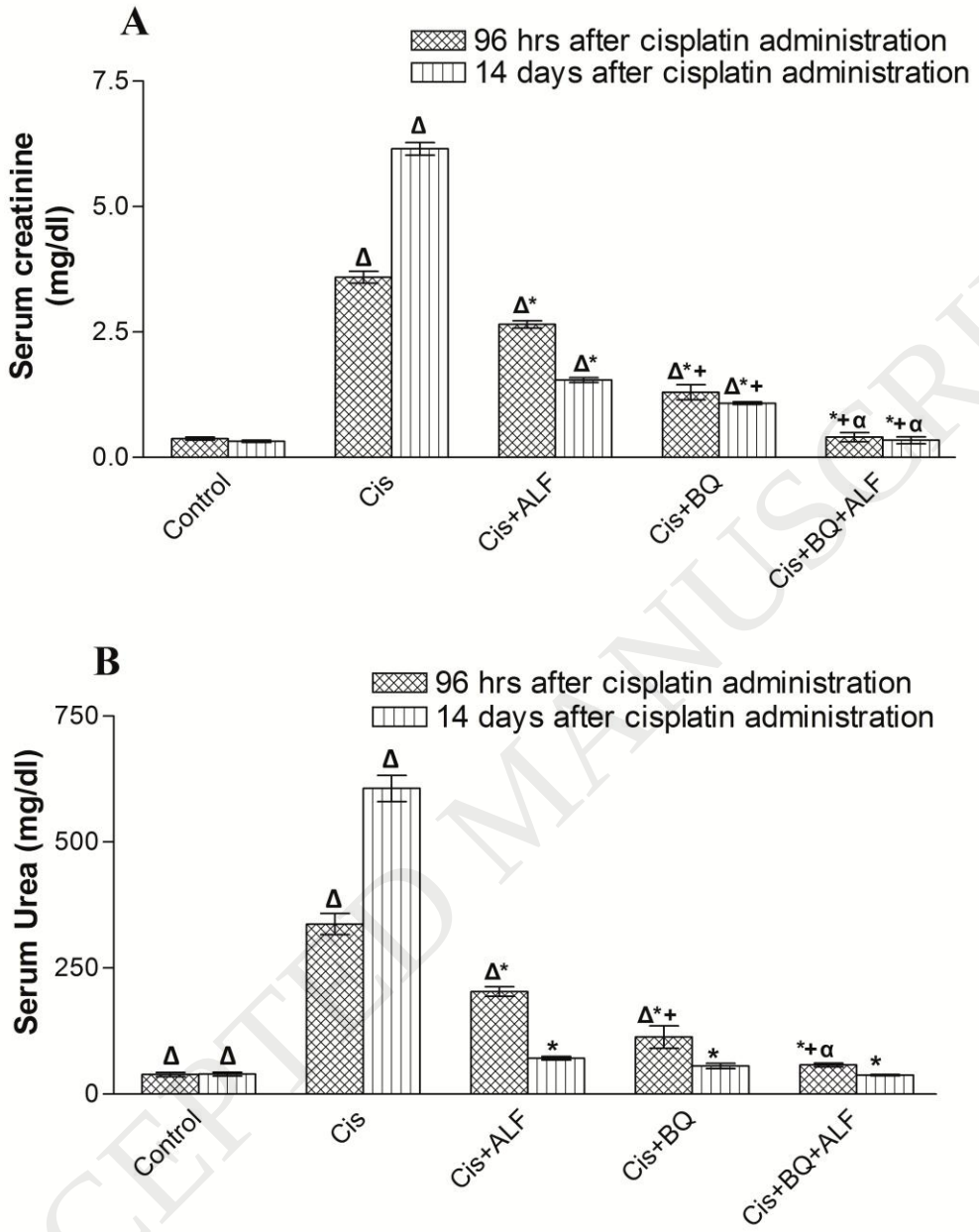


Fig. 2: Effect of ALF and/or BQ on the serum levels of (A) creatinine and (B) urea in Cis-induced nephrotoxicity in rats. The results are expressed as mean ± SD of 8 observations. Statistical analysis was carried out using one-way ANOVA followed by Tukey's multiple comparisons test at ($p < 0.05$), and significance is indicated compared with the control $^{(\Delta)}$, Cis $^{(*)}$, (Cis+ALF) $^{(+)}$ and (Cis+BQ) $^{(\alpha)}$ group. The data were obtained

from rats at 96 hrs and 14 days following single-dose administration of Cis. The animals received alfacalcidol (ALF, 50 ng/kg/day orally) 5 days before and 14 days after cisplatin (Cis, 6 mg/kg, *ip*) insult, BQ-123 (BQ, 1 mg/kg, *ip*) one hour before and one day after Cis insult, or a combination of the two treatments.

