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Effects of vitamin D on doxorubicin-induced lung injury and TRPM2 immunoreactivity in rats

D vitamininin sıçanlarda doksorubisin kaynaklı akciğer hasarı ve TRPM2 immünoreaktivitesi üzerindeki etkileri

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Abstract

Aim: Although doxorubicin (DOX) is a commonly used chemotherapeutic agent, it causes significant toxic side effects on many organs. This study aims to investigate the effects of vitamin D (VD) on DOX-induced lung injury and expression of transient receptor potential melastatin 2 (TRPM2), a Ca^{2+} permeable cation channel.

Methods: A total of 24 Wistar albino rats were separated into four groups of six rats, as follows: The control group received no medications. The VD group received 200 IU/kg VD via an oral dropper (o.d.) for 14-days. DOX group received a single dose of 10 mg/kg DOX intraperitoneally (i.p.) on day 8. DOX+VD group was administered 200 IU/kg VD via an o.d. for 14-days, and a single dose of 10 mg/kg DOX i.p. on day 8. At the end of the experiment, lung and serum samples from all rats were collected. Masson's trichrome staining and streptavidin-biotin-peroxidase complex were applied to the lung tissue sections. Serum total antioxidant status (TAS) and total oxidant status (TOS) were assessed by enzyme-linked immunosorbent assay (ELISA) method.

Results: Histopathological damage was observed in the DOX group compared to the control group, and biochemical and immunohistochemical evaluation revealed that TOS level and TRPM2 expression increased while TAS level decreased significantly (P<0.001). Additionally, compared to the DOX group VD administration significantly reversed these values in the DOX+VD group (P<0.001).

Conclusion: VD showed a protective effect on lung damage induced by DOX. Our data also suggest that TRPM2 channel may have a role in the pathophysiology of DOX-induced lung damage.

Keywords: Doxorubicin, Lung, Oxidative stress, Transient receptor potential melastatin 2, Vitamin D

Öz

Amaç: Doksorubusin (DOX), yaygın olarak kullanılan bir kemoterapötik ajan olmasına rağmen, birçok organda önemli toksik yan etkilere sebep olmaktadır. Bu çalışma, D vitamininin (VD), DOX ile indüklenen akciğer hasarı ve Ca2+ geçirgen katyon kanalı olan, geçici reseptör potansiyeli melastatin 2 (TRPM2) ekspresyonuna etkilerini araştırmayı amaçlamaktadır.

Yöntemler: 24 Wistar albino sıçan dört eşit gruba ayrıldı. Kontrol grubu; hiçbir uygulama yapılmadı. VD grubu; 14 gün boyunca oral damlalıkla (o.d.) 200 IU/kg VD uygulandı. DOX grubu; 8. günde tek doz 10 mg/kg DOX intraperitoneal (i.p.) olarak uygulandı. DOX + VD grubu; 14 gün boyunca o.d. ile 200 IU/kg VD uygulandı, 8. günde tek doz 10 mg/kg DOX i.p. olarak uygulandı. Deney sonunda tüm sıçanlardan akciğer ve serum örnekleri toplandı. Akciğer doku kesitlerine Masson'un trikrom boyama yöntemi ve streptavidin-biotin-peroksidaz kompleks yöntemi uygulandı. Serum toplam antioksidan seviyesi (TAS) ve toplam oksidan seviyesi (TOS) enzime bağlı immünosorbent testi (ELİSA) yöntemi ile değerlendirildi.

Bulgular: DOX grubunda kontrol grubuna göre histopatolojik hasar gözlenirken, biyokimyasal ve immünohistokimyasal değerlendirmede, TOS düzeyinin ve TRPM2 ekspresyonunun anlamlı olarak arttığı, TAS düzeyinin ise anlamlı olarak azaldığı saptandı (P<0,001). Diğer taraftan, VD uygulamasının, DOX grubuna kıyasla, DOX+ VD grubunda bu değerleri önemli ölçüde tersine çevirdiği gözlendi (P<0,001).

