**Original Article**

**Effects of vitamin D on kidney histology and trpv1 channels in doxorubicin-induced nephropathy**

Ali Gurel\(^1\), Hasan Atlıö\(^2\), Nalan Kaya\(^3\), Ebru Onalan\(^4\), Tuncay Kuloglu\(^3\), Bilge Aygen\(^5\)

\(^1\)Nephrology Department, Mengucek Gazi Training and Research Hospital, Erzincan, Turkey; \(^2\)Internal Medicine Department, Firat University Medical School, Elazığ, Turkey; \(^3\)Histology and Embryology Department, Firat University Medical School, Elazığ, Turkey; \(^4\)Medical Biology Department, Firat University Medical School, Elazığ, Turkey; \(^5\)Nephrology Department, Firat University Medical School, Elazığ, Turkey

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**Abstract:** Doxorubicin (DXR) is an antineoplastic agent of the anthracycline group, and may show nephrotoxic effects in animal models and humans. We investigated changes in kidney tissue following doxorubicin treatment and the effects of vitamin D on kidney tissue and TRPV1 channels. In this study, 24 adult male Wistar Albino rats were used. The animals were divided into four groups of six animals. During the 14-day experiment period, Group I did not have any application. 200 IU/day cholecalciferol was administered orally to Group II. Group III received 10 mg/kg single dose of DXR intraperitoneally (IP); and Group IV had a single 10 mg/kg dose of IP DXR and 200 IU/day of oral cholecalciferol. At the end of the experiment, the rats were decapitated, and their kidney tissues were removed. TRPV1 expression and apoptosis were detected in the tissue section by using immunohistochemical, TUNEL and real time-PCR (RT-PCR) techniques. The findings were examined and photographed with BH2 Olympus photomicroscope. As result of immunohistochemical staining, RT-PCR and examination with light microscope, it was found that the TRPV1 immunoreactivity of the DXR group decreased in comparison with the control group, and the vitamin D application did not reverse this effect. Apoptosis detected by the TUNEL method tended to increase in the doxorubicin group and was relatively reversed with the administration of vitamin D. Tissue malondialdehyde (MDA) levels were observed to correlate with the findings of apoptosis. This study showed that vitamin D has anti-apoptotic and antioxidant effects on kidney tissue after DXR-induced injury.

**Keywords:** Doxorubicin, apoptosis, nephropathy, trpv1, vitamin D

**Introduction**

For kidneys, due to the exposure to large amounts of blood, affection by toxic substances is more probable [1]. Anthracyclines are wide spectrum antineoplastics used in the treatment of many solid tumors and leukemias [2-4]. Anthracyclines show their antitumor and cytotoxic effects by interacting with DNA topoisomerase II, a nuclear enzyme that regulates the topology of DNA during the replication, transcription, and recombination processes. DXR is a quinone-containing anthracycline antibiotic [5, 6]. The precise mechanism of the DXR-induced nephrotoxicity (DIN) has not yet been determined. However, according to many researches, DXR-induced cellular damage occurs due to plasma membrane injury caused by free anthracycline radicals [7]. Vitamin D has immunomodulatory effects through the vitamin D receptors on immune system cells, such as macrophages, dendritic cells, and CD4+ and CD8+ T lymphocytes [8, 9]. It is also known that human kidney diseases are associated with deficiency or lack of vitamin D [10]. Studies suggest a relationship between vitamin D deficiency and cardiovascular disorders, such as hypertension and atherosclerosis [11, 12]. TRP channels are non-selective cation channels; however, TRPV channels are highly selective for calcium and magnesium. Transient receptor potential vanilloid 1 (TRPV1)-positive sensory nerves have been shown to play an important role on cardiovascular functions, blood pressure, and body weight. TRPV1 dysfunction causes renal excretion and hemodynamic homeostasis impairments. TRPV1 activation shows protective effects against fibrosis...
in the chronic kidney failure process. In acute kidney failure, TRPV1 activation have renoprotective effects due to reduction of ischemia/reperfusion injury and suppression of inflammatory responses [13].

In vivo or in isolated perfused kidneys, the activation of TRPV1 increases the glomerular filtration rate and renal sodium and water excretion [14]. There are many defense mechanisms in the body that limit the levels and the damages of reactive oxygen species. These are known as ‘antioxidants’. Antioxidants suppress lipid peroxidation by preventing the peroxidation chain reaction and/or collecting reactive oxygen species. MDA is the mainproduct of polyunsaturated fatty acid peroxidation and have been used to detect and to evaluate the in vivo and in vitro oxidative stress [15].