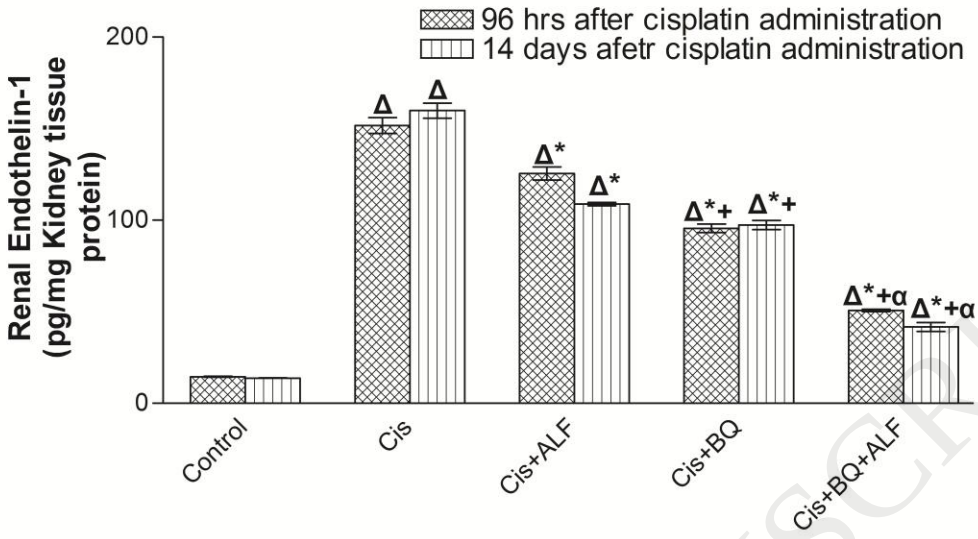


Fig. 3: Effect of ALF and/or BQ on the renal content of endothelin-1 in Cis-induced nephrotoxicity in rats. The results are expressed as mean ± SD of 8 observations. Statistical analysis was carried out using one-way ANOVA followed by Tukey's multiple comparisons test at ($p < 0.05$). Significance is indicated compared with the control $^{(\Delta)}$, Cis $^{(*)}$, (Cis+ALF) $^{(+)}$ and (Cis+BQ) $^{(\alpha)}$ group. The data were obtained from rats at 96 hrs and 14 days following single-dose administration of Cis. The animals received alfacalcidol (ALF, 50 ng/kg/day orally) 5 days before and 14 days after cisplatin (Cis, 6 mg/kg, *ip*) insult, BQ-123 (BQ, 1 mg/kg *ip*) one hour before and one day after Cis insult, or a combination of the two treatments.