Sonuç: VD, DOX'un neden olduğu akciğer hasarı üzerinde koruyucu bir etki gösterdi. Verilerimiz ayrıca, TRPM2 kanalının DOX ile indüklenen akciğer hasarının patofizyolojisinde bir rolü olabileceğini düşündürmektedir.

Anahtar kelimeler: Doksorubusin, Akciğer, Oksidatif stres, Geçici reseptör potansiyeli melastatin 2, D vitamini

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Introduction

Doxorubicin (DOX) (adriamycin) is a potent antineoplastic antibiotic [1] that is used with a wide variety of human cancers, such as breast, ovarian, and hepatocellular carcinoma, and acute lymphoblastic leukemia [2]. However, its side effects limit the clinical use of this drug [3]. The molecular mechanism by which DOX leads to cell death remains unclear [4]. DOX administration decreases the endogenous antioxidants that have important roles in the scavenging of free radicals [5]. DOX-induced release of free radicals may cause oxidative stress, resulting in DNA damage and cell death [6]. Transient receptor potential (TRP) channels are known as non-selective cation channels which are activated by different chemical and physical stimuli such as oxidative stress, heat, and osmotic pressure [7]. Transient receptor potential melastatin 2 (TRPM2) is a heat sensitive, Ca^{2+} permeable cation channel of the melastanine subgroup of TRP superfamily [8]. Oxidative stress stimulates Ca^{2+} flow in TRPM2 channels [9]. Vitamin D (VD) is a steroid hormone that is often obtained by diet and synthesized by the skin when exposed to the sun [10]. It is a membrane antioxidant, and includes Vitamin D_3 (cholecalciferol), 1,25-dihydroxycholecalciferol, 7-dehydrocholesterol (pro-Vitamin D_3) and Vitamin D_2 (ergocalciferol) [11]. VD is both a prooxidative and antioxidative agent [12], and has been reported to reduce chronic inflammation, suppress oxidative stress, and contribute to mitochondrial respiratory function [13].

The present study was devised to investigate the protective utilities of VD in DOX-induced lung tissue injury, and whether the TRPM2 ion channel might be a marker in lung damage.

Materials and methods

Animals

We used 10-12-week-old 24 male Wistar albino rats obtained from Adiyaman University Experimental Animal Production and Research Center. Rats were divided into four equal groups, housed at 22 (2) ºC with a 12 h light/dark cycle and given standard feed and water *ad libitum*.

Experimental design

Animals were treated according to the ethical rules for the care of laboratory animals (Adiyaman University Animal Experiments Local Ethics Committee, protocol no:2020/044). Experimental groups were designed as follows: Control group received no medications. The VD group received 200 IU/kg VD (DEVİT- 3® Deva, Istanbul, Turkey) via an oral dropper (o.d.) for 14-days. DOX group received a single dose of 10 mg/kg DOX (Doxorubicin® Koçak 50 mg, Kocak-Farma, Istanbul, Turkey) intraperitoneally (i.p.) on day 8. DOX+VD group was administered 200 IU/kg VD via an o.d. for 14-days, and a single dose of 10 mg/kg DOX i.p. on day 8. At the end of the experiment, all rats were fasted overnight, and after obtaining intracardiac blood samples under anesthesia with ketamine (75mg/kg, Ketalar®, Eczacıbası; Istanbul, Turkey) and xylazine (10mg/kg, Rompun®, Bayer Turk Chemistry Industry. Ltd. Corp., Istanbul, Turkey), they were decapitated. Next, the lungs were removed rapidly and fixed with 10% formaldehyde for histopathologic examination. Blood samples of rats were centrifuged at 1500 rpm for 15 minutes to separate the serums. Serum samples were placed at -80°C until biochemical analysis.

Light microscopy

Tissue preparation and histopathology

Fixed lung tissues were passed through a graded alcohol series, cleared with xylene, and placed in paraffin. Sections were cut at 5−6 μm and mounted on polylysine slides. After deparaffinization with xylol, sections were passed through descending concentrations of alcohol, and stained with Masson's trichrome. Following evaluation of stained sections, images were obtained using a Leica DM500 microscope (Leica DFC295).

