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Original Article / Özgün Araştırma

Therapeutic Effects of Vitamin D_3 on Motor Functions Following Experimental Spinal Cord Injury

Deneysel Spinal Kord Yaralanmasında Vitamin D_3'ün Motor Fonksiyonlar Üzerine Olan İyileştirici Etkisi

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Summary

Objective: Spinal cord injuries are frequently encountered and is a major health problem due to the severe disability they cause. The neurological damage of acute spinal cord trauma triggers primary mechanisms of injury and cell death activation cascades leading to tissue damage. The aim of this study was to investigate the therapeutic effects of vitamin D_3 in a rat model of spinal cord injury.

Materials and Methods: The study was performed at Haydarpaşa Numune Training Hospital, İstanbul, Turkey. Twenty-one Spraque-Dawley rats were randomly assigned to the following study groups: the control group, undergoing laminectomy procedure; trauma group, undergoing spinal cord injury after laminectomy; and trauma plus vitamin D_3 group, undergoing spinal cord injury after laminectomy and subsequent administration of vitamin D_3 on days 1, 3, 5, 7 intraperitoneally with a dosage of 1 mcg/kg/day. The functional outcome was evaluated by using inclined plane test and Drummond and Moore motor function score on days 1, 7, 14, 21. Spinal cord samples of the control, trauma and trauma plus vitamin D_3 groups were obtained for histopathologic evaluations after clinical examinations and were examined under light microscope.

Results: Inclined plane test scores and motor function scores of vitamin D_3 group were significantly higher than the trauma group at 1st, 2nd and 3rd weeks.

Conclusion: In our study we have investigated the therapeutic effects of vitamin D_3 and found out that it has a positive impact to the recovery process of the spinal cord injury. (Turkish Journal of Neurology 2015; 21:55-61)

Key Words: Experimental spinal cord injury, antioxidant treatment, vitamin D_3

Özet

Amaç: Akut spinal kord yaralanması (SKY) sonrası nörolojik hasar, primer mekanik yaralanma ile olduğu kadar sekonder olarak doku hasarına yol açan hücre ölümü aktivasyon kaskatlarından da kaynaklanmaktadır. Bu çalışmada vitamin D_3'ün sıçanlarda oluşturulan deneysel SKY sonrasında motor fonksiyonlar üzerindeki iyileştirici etkinliği araştırıldı.

Gereç ve Yöntem: Bu çalışma Sağlık Bakanlığı Haydarpaşa Numune Eğitim ve Araştırma Hastanesi'nde yapıldı. Çalışmada 3 grupta, 7'şer adet olmak üzere toplam 21 adet Spraque-Dawley cinsi sıçan kullanıldı. İlk gruba sadece laminektomi yapıldı (kontrol grubu). İkinci gruba laminektomi yapılıp omurilik yaralanması oluşturuldu (travma grubu). Üçüncü gruba laminektomi yapılıp omurilik yaralanması oluşturuldu ve 1., 3., 5., 7. günlerde intraperitoneal olarak 1 mcg/kg/gün dozunda vitamin D_3 enjeksiyonu yapıldı (vitamin D_3 grup). Deneklerin fonksiyonel iyileşmeleri cerrahi işlem sonrası 1., 7., 14. ve 21. günlerde eğik düzlem testi, Drummond ve Moore motor fonksiyon skoru ile değerlendirildi. Kontrol, travma ve vitamin D_3 uygulanan gruplardaki doku parçaları hematoksilen-eozin (HE) boyası ile boyanıp ışık mikroskobunda incelendi.

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Bulgular: Vitamin D_3 grubunda eğik düzlem testi sonuçları ve motor fonksiyon skorları travma grubu ile karşılaştırıldığında 1., 2. ve 3. haftalarda anlamlı olarak daha yüksek bulundu.