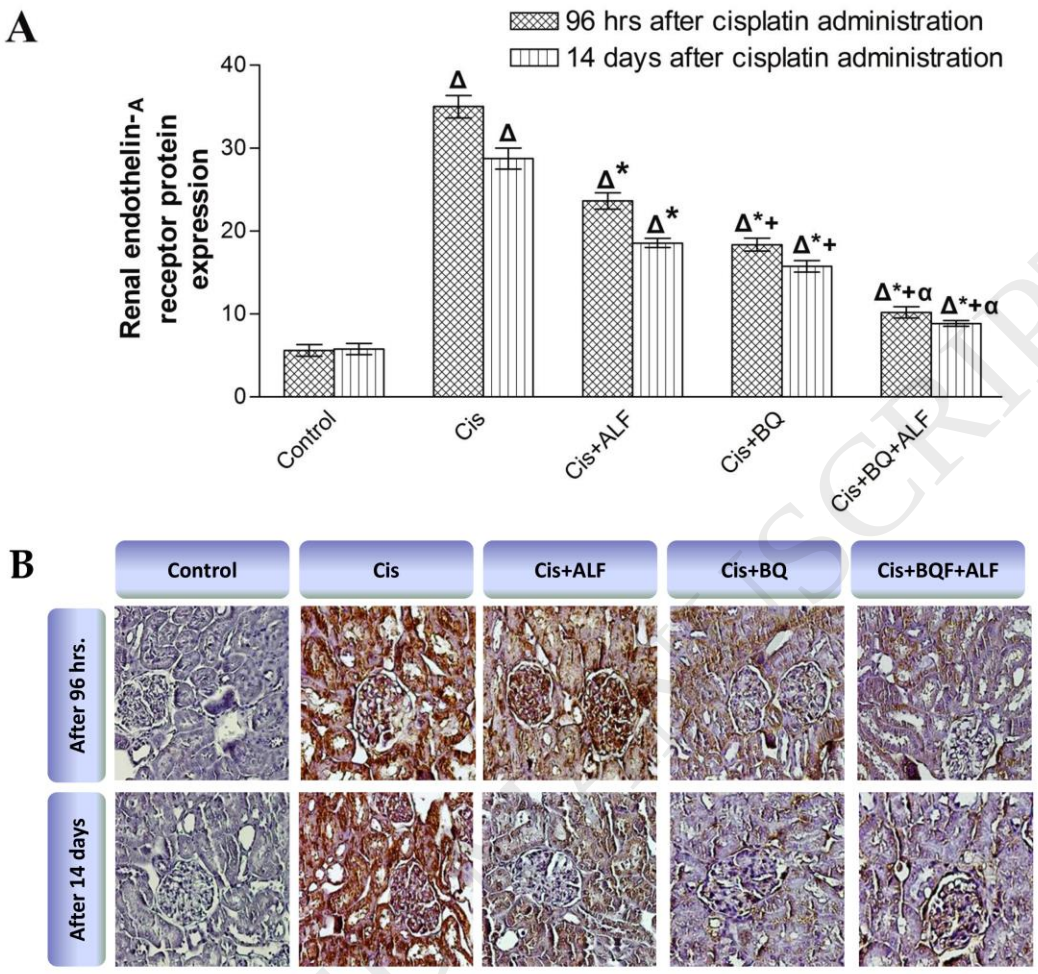


Fig. 4: (A) Effect of ALF and/or BQ on renal immunohistochemical staining of endothelin-$_A$ receptors and (B) representative images of immunostained tissues (400×) in rats with Cis-induced nephrotoxicity. The results are expressed as mean±SD of 8 observations. Statistical analysis was carried out using one-way ANOVA followed by Tukey's multiple comparisons test at ($p < 0.05$). Significance is indicated compared with the control $^{(\Delta)}$, Cis $^{(*)}$, (Cis+ALF) $^{(+)}$ and (Cis+BQ) $^{(\alpha)}$ group. The data were obtained from rats at 96 hrs and 14 days following single-dose administration of Cis. The animals received alfacalcidol (ALF, 50 ng/kg/day orally) 5 days before and 14 days after cisplatin

(Cis, 6 mg/kg, *ip*) insult, BQ-123 (BQ, 1 mg/kg *ip*) one hour before and one day after Cis insult, or a combination of the two treatments.