Biochemical methods

Determining TAS and TOS levels

ELISA method was used for determining the levels of TAS and TOS in serum samples by using TAS (Rat TAS Catalog no: YLA3389Ra YL Biotechnology Co., Ltd, Shanghai, CHINA) and TOS (Rat TOS Catalog no: YLA1392Ra YL Biotechnology Co., Ltd, Shanghai, CHINA) kits according to the instructions of manufacturer. The measurement range of Rat TAS ELISA kit was 1-300 pg/ml, Intra-Assay and Inter-Assay CV values were <10% and <12%, respectively. Sensitivity was 0.54 pg/ml. The measurement range of Rat TOS ELISA kit was 0.02-60 U/ml, Intra-Assay and Inter-Assay CV values were <10% and <12%, respectively. Sensitivity was 0.013 U/ml. The unit of test results is specified in U/ml for serum samples.

Immunohistochemical examination

For antigen retrieval, following rehydration, sections were boiled in a microwave oven (750 W) seven times each in citrate buffer solution, pH 6 for 5 min. The sections were cooled at room temperature for 20 min, washed three times for five minutes each with phosphate-buffered saline (PBS) (P4417; Sigma Chemical Co.), then incubated with hydrogen peroxide block solution (TA-125-HP; Lab Vision Corp. USA) for five minutes to block endogenous peroxidase activity. Sections then were washed three times each with PBS. After five minutes of applying Ultra V Block (TA-125-UB; Lab Vision Corp.), sections were incubated with primary antibody (60 min) for TRPM2 (Anti TRPM2 polyclonal antibody, bs-2888R, Bioss, Inc. USA), secondary antibody (30 min) (biotinylated goat anti-mouse/rabbit Ig G, TP-125-BN; Lab Vision Corp.) and streptavidin peroxidase (30 min) (TS-125-HR; LabVision Corp.). 3-Amino-9-ethylcarbazole (AEC) substrate + AEC chromogen (AEC substrate, TA-015 and HAS, AEC Chromogen, TA-002-HAC; Lab Vision Corp.) solution was dripped on the sections. The sections were washed with PBS. Counterstaining of the sections were performed with Mayer's hematoxylin, passed through PBS, and distilled water, mounted with Large Volume Vision Mount (TA-125-UG; Lab Vision Corp). After evaluation of the sections, images were obtained with a Leica DM500 microscope (Leica DFC295). The histoscore, which reflects the prevalence of immunoreactivity of TRPM2 on the tissue, was based on the rating scale: 0.1, < 25%; 0.4, 26−50%; 0.6, 51−75%; 0.9, 76−100%, and intensity of immunoreactivity: 0, unstained; 0.5, little staining; 1, some staining; 2, moderate staining; 3, strong staining.

Statistical analysis

Statistical analysis was carried out with SPSS 15.0 for Windows (SPSS Inc.). For data with a normal distribution (TAS,

TOS, and immunoreactivity variables), the Shapiro Wilk test was used. For between-group comparisons of TAS, TOS, and immune variables, a one-way analysis of variance (ANOVA) was utilized. Levene's test was performed for testing the homogeneity of variances. Tukey's multiple comparison test was used to reveal differences between groups of significant variables. The results are presented as mean (standard deviation) (SD). $P<0.05$ was considered statistically significant.

Results

Histopathological results

In the histopathological evaluation of lung tissues (Figure 1) the control and VD groups exhibited normal histological structure. Degeneration in the bronchial epithelium, increased collagen fibers at perivascular and peribronchial areas, and alveolar collapse was determined in the DOX group. However, the DOX+VD group demonstrated normal histopathological findings when compared to the samples from the DOX group. Together, our data suggested that VD may protect DOX-induced injuries in the lung tissues.