Sonuç: Bu çalışmamızda, sıçanlarda oluşturulan deneysel SKY'de vitamin D_3'ün tedavi edici etkisi araştırılmış olup klinik olarak iyileşme sürecine anlamlı etkinliği olduğu saptanmıştır. (Türk Nöroloji Dergisi 2015; 21:55-61)

Anahtar Kelimeler: Deneysel spinal kord yaralanması, antioksidan tedavi, vitamin D_3

Introduction

Today, spinal cord injury (SPI) remains as an important topic due to its incidence rate, the magnitude of physical, psychosocial and economic damages it incurs, and the lack of universally accepted treatment protocols. Recent attempts at developing pharmacological treatment methods highlighted the importance of understanding the pathophysiology of the processes following the trauma (1). Even though there is complete loss of function following primary or mechanical trauma in spinal cord injury cases, it is rarely a reason for total transection. It is also known that the biochemical and pathological changes in the cord can worsen the damage (2). Hypoxia on the cord can be seen in the early stage of acute primary injury. Following the primary injury of the tissue, a secondary damage stage involving hypoxia-induced electrolyte imbalance, neural excitation, glutamate release, formation of free radicals and inflammation progresses (3,4,5). In the secondary injury, the cascade of events that take place is a product of the activation of endogenous cell-death pathways. Despite the promising therapeutic effects of many agents, only methylprednisolone was shown to be effective in treatment in large-scale clinical studies (6).

Vitamin D is not really a vitamin but a secosteroid hormone and it has two types that come from different sources but have similar structure and composition. Ergocalciferol (vitamin D_2) and cholecalciferol (vitamin D_3) can be absorbed from nutrients but the main source of vitamin D is first synthesized on the skin with UVB rays and then reaches its active form after being hydroxylated in the liver and kidneys. In liver, vitamin D_3 turns into its active form calcidiol and vitamin D_2 turns into its active form 25(OH)D_2 (7,8,9). The biological functions of Vitamin D_3 include the regulation of calcium balance, cell differentiation in the nervous system, release of neutrophyls and the activation of the enzymes and genes that play key roles in neurotransmitter synthesis (10). Brain is a target tissue for the effects of vitamin D_3. It was shown that vitamin D receptor (VDR) which is a member of the nuclear hormone receptor superfamily is localized in brain, spinal cord and glial cells (11,12). In addition, the genes coding the enzymes associated with vitamin D_3 metabolism are expressed by the central nervous system (CNS) cells. The wide-spread distribution of VDR in different regions of the sensory, motor and limbic systems suggests that vitamin D_3 may possess many additional functional properties (13).

Experimental neurodegeneration models suggested vitamin D_3 to be neuroprotective (14,15,16,17). There is scientific evidence suggesting that vitamin D_3 also regulates immune system and brain functions (18). Studies showed that through VDR mediation vitamin D_3 prevents cytotoxicity in cortical neurons by regulating inducible nitric oxide synthesis (iNOS) and it inhibits the iNOS expression in macrophage, active microglia and astrocytes (19,20). Another study showed that the blocking of iNOS through selective inhibitors decreases blood-spinal cord barrier damage induced by spinal injury, edema formation and cell reactions, therefore imposing a restorative influence over motor functions (21,22). Based on the neuroprotective efficacy of the pharmacological analogues of vitamin D_3 in neurodegenerative and neuroimmune diseases, this study looked at the restorative effects of vitamin D_3 on neurological functions in experimentally induced spinal cord injuries.

Materials and Methods

This study was conducted in Ministry of Health Haydarpaşa Numune Training and Research Hospital under the approval of animal rights ethical board. Three groups of 7 Spraque-Dawley rats (21 in total) were used in the study. The weights of the rats in each group were between 180-220. Group 1 (n=7 rats): Laminectomy-only (control) group, group 2 (n=7 rats): Laminectomized and spinal cord trauma-induced group (traumatized group), group 3 (n=7 rats): Laminectomized, spinal cord traumatized and vitamin D_3-administered group (on the 1st, 3rd, 5th, and 7th days, intraperitoneally, once a day 1 mcg/kg/day of Calcijex amp. 1 mcg/1 mL, Abbott) (vitamin D_3 group) (23).