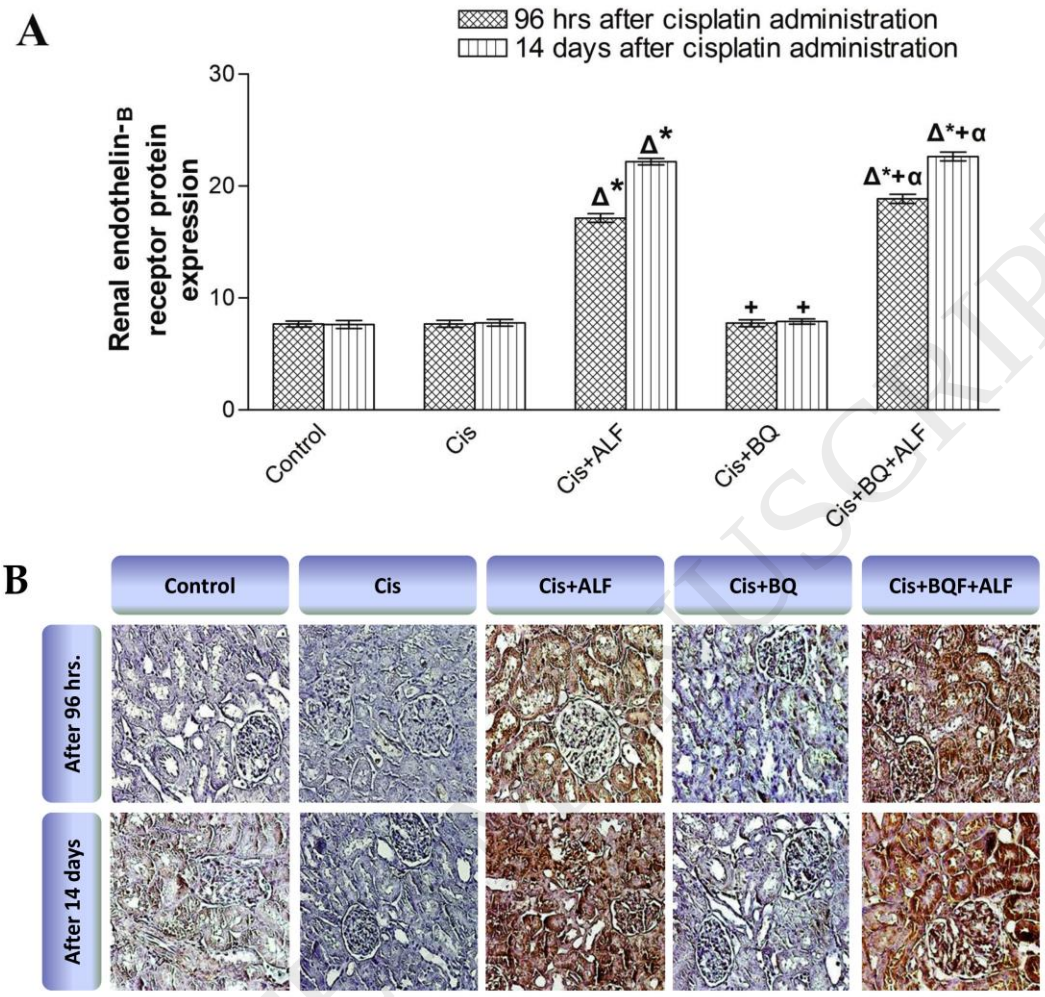


Fig. 5: (A) Effect of ALF and/or BQ on renal immunohistochemical staining for endothelin-B receptors and **(B) representative images of immunostained tissues (400×) in rats with Cis-induced nephrotoxicity.** The results are expressed as mean±SD of 8 observations. Statistical analysis was carried out using one-way ANOVA followed by Tukey's multiple comparisons test at ($p < 0.05$). Significance is indicated compared with the control $^{(\Delta)}$, Cis $^{(*)}$, (Cis+ALF) $^{(+)}$ and (Cis+BQ) $^{(\alpha)}$ group. The data were obtained from rats at 96 hrs and 14 days following single-dose administration of Cis. The animals

received alfacalcidol (ALF, 50 ng/kg/day orally) 5 days before and 14 days after cisplatin (Cis, 6 mg/kg *ip*) insult, BQ-123 (BQ, 1 mg/kg *ip*) one hour before and one day after Cis insult, or a combination of the two treatments.