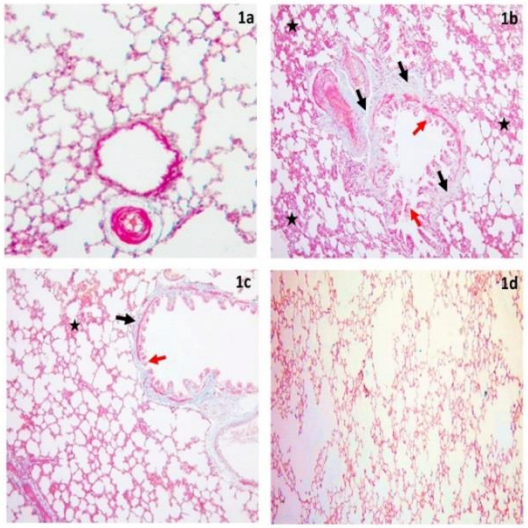

Figure 1: Masson trichrome stained lung tissues, magnification 200×. Normal histological appearance of control (1a) and VD (1d) groups. Degeneration in bronchial epithelium (red arrow), increased collagen at perivascular and peribronchial areas (black arrow) and alveolar collapse (black asterix) in DOX group (1b). Decreased degeneration in bronchial epithelium (red arrow), reduced collagen at perivascular and peribronchial areas (black arrow) and decreased alveolar injury (black asterix) in the DOX+VD group (1c).

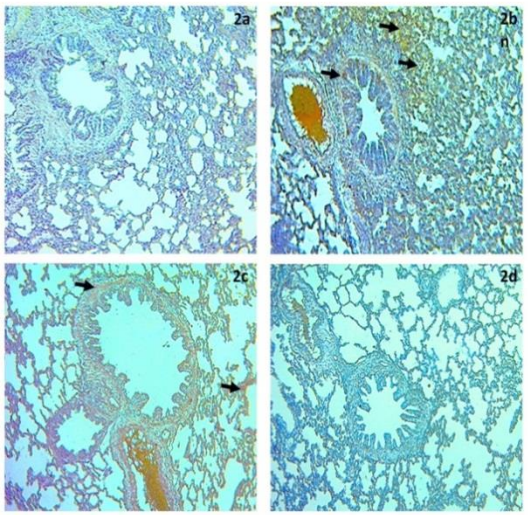

Figure 2: Lung tissues stained with Streptavidin biotin peroxidase complex method with Mayer's Hematoxylin counterstain for TRPM2 immunoreactivity, magnification 200×. Slightly TRPMP2 immunoreactivity in lung tissue of the control group (2a) and the VD group (2d). Increased TRPMP2 immunoreactivity (black arrow) in lung tissue of the DOX group (2b). Decreased TRPMP2 immunoreactivity (black arrow) in lung tissue of the DOX+VD group (2c).

Immunohistochemical results

Slight TRPM2 expression was observed in the control and VD groups ($P=0.097$) (Figure2). However, it was significantly increased in the DOX group when compared with the control group ($P<0.001$). Interestingly, when combined with DOX, VD decreased the expression of TRPM2 significantly in the DOX+VD group ($P<0.001$) (Table 1).

Table 1: Histoscore of TRPMP2 immunoreactivity in lung tissue

Groups	TRPM2 immunoreactivity
Control	0.79^{a} (0.07)
DOX	1.62^{c} (0.11)
DOX+ VD	1.19^{b} (0.04)
VD	0.68^{a} (0.06)
P-value*	<0.001

[a b c] Means within the same row with differing superscripts are significantly different ($P<0.05$, Tukey's test) *: One Way ANOVA

Biochemical results

TAS and TOS levels were similar in the control and VD groups ($P=0.124$, and $P=0.582$, respectively). In contrast, we observed significantly decreased TAS levels and increased TOS levels in the DOX group compared to the control samples ($P<0.001$, and $P<0.001$, respectively). TAS and TOS levels were reversed when we administered DOX in combination with VD in the DOX+VD group ($P<0.001$, and $P<0.001$, respectively) (Table 2).