For general anesthesia, 2 mg/kg ketamine HCI (Ketalar, Parke-Davis Eczacıbaşı-İstanbul) was given intraperitoneally before the surgical operation. Rats were positioned on special boards in prone position to enable comfortable access the during surgical operation. Thoracic regions were first sterilized with PVD iodine and then shaved. The region was sterilized once more with PVD iodine after shaving. Body temperature was fixed at 37 °C during the operation until the anesthetic effect wore off. Taking the interscapular distance as a reference, a 2 cm incision at the level of T5-T12 was made which penetrated dermis, epidermis and cleared away paravertebral muscles, eventually revealing the laminae. After the T7-8-9 laminectomy, standard spinal cord injury was induced by using modified Allen weight-drop model (24). Following hemostasis, primary suturing was applied on paravertebral muscles and skin using 3/0 vicryl in a way that preserved the anatomical layering. The rats were woken normally in room temperature. In the study, the animals that received T 7-8-9 total laminectomy all had intact dura matter. Using the modified Allen weight-drop model, SCI was induced by dropping a 10 g weight through a 10 cm long glass tube with 0.5 diameter. The rats that were given SCI were woken up in room temperature and their motor functions were examined. Fourteen rats on which trauma was induced (the trauma and

vitamin D_3 groups) were seen to be paraplegic. Seven rats that received laminectomy only (control group) all retained their full motor strength. Rats were placed in individual cages that were kept in room temperature and were given standard rat food. Paraplegic 14 rats were fed using orogastric tube during the early post-op stage. Bladder functions were monitored using the urinary catheters. Vitamin D_3 (Calcijex 1 mcg/1 mL) was acquired from Abbott Pharmaceuticals (İstanbul). Seven rats in the third group that had laminectomy and spinal injury using the weight-drop model received 1 mcg/kg/day vitamin D_3 intraperitoneally once a day on the 1st, 3rd, 5th and 7th days.

Evaluation of Functional Recovery

a) Inclined plane test (25): Functional recovery of the rats were evaluated by the inclined plane test developed by Rivliv and Tator which is commonly used in experimental acute spinal cord injury. Rats in all three groups went under inclined plane test on the 1st, 7th, 14th and 21st days following the surgery.

b) Clinical motor exam: The rats in the study went under motor examination in order to assess the functional recovery. The motor functions of the rats were evaluated on the 1st, 7th, 14th and 21st days following the surgery with the inclined plane test using Drummond and Moore criteria (26). Inclined plane test and clinical motor exam results were compared between groups, and within groups on the 1st, 7th, 14th and 21st days.

Histopathological Examination

Light microscopy examinations in our study were conducted in Ministry of Health Haydarpaşa Numune Training and Research Hospital Pathology Laboratory. At the end of 3 weeks, rats were anesthetizes using 2 mg/kg intraperitoneal ketamine HCI and put in a supine position. After the anesthesia, the old incisions were reopened and the 30 mm spinal cord segments at the level of T 7-8-9 were examined under light microscopy. All of the tissue segments of control, trauma and vitamin D_3 groups were examined. Following the routine tissue examination, paraffin blocks were cut in 5-micron thickness and dyed with hematoxylin-eosin (HE) before being examined under the light microscope.

Statistical Analysis

The data was analyzed using SPSS (Statistical Package for Social Sciences) for Windows 10.0. The quantitative data in the study did not follow a normal distribution. Therefore we used Kruskal Wallis test to compare the groups in terms of dependent variables, and Mann Whitney U test to identify the different group. Within-group tests were conducted using Wilcoxon signed test. The results were evaluated within 95% confidence interval and $p<0.05$ significance level.

Results

Evaluation of the Inclined Plane Test Results

The levels of inclined plane test for each group on the 1st day, 1st week, 2nd week and 3rd week are shown in Table 1.

Rating of the Motor Functions (according to Drummond and Moore criteria)

The motor function scores for each group on the 1st day, 1st week, 2nd week and 3rd week are shown in Table 1.

Light Microscopy

The spinal cord cross-section of the control group, which did not receive trauma following laminectomy, is shown in Figures 1 and 2. In the trauma group cross-sections, there were liquefactive necrosis causing scattered cavities, dense histiocytes, and polymorphic leukocyte infiltration. Additionally, the surrounding tissue was infarction-like with axonal swelling and sparse axonal spheroids (Figures 3 and 4). The extent of the damage in vitamin D_3 group cross-sections covered a larger area than the trauma group, which did not receive the medication, and the morphological changes in the damaged region were at least as severe as the trauma group (Figures 5 and 6).