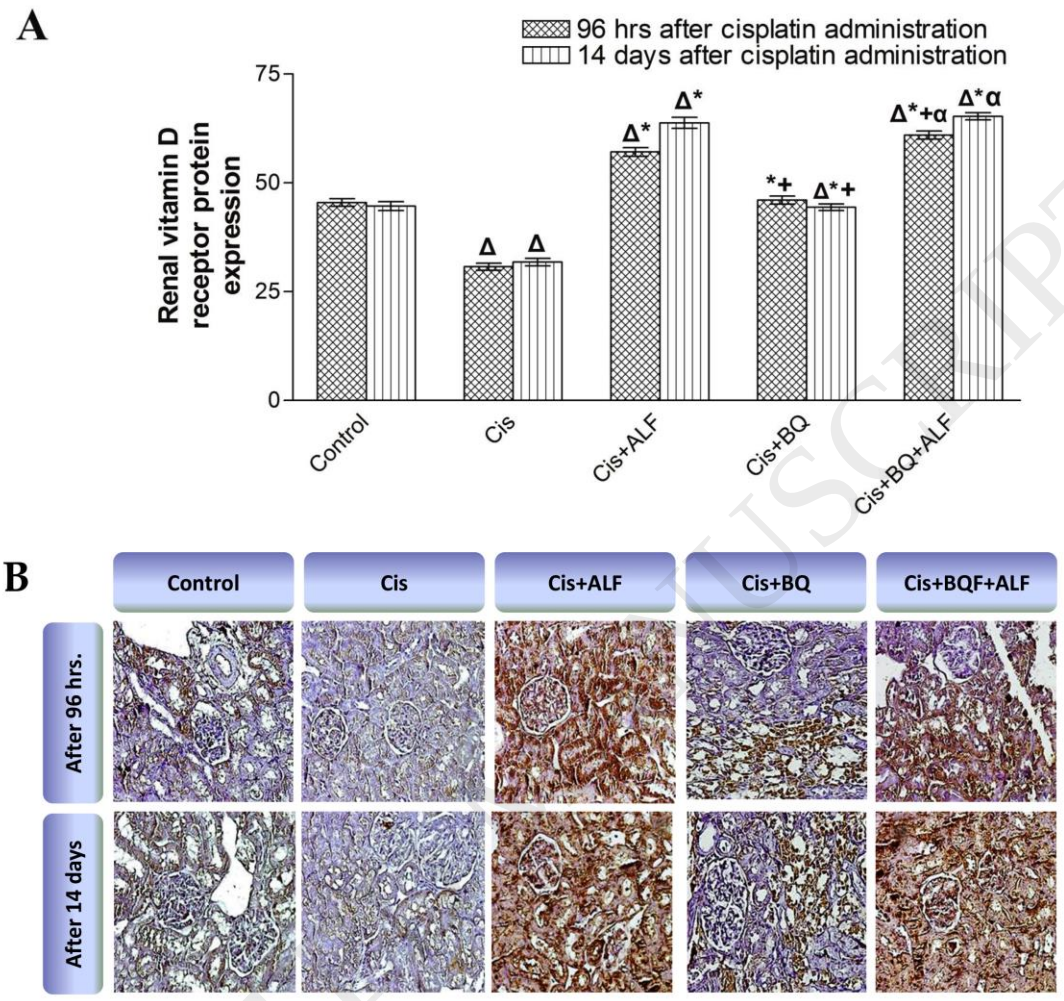


Fig. 6: (A) Effect of ALF and/or BQ on renal immunohistochemical staining for vitamin D receptors and (B) representative images of immunostained tissues (400×) in rats with Cis-induced nephrotoxicity. The results are expressed as mean±SD of 8 observations. Statistical analysis was carried out using one-way ANOVA followed by Tukey's multiple comparisons test at ($p < 0.05$) Significance is indicated compared with the control $^{(\Delta)}$, Cis $^{(*)}$, (Cis+ALF) $^{(+)}$ and (Cis+BQ) $^{(\alpha)}$ group. The data were obtained from rats at 96 hrs and 14 days following single-dose administration of Cis. The animals

received alfacalcidol (ALF, 50 ng/kg/day orally) 5 days before and 14 days after cisplatin (Cis, 6 mg/kg *ip*) insult, BQ-123 (BQ, 1 mg/kg *ip*) one hour before and one day after Cis insult, or a combination of the two treatments.

