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Original Article *Özgün Araştırma*

THE INVESTIGATION OF THE EFFECTS OF VITAMIN D AND OMEGA 3 ON VENA CAVA

Vitamin D ve Omega 3'ün Vena Kava Üzerine Etkilerinin Araştırılması

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ABSTRACT

Objective: Vitamin D has a protective role in the cardiovascular system and it affects blood pressure. Omega-3 fatty acids are dietary fats gained from fish and plant oils and involve in coronary heart disease and other cardiovascular complications. The aim of the study was to investigate the effects of Vitamin D and Omega 3 on the vascular structure at the cellular level.

Material and Methods: In the current study, a total of 24 rats were divided into 4 groups. Each group contained 6 animals. The groups are as follows; control, vitamin D, Omega 3; and combined Vitamin D and Omega 3. Vena cava samples from all groups were obtained and stained with hematoxylin and eosin (H&E) for histological alternations. Additionally, endothelial and vascular functions were investigated immunohistochemically.

Results: The H&E staining revealed that the treatment of either Vitamin D or Omega 3 did not cause histomorphological changes in the structure of the vena cava under normal conditions. The immunoexpression of inducible nitric oxide synthase was decreased and vascular endothelial growth factor was increased in the vena cava of rats with the combined treatment of Vitamin D and Omega 3.

Conclusion: In conclusion, combined supplements of Vitamin D and Omega 3 did not have harmful effects on the blood vessel however further studies should be performed to determine the beneficial effects of these supplements.

Keywords: *Vitamin D, Omega 3, histopathology, vena cava, inducible nitric oxide synthase, vascular endothelial growth factor*

ÖZ

Amaç: D vitamini kardiyovasküler sistem üzerinde koruyucu bir role sahiptir ve kan basıncını etkiler. Omega-3 yağ asitleri balık ve bitki yağlarından elde edilir ve koroner kalp hastalığına ve diğer kardiyovasküler komplikasyonlarda önemli rolü vardır. Çalışmamızın amacı, D vitamini ve Omega 3'ün damar yapısı üzerindeki etkilerinin hücresel düzeyde araştırmaktır.

Gereç ve Yöntemler: Mevcut çalışmada toplam 24 sıçan kullanıldı. Her biri 6 hayvandan oluşan kontrol, D vitamini, Omega 3; ve kombine D Vitamini ve Omega 3 tedavisi uygulanan olmak üzere 4 grup belirlendi. Deney sonunda tüm gruplardan vena cava örnekleri alındı. Dokular histolojik değişimler için analiz edildi. Endotelyal ve vasküler fonksiyonlar immünhistokimyasal yöntem ile incelendi.

Bulgular: D Vitamini veya Omega 3 kullanımının normal koşullar altında vena cava yapısında histomorfolojik değişikliklere yol açmadığı tespit edildi. Vitamin D ve Omega 3'ün birlikte kullanımı sonucunda sıçanların vena cava örneklerinde indüklenebilir nitrik oksit sentazın immünoekspresyonu azaltırken, vasküler endotelyal büyüme faktörünün arttığı tespit edildi.

Sonuç: Sonuç olarak, D vitamini ve Omega 3'ün kombine takviyelerinin hücresel düzeyde damar yapısı üzerine zararlı bir etkisi olmadığına ancak faydalarının tespit edilmesi için daha kapsamlı çalışmalar yapılması kanaatine varıldı**.**

Anahtar Kelimeler: *Vitamin D, Omega 3, histopatoloji, vena kava, indüklenebilir nitrik oksit sentaz, vasküler endotelyal büyüme faktörü*

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INTRODUCTION

When exposed to ultraviolet radiation, the skin produces vitamin D, which is then synthesized non-enzymatically and processed in the liver and kidney (1).The metabolism of calcium, phosphorus, and the remodeling of bone tissue are all regulated by vitamin D. It functions as a regulator in cell differentiation, cell growth inhibition, and immunological modulation (2). Vitamin D has been discovered to affect 3% of the human genome, and its analogs alter the genomic regulation of several processes, including cellular differentiation, proliferation, apoptosis, and angiogenesis (3).Low vitamin D levels have been associated with an increased risk of cardiovascular disease (CVDs), such as artery disease (4) myocardial infarction (5), heart failure (6), cardiomyopathy (7), and fibrosis (8). However, there was insufficient knowledge regarding how vascular cells are affected by vitamin D and its analogs.