Table 1. Evaluation of the inclined plane degrees of the groups on the 1st day, 1st, 2nd, and 3rd weeks

Inclined Plane	Group 1 (Control) Mean ± SD (Median)	Group 2 (Trauma) Mean ± SD (Median)	Group 3 (Vitamin D_3) Mean ± SD (Median)	p
1st day	60.00±0.00 (60)	10.00±0.00 (10)	10.00±0.00 (10)	0.001**
1st week	60.00±0.00 (60)	11.43±2.44 (10)	13.57±2.44 (15)	0.001**
2nd week	60.00±0.00 (60)	14.28±3.45 (15)	22.86±2.67 (25)	0.001**
3rd week	60.00±0.00 (60)	25.71±3.45 (25)	42.86±14.54 (45)	0.001**

Kruskal Wallis Test, **p<0.01

Table 2. Evaluation of the motor function of the groups on the 1st day, 1st, 2nd, and 3rd weeks

Motor Fonksiyon Skorları	Group 1 (Control) Mean ± SD (Median)	Group 2 (Trauma) Mean ± SD (Median)	Group 3 (Vitamin D_3) Mean ± SD (Median)	p
1st day	4.00±0.00 (4)	0.00±0.00 (0)	0.00±0.00 (0)	0.001**
1st week	4.00±0.00 (4)	0.28±0.49 (0)	0.57±0.53 (1)	0.001**
2nd week	4.00±0.00 (4)	1.00±0.76 (1)	1.50±0.41 (1.5)	0.001**
3rd week	4.00±0.00 (4)	1.50±0.50 (1.5)	2.43±0.45 (2.5)	0.001**

Kruskal Wallis Test, **p<0.01

Discussion

In the past 20 years, researchers have been focusing on the pathophysiological mechanisms of acute spinal cord injury in order to fix the neurological functions (27,28,29,30,31). Neurological damage following SCI is caused by the cell death activation cascades (secondary injury) as much as the 'primary mechanical injury' (32). Secondary spinal cord injury is the damage resulting from a series of pathophysiological processes triggered by the primary injury in the following hours or days (33,34,35). Numerous experimental studies showed a dose and severity dependent decrease in spinal cord blood-flow that gradually worsens following the injury (36). An interesting finding is that ischemia may be preventable if it is treated during the first few hours of the progressive decline in the post-traumatic ischemia (37,38). An important mechanism responsible for the advancement of the secondary damage is the increase in the NO (nitric oxide) synthesis (39,40). With the increased concentration, NO becomes neurotoxic and facilitates the secondary damage as a free radical. Being one of the three enzymes playing a role in the nitric oxide synthesis, iNOS does not exist in healthy tissues but causes NO synthesis after being stimulated by the inflammatory mediators and cytokines during the pathological processes (41,42,43). NOS inhibition in the early stages of SCI provides the benefit of improving neurological functions and histopathological changes (44).

Even though it was initially assumed that calcitriol, the active form of vitamin D, was produced by the liver and kidneys, it was later discovered that many other organs including the brain also express vitamin D1 hydroxylase (45). Additionally, VDRs propagate over large areas of both human and rat brains (46,47). Studies showed that through VDR, vitamin D regulates brain-based neurotropic factor, nerve growth factor and the gene expression coding neurotrophin 3 (48). It was shown that addition of vitamin D_3 in cell cultures increases neurotrophin expression

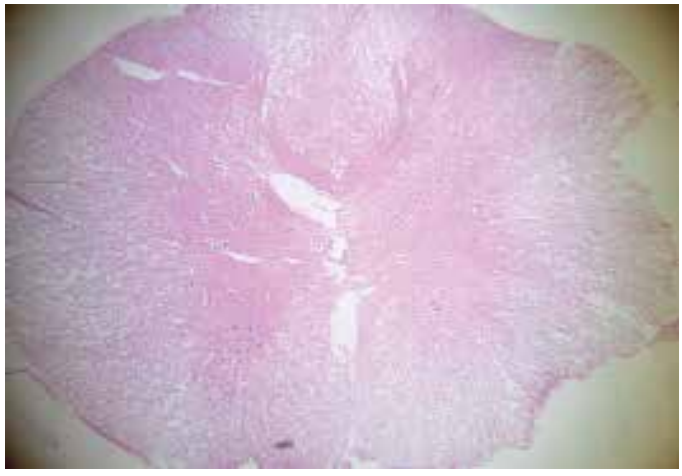

Figure 1. Spinal cord in the control group following laminectomy, without trauma (HE, x40 magnified)