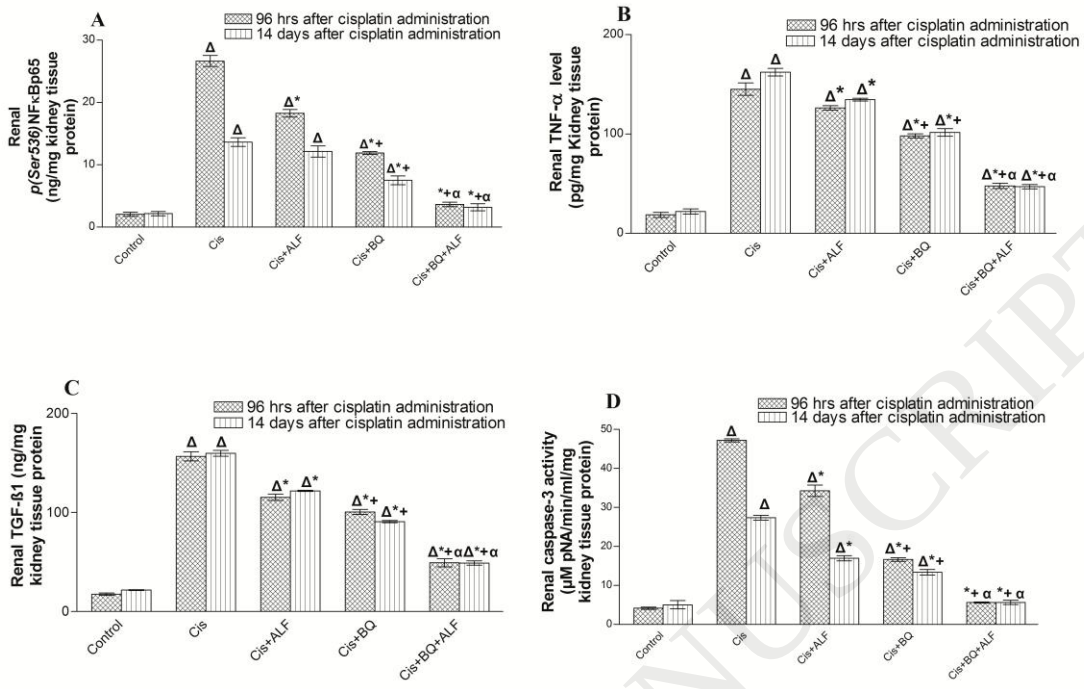


Fig. 7: Effect of ALF and/or BQ on the renal content of (A) *p*NF-κBp65, (B) TNF-α, and (C) TGF-β1 and (D) the activity of caspase-3 in Cis-induced nephrotoxicity in rats. The results are expressed as mean ± SD of 8 observations. Statistical analysis was carried out using one-way ANOVA followed by Tukey's multiple comparisons test at ($p < 0.05$) Significance is indicated compared with the control $^{(\Delta)}$, Cis $^{(*)}$, (Cis+ALF) $^{(+)}$ and (Cis+BQ) $^{(\alpha)}$ group. The data were obtained from rats at 96 hrs and 14 days following single-dose administration of Cis. The animals received alfacalcidol (ALF, 50 ng/kg/day orally) 5 days before and 14 days after cisplatin (Cis, 6 mg/kg *ip*) insult, BQ-123 (BQ, 1 mg/kg *ip*) one hour before and one day after Cis insult, or a combination of the two treatments.