The vitamin D treatment enhanced endothelial cell (EC) function, according to a study on individuals with elevated cardiovascular risk. To certain the effects of vitamin D on angiogenesis at the cellular level, numerous research has concentrated on vascular ECs. The fundamental biological component of blood vessels, vascular smooth muscle cells, is crucial for maintaining vascular integrity and function (9).

Endothelial cells produce nitric oxide (NO), which is important for maintaining vascular health and function. In hypercholesteremic individuals or experimental atherosclerosis animal models, the decreased NO production is linked to endothelial dysfunction. On the other hand, in situations of persistent inflammation, endothelial and other cell types exhibit localized expression of the Inducible nitric oxide synthase (iNOS) (10).

The two primary types of omega-3 fatty acids are eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (11). EPA and DHA are transformed into the liver and converted into triglycerides and released into circulation. Omega-3 fatty acids are bound to albumin, and only a small percentage of them are free. Omega-3 fatty acids play a significant function in the prevention of diseases such as arrhythmia, chronic heart failure, autoimmune diseases, rheumatoid arthritis, and carcinogenesis(12).

There is no evidence about the effects of omega-3 and vitamin D supplements on blood vessels therefore the study's goal was to find out how Vitamin D and Omega 3 fatty acids affected vascular cells.

MATERIALS AND METHODS

Animals and Experimental Design

In our study, twenty-four, 21 days old, female pre-pubertal Wistar albino rats were used During the experiment, the animals were given ad libitum standard rodent feed and tap water. The animals were housed in polypropylene cages, 12 hours light / 12 hours dark rooms, 22±0.50C temperature, and appropriate humidity. The animals were divided into four groups each consisting of 6 animals. Group I (Control): used as the control group. Group II (Vitamin D): 120ng/100g/week 1,25(OH)2D3 (Calcijex ampule, Abbott) injected into the rats subcutaneously (13). Group III (Omega 3): rats were injected with 1 ml/kg/day Omegaven (Fresenius Kabi, Austria) (100 mL Omegaven contains 1.25–2.82 g of eicosapentaenoic acid (EPA) and 1.44–3.09 g of docosahexaenoic acid (DHA)] intraperitoneally for 28 days (14), Group IV (Vitamin D+Omega 3): The rats were injected with 120ng/100g/week 1,25(OH)2D3 and 1 ml/kg/day Omegaven for 28 days. At the end of the 28th day, the vena cava tissues were obtained for histo-pathological and immunohistochemical examinations.

Histological examinations

The vena cava samples were fixed in 10% formalin after that standard histological methods were performed and the tissues were embedded in paraffin. From each paraffin block, 5 µm-thick slices were taken and hematoxylin-eosin (H&E) staining was performed. The slides were examined histologically with a light microscope. (Leica DM4000, Wetzlar, Germany).

Immunohistochemical examinations

5 µm-thick vena cava tissue sections were obtained from paraffin blocks. The paraffin sections underwent xylene

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and rehydration in a progressive decrease of ethanol. Antigen retrieval was performed. Endogenous peroxidases were quenched with 0.3% hydrogen peroxide in methanol for 5 min at room temperature. The sections were blocked in blocking serum (Ultra V Block, ScyTek Laboratories, Utah, USA) for 5 min. Then, slides were incubated with anti-iNOS (antibody (SP126) ab115819) and Vascular endothelial growth factor (VEGF)(PA5-85171,thermo) for overnight at 4 °C. The tissues were washed with PBS for 3x5 minutes after the application of the primary antibodies and incubated with biotinylated anti-mouse (BA-9200; 1:400 Dilution; Vector Laboratories, Burlingame, CA) secondary antibodies for 10 min. The tissues were washed again with PBS for 3x5 minutes after the application of the secondary antibody and incubated with streptavidin peroxidase for 10 min and then taken into PBS. The DAB Substrate Kit solution was applied for the reaction. The slides were counterstained with Mayer's hematoxylin and covered with entellan. The slides were examined and photographed under a light microscope (LeicaDM4000,Wetzlar,Germany).H-SCORE analyses were conducted for the immunohistochemistry evaluation as previously described (15).