In this study we aimed to investigate the effects of vitamin D, an antioxidant and anti-inflammatory molecule, on histopathological changes, TRPV1 channels and oxidative stress on the kidney tissues of rats with DIN.

Materials and methods

Ethical approval required for conducting the study was obtained from local ethics committee of animal experiments.

Study protocol

Eight-week old Wistar Albino male (250-350 g) rats were used in the study. Rats were kept at a temperature of 21°C for 12 hours of light and 12 hours of darkness and their cages were cleaned daily. Baits were provided in steel containers, and water was available in glass bottles ad libitum. The rats were fed with rat chow prepared in specially shaped pellets.

Four experimental groups were established as follows: The control group (n = 6): There were no transactions during the 14-day study. Vitamin D group (n = 6): Throughout the experiment 200 IU/day cholecalciferol were administered orally each day (Devit-3 drops, 50,000 IU/15 ml, Deva, Istanbul). DXR group (n = 6): A single dose of 10 mg/kg intraperitoneal (IP) doxorubicin hydrochloride (C27H29NO11HCl) (Doxo Teva 50 mg vial) was administered on the first day of the study and 200 IU cholecalciferol were administered orally each day during the experiment.

At the end of the experiment, all rats were decapitated under an IP anesthesia procedure. The kidney tissues were removed quickly after decapitation. A portion of the removed kidney tissues was fixed in 10% formalin solution for histological studies. For MDA and TRPV1 mRNA analysis, other sections of tissues were stored at -80°C.

Determination of MDA levels

For MDA analysis, a buffer solution was prepared with 0.42 g Tris-base + 1.43 Tris-HCl + 3 g KCl, and 0.5 ml Tween 20 in 250 ml distilled water. This buffer was used to homogenize the samples. Next, 5 ml of the buffer were added to 1 g of tissue, and the tissue was completely disrupted with the homogenizer (Ultra-Turax T25, IKA-Labortechnik). The homogenate was centrifuged at 5000 rpm for 5 minutes, and 1 ml of the supernatant fraction was transferred to a new tube. After that, 1 ml of 10% Tri-chloro acetic acid (TCA), 1 of ml 0.067% 2-thio barbituric acid (TBA), 1 ml of distilled water, and 0.5 ml of 4% HCl were added to 1 ml of the sample. The prepared mixture incubated at 90°C for 120 minutes. After incubation, the tubes were cooled at room temperature, and the mixture was vortexed after the addition of 3 ml butanol. The tubes were centrifuged at 5000 rpm for 5 minutes, and the resulting supernatant was analyzed using spectrophotometry against butanol at 532 nm.

TUNEL method

Sections 4-6 μm in thickness obtained from paraffin blocks were applied to polylysine slides. Apoptotic cells were identified using Apop Tag Plus Peroxidase In Situ Apoptosis Detection Kits (Chemicon, catno: S7101, USA) in accordance with the manufacturer’s instructions. Preparations were reviewed, evaluated, and photographed with the research microscope (Olympus BH-2). In the evaluation of TUNEL staining, nuclei stained with Harris hematoxylin blue were normal, while brown nuclear staining was interpreted as representing apoptotic cells. At 10 times magnification in
randomly selected areas, at least 500 normal and apoptotic cells were counted. The proportion of apoptotic cells compared with the total (normal/apoptotic) number of cells was calculated as the apoptotic index (AI). The scale bar was 50 μm.