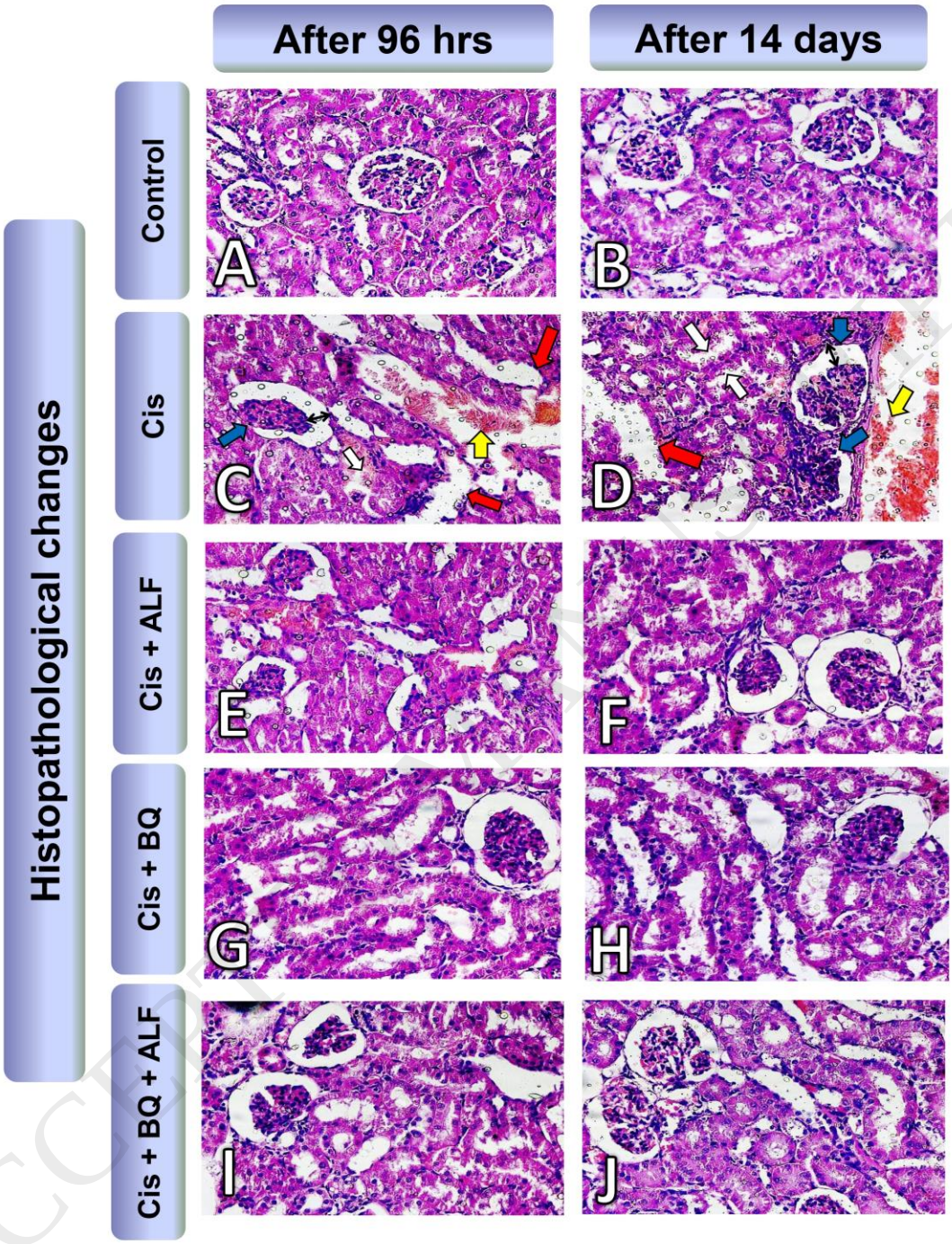
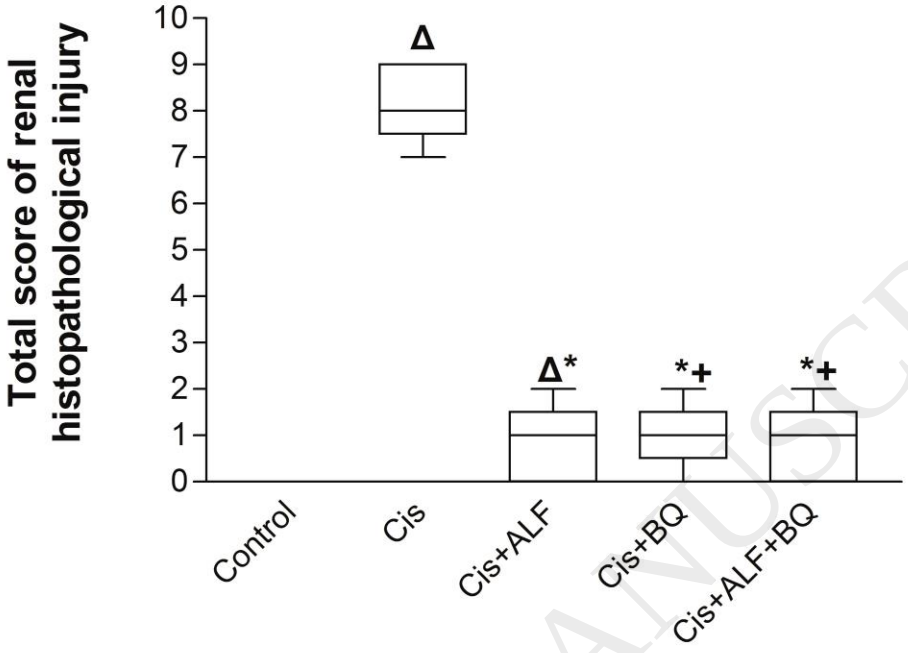