Statistical analyses

Immunocytochemistry expressions were evaluated using GraphPad Prism software (GraphPad, San Diego, CA). Data between groups were analyzed for significance using an ANOVA followed by Turkey's multiple comparison tests. The statistical significance level was accepted as $p<0.05$.

RESULTS

Histomorphological Results

Figure 1 displays the results of the H&E staining. All groups have a normal histomorphological structure of the vena cava. Vena cava sections showed an appropriate structure of tunica intima consisting of a single layer of flattened cells. It was difficult to distinguish tunica adventitia from the tunica media. Tunica media consisted of smooth muscle cells. The tunica adventitia was made of loose connective tissue.

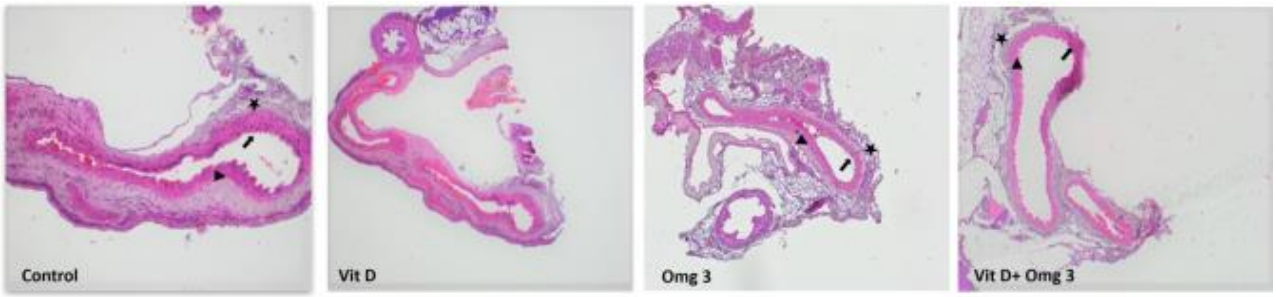

Figure 1: Images of the vena cava stained with hematoxylin-eosin Arrow: tunica intima (endothelium), arrow head: tunica media and star: tunica adventitia. (H&E staining; Magnification: 10×)

Immunohistochemistry Assay Results

The vena cava from the control group showed weak iNOS staining. Vena cava sections treated with vitamin D, omega 3, or both vitamin D+Omega 3 showed slight staining with iNOS (Figure 2A). Even though all groups showed immunoexpression of VEGF the Vitamin D + Omega 3 group's combined treatment showed intense staining in the vena cava (Figure 2B).

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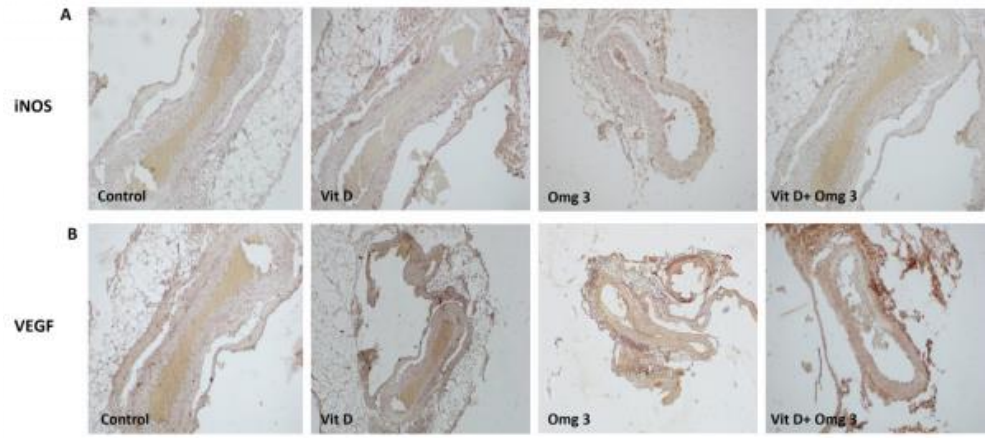