**Immunohistochemistry method**

Sections 4-6 μm in thickness taken from paraffin blocks were collected on polylysined slides. Deparaffinized tissues were passed through a graded alcohol series and boiled in a citrate buffer at a pH of 6 in a microwave oven (750 W) for 7 + 5 minutes. The tissues were cooled for about 20 minutes at room temperature after boiling and then were washed with Phosphate Buffered Saline (PBS, P4417, Sigma-Aldrich, USA) for 3×5 minutes. The tissues were incubated in hydrogen peroxide (Hydrogen peroxide Block, TA-125-HP, LabVision Corporation, USA) for 5 minutes to block endogenous peroxidase activity. After washing with PBS for 3×5 minutes, Ultra V Block (TA-125-UB, LabVision Corporation, USA) was administered for 5 minutes in order to prevent background painting. Tissues were incubated in 1/200 diluted primary polyclonal antibody (Rabbit-polyclonal TRPV1 primary antibody, bs-1931R, Biossantibodies, Boston, USA) in a humid environment for 60 minutes at room temperature. Tissues were washed with PBS for 3×5 minutes after the application of the primary antibody and incubated with the secondary antibody (Biotinylated Goat anti-Polyvalent (anti-mouse/rabbit IgG), TP-125-BN, LabVision Corporation, USA) in a humid environment for 30 minutes at room temperature. Tissues were washed with PBS for 3×5 minutes after application of the secondary antibody and incubated with Streptavidin Peroxidase (TS-125-HR, LabVision Corporation, USA) in a humid environment for 30 minutes at room temperature. 3-amino-9-ethylcarbazol (AEC) Substrate + AEC Chromogen (AEC Substrate, TA-015 and HAS, AEC Chromogen, TA-002-HAC, LabVision Corporation, USA) were added dropwise to the tissue after the video signal was received by the light microscope. Counterstaining textures of tissues were created using Mayer’s hematoxylin staining then rinsed to PBS and distilled water closed with proper closing solution (Large Volume VisionMount, TR-125-UG, LabVision Corporation, USA). Preparations were examined, evaluated and photographed with an Olympus BX 50 microscope. The immunohistochemical histo-score was created on the basis of immunoreactivity prevalence (0.1: <25%, 0.4: 26-50%, 0.6: 51-75%, 0.9: 76-100%) and severity (0: no, +0.5: very little, +1: little, +2: medium, +3: severe). Histo-score = prevalence × severity.

**Quantitative RT-PCR analysis**

Total RNA was isolated from kidney matter using Trizol reagent (Invitrogen, Carlsbad, CA). Random-primed cDNAs were generated by reverse-transcription of total RNA samples with a High Capacity RNA to cDNA Synthesis kit (Invitrogen, Carlsbad, CA). PCR reactions were prepared, in triplicate, and heated to 50°C for 2 min, followed by 40 cycles of denaturation at 95°C for 10 min, 95°C for 15 s and 60°C for 1 min. Standard curves (cycle threshold values versus template concentration) were prepared for each target gene and for the endogenous reference (Glyceraldehyde 3-phosphate dehydrogenase; GAPDH, Cat No: Rn 1775761_g1) in each sample. A real-time PCR analysis was performed with the ABI Prism 7500 Fast Real Time PCR Instrument (Applied Biosystems, Foster City, CA) using Tag Man Master Mix (Applied Biosystems, Foster City, CA). All results were standardized to the levels of GAPDH. Primer and probe oligonucleotides used for real-time quantitative RT-PCR were purchased Applied Biosystems (Cat no: Rn 00583117_m1) (Applied Biosystems, Foster City, CA). The samples were quantified for TRPV1 gene using the comparative Ct (DDCt) method, as described in the Assays-on-Demand Users Manual (Applied Biosystems, Foster City, CA).

**Statistical analysis**

Data were analyzed using a commercially available statistics software package (SPSS® for Windows, version 22.0). Distributions of the groups were tested using the one sample Kolmogrov-Smirnov test. Measured parameters for histopathological changes were normally distributed, and parametric statistical methods were used to analyze the data. One-way ANOVA test was performed, and post hoc multiple comparisons were done with Bonferroni. When biochemical values were not normally distributed, the nonparametric Kruskal-Wallis test of multiple independent groups was used.
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Results

MDA levels

MDA levels, measured spectrophotometrically, were similar in the control and vitamin D groups. The MDA levels of the DXR group were found to be significantly increased in comparison with the control group (P<0.05). In the DXR+ vitamin D group, MDA levels were significantly decreased in comparison with the DXR group (P<0.05, Table 1).

TUNEL findings

Results of TUNEL staining in order to determine apoptotic cells under light microscopy revealed that TUNEL positivity in kidney tissue was similar in the control and vitamin D groups (Figure 1A and 1B). The DXR group had increased staining in comparison with the control group (Figure 1C) (P<0.05). The DXR+ vitamin D group had less staining than the DXR group (Figure 1D) (P<0.05, Table 1).

Immunohistochemical findings- TRPV1 immunoreactivity

Immunohistochemistry results for TRPV1 immunoreactivity examined under light microscopy revealed that TRPV1 immunoreactivity was observed only in the glomeruli (G) of the kidney tissue. TRPV1 immunoreactivity was evident in the control group (Figure 2A). The vitamin D (Figure 2B) and DXR (Figure 2C) groups had significantly decreased TRPV1 immunoreactivity in comparison with the control group (P<0.05). In the DXR+ vitamin D group (Figure 2D), there was a significantly decreased TRPV1 immunoreactivity in comparison with the DXR group (P<0.05, Table 1).