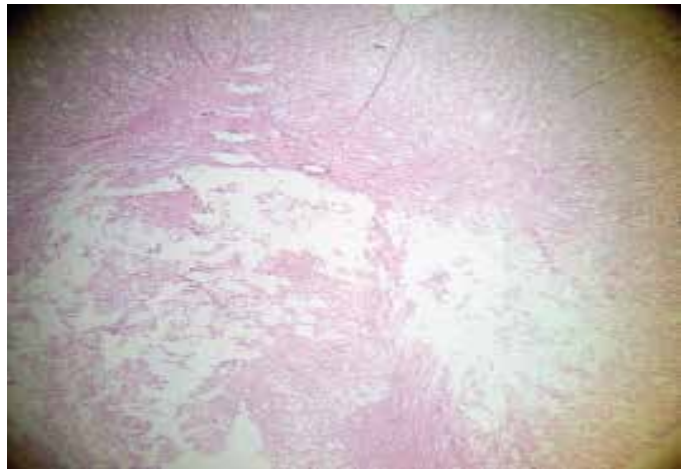

Figure 3. Trauma group. Spinal cord following laminectomy and trauma (HE, x40)

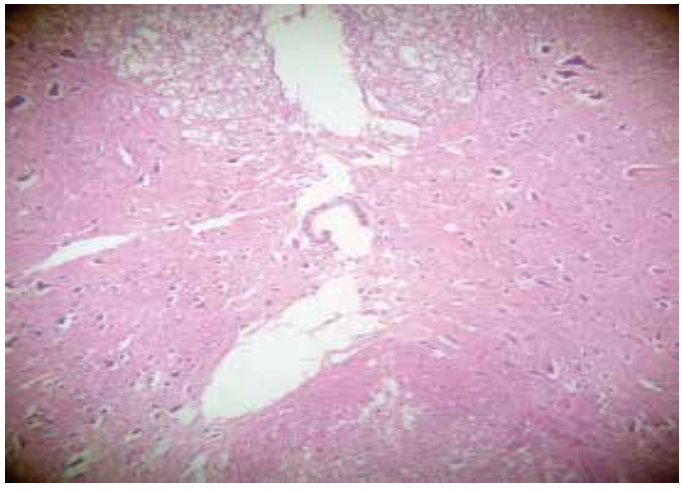

Figure 2. Control group (HE, x100)

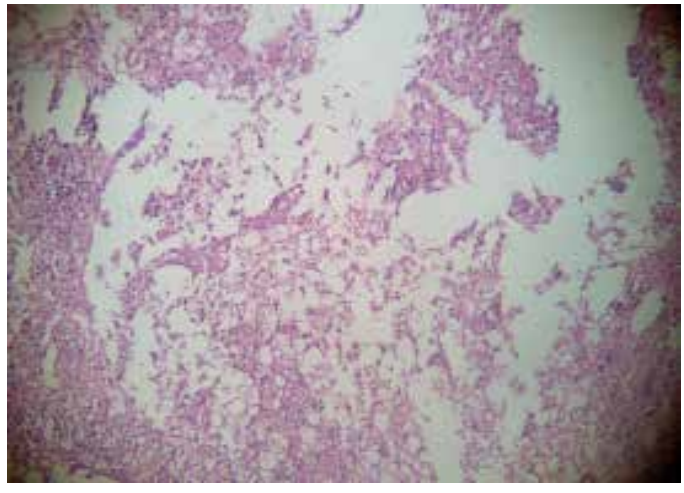

Figure 4. Trauma group (HE, x100)