Figure 2: Immunohistochemical distribution of iNOS (A) and VEGF (B) in rat vena cava sections

There was a weak immunostaining of iNOS in control group. Low expression were detected in the Vitamin D, Omega 3 and Vitamin D combined Omega 3 groups (Upper panel A:Immunostaining of iNOS; Vit D and Omg 3 magnification: 10×; Control, Vit D + Omg 3 magnification: 20×). All the groups showed positive immunostaining for VEGF. The intense staining was observed in Vitamin D combined Omega 3 group (Lower panel B: Immunostaining of VEGF; Vit D, Omg 3, Vit D + Omg magnification: 10×; Control magnification: 20×).

The H-SCORE analyses of immunoexpression levels of iNOS and VEGF were shown in Figure 3. The immunoexpression of iNOS was decreased in the combined treatment of Vitamin D and Omega 3 group compared to the control (p=0.353) (Figure 3A). The immunoexpression level of VEGF was increased in Vitamin D (p=0.142), Omega 3 (p=0.799), and Vitamin D and Omega 3 (p=0.010) groups compared to the control. A significantly increased was seen in the combined treatment of Vitamin D and Omega 3 group compare to the control (p=0.010). (Figure 3B).

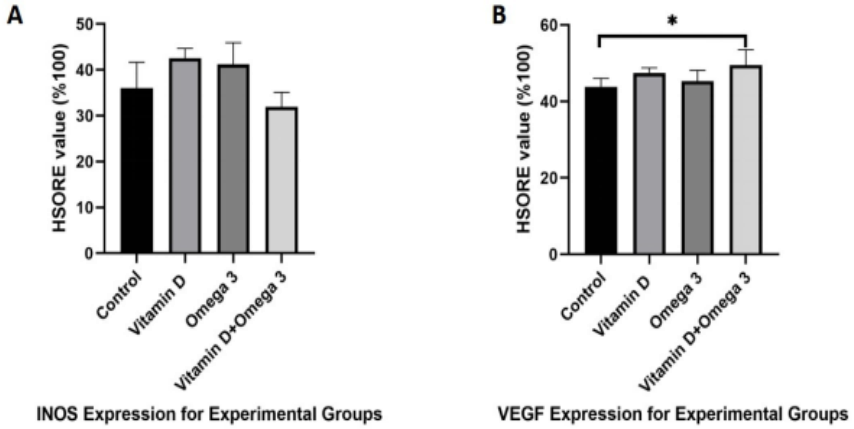

Figure 3: H-scores of iNOS and VEGF immunostaining intensities in the ven a cava samples from experimental groups

The H-score analyses revealed the immunoexpression of iNOS was decreased (p=0.3539) compare to control(A). The significantly increased of VEGF immunoexpression was seen in the combined treatment of Vitamin D and Omega 3 group compare to control ($p < 0.05$).(Data are presented as mean ± standard error. $p < 0.05$ values were considered as a significant. (: p=0.0102)).

DISCUSSION

Vitamin D is crucial for calcium and bone homeostasis, due to its ability to control the activation of the vitamin D receptor transcriptional factor. Studies on the processes of vitamin D's cellular protection in avoiding a variety of illnesses, such as osteoporosis, cancer, autoimmune disorders, and cardiovascular diseases (16).

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Omega-3 fatty acids are recognized to provide advantages for the heart. Increased omega-3 fatty acid consumption may reduce cardiovascular mortality, according to studies (17). In individuals with coronary artery disease, omega-3 fatty acids, in particular EPA, improve the equilibrium between the vasodilator nitric oxide, which has positive effects on endothelial function (18).