RT-PCR analysis findings

The vitamin D group had significantly higher TRPV1 mRNA levels in comparison with the control group, and in DXR cases, this elevation was more pronounced. In comparison with the DXR group, the DXR+ vitamin D group had significantly reduced TRPV1 mRNA levels (P<0.05, Table 1; Figure 1).

Discussion

The kidneys produce 1.5-2.5 liters of urine per day via filtration, reabsorption, and excretion functions, from the large amounts of blood that they are exposed to. Because they are highly vascularized organs, they are more likely to be affected by any toxic substances present in the blood.

Antineoplastic drugs used in chemotherapy have toxic effects on rapidly reproducing cells such as gastrointestinal, hematopoietic system, and testis cells. Many antineoplastic agents are known to have toxic effects on kidney tissue as well. Anthracyclines are wide spectrum and effective agents used in the treatment of solid and hematologic malignancies. Nephrotoxicity is one of the major side effects of anthracycline antibiotics. The mechanism of DIN is not fully known. DIN likely arises due to severe plasma membrane damage from free antracycline radicals generated by DXR. Many studies have demonstrated that reactive oxygen metabolites are involved in the primary pathogenic mechanism of DIN in rats. An alternative hypothesis is that nephrotoxicity of DXR may be caused via an immune-mediated mechanism. It has recently been reported that CD4+ CD25+ regulatory T cells, normally associated with immune-mediated glomerulonephritis and

<table>
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<tr>
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<th>MDA (mean ± standard deviation)</th>
<th>Apoptotic index (%)</th>
<th>TRPV1 mRNA (PCR)</th>
<th>TRPV1 immunoreactivity Histoskor</th>
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<tbody>
<tr>
<td>Control</td>
<td>130.32±7.81</td>
<td>2.16±0.40</td>
<td>1</td>
<td>1.95±0.36</td>
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<tr>
<td>Vit D</td>
<td>145.84±23.62</td>
<td>2.33±0.51</td>
<td>2.2a</td>
<td>0.56±0.08a</td>
</tr>
<tr>
<td>DXR</td>
<td>253.30±1.99a</td>
<td>12.83±0.98a</td>
<td>6a, b</td>
<td>1.06±0.20a,b</td>
</tr>
<tr>
<td>DXR+ vit D</td>
<td>149.43±8.40b</td>
<td>4.50±0.833a,b</td>
<td>3</td>
<td>0.53±0.10a,c</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± standard deviation, *in comparison with the control group, ^in comparison with the vitamin D group, &in comparison with the DXR group (P<0.05).
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There are many studies about DIN on the literature. A current experimental study revealed that doxorubicin treatment causes an increase in pre-apoptotic bax and caspase-3 immunoreactivity and lipid peroxidation with increasing MDA levels in kidney tissue [4]. Nicotinamide administration before DXR treatment has been observed to protect kidney tissue against DIN, probably due to its antioxidant and anti-inflammatory effects [17]. Curcumin was determined to have a protective effect against proteinuria, albuminuria, hypoalbuminemia, and hyperlipidemia due to DIN and also was found to inhibit an increase of urinary N-acetyl-beta-D-glucosaminidases (tubular damage marker), fibronectin, glucosamineglucan, and the increase in plasma cholesterol caused by DIN. Additionally curcumin partially corrected the decreased GFR due to DIN by suppressing the oxidative damage and increasing kidney glutathione content and glutathione peroxidase activity [18]. The lycopene molecule, which was obtained from tomatoes, was found to decrease urea and creatinine levels, increase glutathione and other antioxidants, and also improve histopathological changes in rats with DIN [19]. Luffa acutangula extract was also observed to improve biochemical and histopathological parameters via its antioxidant effects [20], in another experimental model, coenzyme-Q10 also led to the recovery of biochemical and histopathological parameters by reducing the levels of oxidants in DIN [21]. Rashikh et al. detected an increase in blood pressure, plasma renin activity, plasma urea and creatinine levels, and tissue MDA levels but a decrease in plasma albumin, total protein, renal tissue antioxidant molecules such as glutathione, superoxide dismutase, and catalase in rats given DXR. They also observed histopathological changes, such as contraction of podocyte foot processes, expansion of glomerular basement membranes, and reduced diameter of slit diaphragms. The direct renin inhibitor aliskiren was found to prevent these pathological processes [22]. In another study, telemisartan and captopril were found to have nearly equal protective effects against DIN by reducing the expression of iNOS and their antioxidant properties [23]. Zingiber officinale roscoe (ginger) has protective effects against DIN by increasing the renal GSH levels [24]. The application of silymarin has similar effects via inhibiting lipid peroxidation and increasing renal GSH levels [25]. The apoptotic effect of DXR is likely mediated by p53 protein. Sabuncuoglu showed that...
DXR causes apoptosis in lung cells; the administration of aminoguanidine, an antioxidant, was found to reduce apoptosis [26]. Zhao D et al. [27] demonstrated in their current study that DXR increases apoptosis in osteosarcoma cells by increasing apoptosis-related proteins caspase 3 and cytochrome C levels and reducing the mitochondrial membrane potential.