and nerve growth (49). Another study found that neuronal growth factor expression decreases in the rat brain when vitamin D_3 concentration in the environment is decreased (50). Chabas et al. found that vitamin D (especially vitamin D_3) plays an important role in axogenesis and myelination through the genes associated with calcitriol (51,52). In many experimental neurodegeneration models, vitamin D_3 was also shown to be neuroprotective (53,54,55). Studies have shown that vitamin D_3 decreases the formation of nitrite, reduces oxidative stress by increasing gamma glutamyl transpeptidase (56). In addition, in vivo studies showed that it decreases cortical infarcts after middle cerebral artery clogging (57). In the present study, which was motivated by these findings, our functional recovery measurements using inclined plane test showed that the drug group which received vitamin D_3 showed improvement following the first week. It was seen that the rats received vitamin D_3 and those that simply received the trauma had started to differ in statistically significant amounts after two weeks in terms of incline plane degrees. The analysis of motor function scores using Drummond and Moore criteria showed that the vitamin D_3 group showed improvement in motor functions after two weeks. A statistically significant difference in motor functions between the vitamin group and the trauma-only group developed on the third week. These findings were interpreted as vitamin D_3 inducing neuronal growth factors and contributing to axonogenesis and additionally facilitating the healing process through its antioxidant effect. Additionally another study showed an increase in the muscle mass and strength in hemiplegic patients following vitamin D treatment, which was interpreted as vitamin D treatment increasing physical performance. These results also explain the clinical improvement seen in our study (58,59).

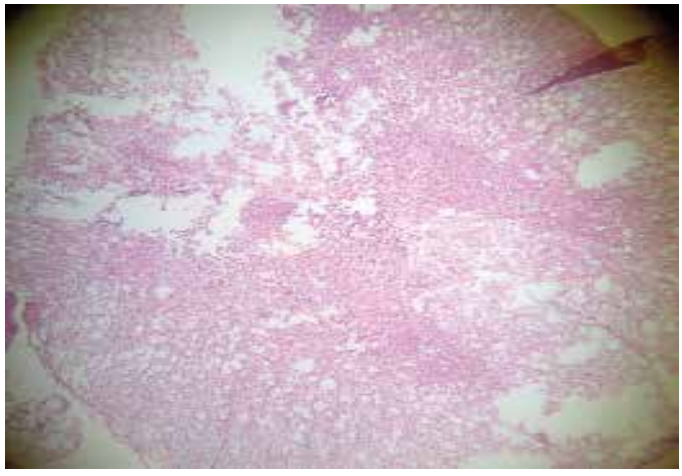

Figure 5. Traumatized, post-op on the 1st, 3rd, 5th and 7th days 1 mcg/ml dose vitamin D_3 group (HE, x40)

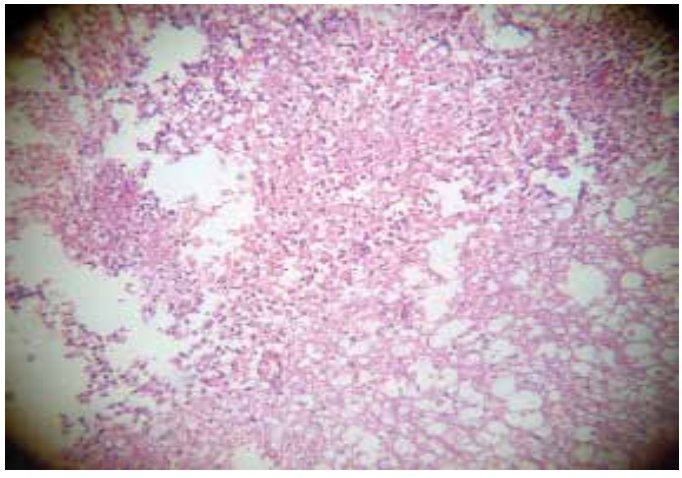

Figure 6. Traumatized and given 1 mcg/ml dose vitamin D_3, post-op on the 1st, 3rd, 5th and 7th days (HE, x100)

Our light microscopy study showed regional cavities caused by liquefactive necrosis, dense histiocytes and polymorph leukocyte infiltration accompanied by infarction-like appearance in the surrounding tissues, axonal swelling and sporadic axonal steroids in the trauma group. Vitamin D_3 group's slices showed a similar appearance in the morphological damage area. The reason why the clinical improvement was not paralleled by the histology may be that the precise time frame for the destructive effects of the secondary injury is unknown. The effect of the damage can extend to as much as 3-6 weeks and it was assumed that these effects were still in progress during our study (60,61).

In this study, we investigated the neuroprotective effects of vitamin D_3 in SCI created by modified Allen weight-drop model. Even though there was clinical improvement in the neurological functions, microscopy studies did not show meaningful changes. Further studies are needed for the clinical use of vitamin D_3.

Ethical Approval: The manuscript was derived from a thesis. Ethical approval for the study was given by the Ministry of Health Haydarpaşa Numune Training and Research Hospital Ethical Board

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