The current study investigated the effects of vitamin D and Omega 3 on the vena cava by histological methods. First, our H&E data demonstrated that treatment with vitamin D and Omega 3 either alone or combined, did not affect the histomorphological structure of the vena cava under normal conditions.

Despite numerous studies on the protective effects of vitamin D and omega 3 under stressful circumstances or other experimental ischemia/reperfusion animal models, as well as studies on thrombosis on vessels, in our study under normal circumstances, there was no difference at the cellular level between the groups.

Vitamin D supplements can be used to treat vitamin D deficiency, which is linked to cardiovascular disease (19). Studies that have been published show a connection between low vitamin D levels and arterial endothelial dysfunction (20).

Similar results were found in a study on vitamin D's impact on endothelial dysfunction in diabetic rats. It has been shown that STZ-induced diabetes causes endothelial function to deteriorate and that vitamin D treatment greatly improved diabetes-related endothelial dysfunction. Additionally, vitamin D supplementation was reported to lower aortic iNOS activity levels. The results of this investigation indicated that diabetic rats' vascular function was enhanced by vitamin D supplementation (21).

The primary indicator of inflammatory processes is iNOS expression. Specific inhibition of iNOS gene expression or inactivation of iNOS enzyme is regarded as a target for therapeutic intervention in inflammatory diseases (22). iNOS expression in cortical neurons has been examined in a study on the inflammatory process in Alzheimer's disease (AD) following the administration of amyloid- β (Aβ), vitamin D, and Aβ combined with vitamin D, respectively. They have shown that Vitamin D-treated cortical neurons results in a low level of iNOS mRNA and protein expressions. These data indicated the key role of vitamin D in regulating iNOS and possibly NO(23).

Another study focused on the function of vitamin D in an experimental mouse model of persistent alcohol-induced liver damage. The study's findings demonstrated that a vitamin D deficit led to pathological harm and dysfunction in the liver caused by alcohol consumption. Reactive oxygen species (ROS) were randomly produced along with alcohol metabolism and NO is representative of ROS, which was induced by iNOS. The same study also revealed that vitamin D deficiency aggravated alcohol-induced increase of iNOS (24).

Angiogenic growth factors are used to treat peripheral vascular and ischemic heart diseases. Vascular endothelial growth factor has been shown to increase angiogenesis in investigations of cardiac ischemia in both humans and animals (25). Furthermore, it is well recognized that vitamin D can affect VEGF levels, however, there is conflicting evidence in the literature. Various pieces of evidence contend that vitamin D either stimulates or inhibits the secretion of VEGF (26).

The researchers have investigated the effects of Vitamin D on hypoxia-induced bronchopulmonary dysplasia (BPD) in neonatal rats. The study's findings demonstrated that vitamin D3 therapy elevated serum 25OHD and upregulated VDR in lung tissues, whether or not there was hypoxia present. The researchers also discovered that vitamin D3 therapy enhanced VEGF expression and decreased alveolar simplification in lung tissue that had been affected by hypoxia. By controlling vitamin D-VDR signaling pathways, vitamin D has been shown to have protective effects on hypoxia-induced BPD in newborn rats (27).

The immunoexpression of iNOS and VEGF, which are related to endothelial functions and vascular smooth muscle cells, respectively, was examined in the present work. Although all the groups were positive for the

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iNOS and VEGF, the lower expression of iNOS and higher expression of VEGF were detected in the combined treatment of Vitamin D and Omega 3.

In conclusion, we can say that either Vitamin D or Omega 3 supplements may have beneficial effects on people's health at the vascular level.

Conflict of Interest: The author has no conflict of interest to declare.

Researchers' Contribution Rate Statement:

Concept/Design: BK; Analysis/Interpretation: EA; Data Collection: MFB, US; Writer: BH, EA; Critical Review: MNB, HD, US; Approver: BH,EA

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Ethics Committee Approval: This study was approved by the Afyon Kocatepe University Animal Experiments Local Ethics Committee (Decision number: 49533702/113).

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