In our study, the apoptotic index of the DXR group was significantly higher in comparison with the control group, while vitamin D application to the DXR group caused a significant reduction of the apoptotic index in comparison with the DXR group.

There are many current studies about the relationship between kidney disease and vitamin D deficiency. Active vitamin D is produced in the kidney, and vitamin D deficiency develops early in CKD. Much clinical evidence suggests the renoprotective effects of vitamin D [28]. Hojs et al. [29] found a significant decrease in 24-hour urine albumin and quantitative proteinuria in pre-dialysis CKD patients with secondary hyperparathyroidism who received 1 μg/day paricalcitol over a 6 month follow-up period. Similarly, de Borst et al. [30] found a significant decrease in proteinuria with active vitamin D therapy in this patient population. He et al. [31] reported that paricalcitol decreases podocyte damage, proteinuria and kidney damage by inhibiting the Wnt/β-catenin signaling pathway and reducing proinflammatory cytokines. In another study on this subject, Panichi et al. [32] found that 1, 25 (OH)₂D₃ treatment decreased the apoptotic process in the kidneys through the reduction of glomerular hypercellularity and inflammatory cell infiltration in an experimental model of mesangio proliferative glomerulonephritis termed anti-Thy-1.1 nephritis. The use of active vitamin D and its analogs has been shown to prevent podocyte damage significantly in a puromycin aminonucleoside nephrosis model [33]. Dabak et al. [16] found that vitamin D application partially reversed the histopathologic changes of DIN.

Similar to other studies in the literature, our study revealed that MDA, an indicator of lipid peroxidation, was present in significantly higher concentrations in the DXR group than in the control group. MDA levels of the DXR+ vitamin D group were significantly lower than those of the DXR group. All these findings support the possibility of protective effects of vitamin D against DIN by interfering with antioxidant systems.

The JBP485 dipeptide is excreted by organic anion transporters in rat kidney tissue and
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plays a protective role in the liver and gastrointestinal tract. After the active vitamin D application plasma concentration of JBP485 was found to increase, the renal clearance decreased; this effect is probably due to a decrease in organic anion transporter expression in renal tissue [34].

In the literature, many experimental studies have reported that the activation of TRPV1 channels prevents adipogenesis and obesity; intrathecal administration of TRPV1-shRNA increases blood pressure, and in the case of myocardial infarction, both a serotonin and a TRPV1 increase can trigger sudden bradycardia and hypotension (Bezold-Jarisch reflex) [11, 12]. In isolated perfused kidneys, TRPV1 activation reduces renal perfusion pressure and sustains the glomerular filtration rate and renal sodium and water excretion possibly via simultaneous calcitonin gene-related peptide and substance P receptor stimulation. These findings suggest that TRPV1 plays a key role on renal hemodynamics and excretion functions [14].

TRPV1 agonists have been demonstrated to have protective and anti-inflammatory effects on the kidneys against ischemia/reperfusion injury [35]. Another study showed that impaired renal function after acute renal failure was significantly alleviated after the activation of TRPV1 [36].

In this study, kidney tissue TRPV1 immunoreactivity decreased with DXR application and was not significantly reversed by vitamin D therapy. Moreover, kidney damage induced by DXR has considerably improved with vitamin D application. This improvement is observed by decreased TUNEL immunoreactivity, which is a marker of apoptosis. This effect of vitamin D may likely be associated with its antioxidant effects. Additionally, we determined that DXR application increased apoptosis and MDA levels in kidney tissue, while vitamin D partly reversed these effects. In light of these findings, further studies will be needed to determine whether vitamin D may be considered an alternative treatment option to reduce the toxic effects of chemotherapeutics, including DXR.

Disclosure of conflict of interest

None.

Address correspondence to: Ali Gurel, Nephrology Department, Mengueck Gazi Training and Research Hospital, Erzincan, Turkey. E-mail: draligurel@gmail.com

References

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