Fig. 8: Renal photomicrographs showing the effect of ALF and/or BQ on Cis-induced nephrotoxicity in rats. The sections (A & B) of normal renal glomeruli and tubules show normal intact structures, whereas sections obtained (C) 96 hrs after or (D)

14 days after Cis administration show condensed atrophic features of glomeruli (light blue arrows) with widening of Bowman's space (↔ arrows), tubular cell necrosis, tubular dilatation, sloughing of renal epithelial cells (red arrows), haemorrhage (yellow arrows) and inflammatory cell infiltration (white arrows). Significant reductions in all types of Cis-induced structural renal damage are noticeable to various extents in the sections from rats treated with ALF (E & F) or BQ (G & H). However, the sections from rats receiving the combination regimen (I & J) show a marked attenuation in Cis-induced renal injury after both time intervals 96 hrs and 14 days, respectively; (H&E, 400×). The data were obtained from rats at 96 hrs and 14 days following single-dose administration of Cis. The animals received alfacalcidol (ALF, 50 ng/kg/day orally) 5 days before and 14 days after cisplatin (Cis, 6 mg/kg, *ip*) insult, BQ-123 (BQ, 1 mg/kg *ip*) one hour before and one day after Cis insult, or a combination of the two treatments.

A



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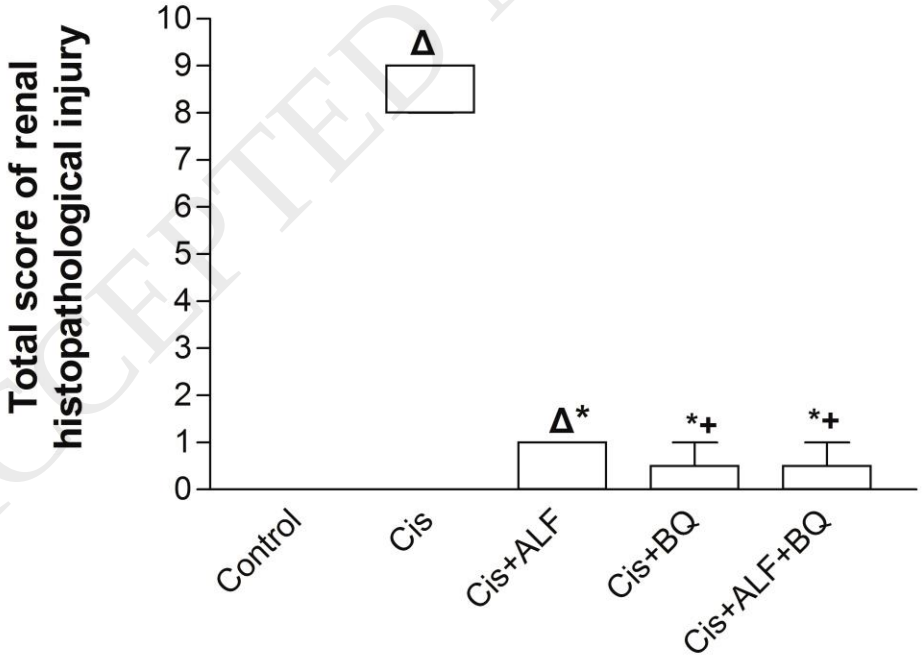

Fig. 9: Effect of ALF and/or BQ on total renal histopathological injury scores at (A) 96 hrs and (B) 14 days following Cis-induced nephrotoxicity in rats. The values are presented as median (min-max), and the statistical analysis was performed using the Kruskal-Wallis test followed by Dunn's multiple comparisons test. Differences with values of ($p < 0.05$) were considered statistically significant compared with the control $^{(\Delta)}$ and Cis $^{(*)}$ groups. The animals received alfacalcidol (ALF, 50 ng/kg/day orally) 5 days before and 14 days after cisplatin (Cis, 6 mg/kg, *ip*) insult, BQ-123 (BQ, 1 mg/kg *ip*) one hour before and one day after Cis insult, or a combination of the two treatments.