Table 2: Serum levels of TAS and TOS

Groups	TAS (U/ML)	TOS (U/ml)
Control	1.48^{ab} (0.06)	15.67^{a} (0.44)
DOX	1.10^{c} (0.05)	20.64^{c} (0.97)
DOX+ VD	1.40^{b} (0.06)	18.04^{b} (0.29)
VD	1.53^{a} (0.05)	16.10^{a} (0.36)
P-value*	<0.001	<0.001

[a b c] Means within the same row with differing superscripts are significantly different ($P<0.05$, Tukey's test) *: One Way ANOVA

Discussion

Injuries induced by oxygen radicals in membrane lipids is the most important cause of DOX-induced toxicity [14]. Over-production of these radicals, especially the hydroxyl radicals, cause injury to macromolecules such as proteins, DNA, and membrane phospholipids [15].

DOX causes an increase in the collagen fibers on the alveolar wall of lung, degeneration of some cellular organelles [16], arterial endothelial and alveolar epithelial necrosis along with edema at periarterial and subpleural areas, and emphysema [17]. Consistent with the published reports, in the current study, DOX administration caused degeneration in the bronchial epithelium, increased collagen fibers at perivascular and peribronchial areas, and alveolar collapse in lung tissues of rats. These findings support the data that DOX is toxic to lung tissue due to increased lipid peroxidation and ROS production, which induces oxidative stress [16].

Deficiency of VD is related to decreased lung function, obstructive lung diseases such as chronic obstructive pulmonary disease and asthma [18]. VD is effective on the protection of pulmonary injury and tissue repair [19], and in preventing oxidative stress [20]. In this study, VD administration exhibited ameliorative utility by reversing the tissue structure, similar to the control group, as VD reduces tissue injury [21].

In 2017, Dietrich et al. [22] reported that as toxic sensors and effectors, TRP channels have roles in asthma, lung inflammation, fibrosis, and edema, along with Chronic Obstructive Pulmonary Disease (COPD). TRPM2 is an ion

channel located on the cell membrane. Oxidative stress [23] causes TRPM2 ion channels to open and increases intracellular Ca2+ ions [24]. In this process, intracellular influx of Ca2+ is considered the initiator of pathophysiological events that can lead to cell death [25]. In diabetic kidney tissue, increased TRPM2 immunoreactivity has been shown to decrease following administration of antioxidants, enalapril [26], and losartan [26]. Similarly, melatonin, a strong antioxidant, inhibits TRPM2 channel gated Ca2+ influx [28]. In the current study, increased TRPM2 expression in the DOX group could be related to DOX-induced oxidative stress. VD administration may have decreased the TRPM2 expression due to its antioxidant effect, in line with the literature.

An imbalance between ROS and the antioxidant defense system leads to oxidative stress, which is related to the pathogenesis of acute and chronic lung injury [29]. Oxidative stress occurs due to a disequilibrium between oxidants and antioxidants and causes tissue injury [30]. The total redox statuses of individuals were best evaluated with TAS and TOS levels combined [31,32]. DOX treatment has been reported to lead to an increase in TOS level and a decrease in TAS level in testis [33], and heart [34], and tissues. These findings show that DOX disrupted the oxidant-antioxidant balance and led to tissue damage. In the current study, a significant decrease in TAS level and a significant increase in TOS level in serum was observed in accordance with the literature, while VD administration decreased serum TOS and increased serum TAS levels in diabetic rats significantly [35]. Similarly, we observed decreased TOS level and significantly increased TAS level in the DOX+VD group. This restorative effect of VD could be attributed to antioxidative utility of VD [11,12].

Conclusion

VD could be a potential therapeutic agent for DOX-induced pulmonary toxicity. We suggest that increased TRPM2 immunoreactivity in the DOX group could be involved in the pathophysiological mechanism of pulmonary cytotoxicity and should be focused on as a therapeutic option to prevent chemotherapy induced cytotoxicity. Further studies are expected to explain the efficacy and role of VD and TRPM2 on chemotherapy induced cytotoxicity.

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