Serum total oxidant/anti-oxidant status, ischemia-modified albumin and oxidized-low density lipoprotein levels in patients with vitamin D deficiency

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ABSTRACT

Objective: Oxidative damage may be responsible for the pathogenesis and complications of many diseases. Vitamin D deficiency has been suggested as a potential mediator of various extra-skeletal pathologies. However, there are limited data on anti-oxidant properties of vitamin D. **Materials and methods:** Forty-one subjects with vitamin D deficiency and 30 healthy controls were enrolled into the study. The levels of total anti-oxidant status (TAS), total oxidant status (TOS), ischemia-modified albumin (IMA), oxidized-low density lipoprotein (ox-LDL), high-sensitivity C-reactive protein (hs-CRP) and fibrinogen were measured in both groups. The measurements were repeated in 17 patients after the replacement of vitamin D. **Results:** Serum IMA and TOS levels were significantly higher (p < 0.001 and p = 0.035, respectively), while TAS levels were significantly lower in patients, compared to controls (p < 0.001). Additionally, fibrinogen was significantly higher in patients than controls (p = 0.003), while ox-LDL and hs-CRP levels were similar between two groups. After the replacement of vitamin D, TAS level significantly increased (p = 0.037), and TOS and fibrinogen levels significantly decreased (p = 0.043 and p = 0.010, respectively). Vitamin D levels were negatively correlated with IMA and fibrinogen levels (r = -0.500, p < 0.001 and r = -0.391, p = 0.002, respectively), although positively correlated with TAS levels (r = 0.430, p < 0.001). No correlation was found between vitamin D levels, and the TOS, ox-LDL and hs-CRP levels. **Conclusions:** In this study, while serum IMA, TOS and fibrinogen levels were increased, TAS levels were seen to be decreased in patients with vitamin D deficiency. These results suggest that oxidative/anti-oxidative balance shifts in favours of oxidative status in vitamin D deficiency. Arch Endocrinol Metab. 2015;59(4):318-24

Keywords
Vitamin D; total anti-oxidant status; total oxidant status; ischemia-modified albumin; oxidized-low density lipoprotein

INTRODUCTION

Vitamin D has a major impact on the regulation of calcium (Ca) and phosphorus (P) balance in human body, and so in the regulation of bone homeostasis (1). Additionally, vitamin D plays a role in the homeostasis of different tissues such as skeletal muscle, vascular smooth muscle, myocardium and endothelium with a beneficial effect on cardiovascular function (1,2). Vitamin D deficiency was suggested as a potential mediator of many extra-skeletal pathologies, including cardiovascular diseases.

Reactive oxygen species (ROS) are such highly reactive molecules that when present in excess, they overwhelm the protective systems, and so result in cell damage and lipid peroxidation (3). ROS are constituted in oxidative processes that normally occur at relatively low levels in all cells and tissues (3). In normal situations, a number of anti-oxidant mechanisms serve to control ROS production (4). The imbalance between ROS production and anti-oxidant power is defined as oxidative stress. The measurements of total anti-oxidant status (TAS) and total oxidant status (TOS) are used to predict oxidative status (5). Especially the measurement of TAS reflects the overall anti-oxidant state in an organism.

Initially emerging as a marker of ischemia, ischemia-modified albumin (IMA) is considered to be beneficial in the identification of acute coronary syndromes. However, IMA is seen as elevated in individuals undergoing oxidative stress other than cardiac

ischemia because it is not tissue specific. The production of IMA seems to be associated with the production of ROS modifying the metal-binding sites of albumin (6). IMA is considered a non-specific biomarker in the evaluation of oxidative status or atherosclerosis burden.

Oxidized-low density lipoprotein (ox-LDL) arises from the binding of low density lipoprotein cholesterol (LDL-C) with unsaturated fatty acids. Increasing evidence shows that accentuated oxidative stress favors oxidative modifications of LDL-C and plays an important role in the development of atherosclerosis (7). LDL-C oxidation mainly occurs in the intima layer of vessels, but some of the ox-LDL in the intima re-enters the general circulation. Therefore, the determination of ox-LDL levels in serum provides information for oxidation taking place in the vessel (8).

Although oxidative stress is involved in the pathophysiology of several chronic conditions, limited data related to the effect of vitamin D deficiency and vitamin D supplementation on oxidative stress are available. To the best of our knowledge, no studies associated with the investigation of oxidative status via the measurements of TAS, TOS, IMA and ox-LDL in patients with vitamin D deficiency are present. Therefore, we aimed at evaluating oxidative status via these measurements in patients with vitamin D deficiency.

MATERIALS AND METHODS

Subjects

Forty-one patients with vitamin D deficiency were included into the study. Plasma 25-hydroxyvitamin D [25(OH)D] levels were measured between January and May. The level of 25(OH)D below 20 ng/mL was accepted as vitamin D deficiency. The patients were compared with age- and sex-mathched 30 healthy subjects with normal 25(OH)D level (> 30 ng/mL). Patients with the history of *diabetes mellitus*, hypertension, hypothyroidism, hyperthyroidism, liver or pulmonary diseases, malignancy, renal, coronary heart or rheumatologic diseases, and those using drugs affecting oxidative status, taking a thyroid medication and cigarette smokers constituted our exclusion criteria. The study was approved by the ethical board of the institution, and informed consent was obtained from all participants (Approval date and number: 9th January 2013-2013/81).

Biochemical measurements

Blood samples were drawn from the antecubital vein after overnight fasting. Venous blood samples were centrifuged at 3000 rpm for 10 min, and samples were stored at -80 °C until analysis. Plasma 25(OH)D levels were measured with liquid chromatography-tandem mass spectrometry (LC-MS/MS) method (WATERS auto-analyser LIKROM System). The reference ranges were between 25-80 ng/mL for 25(OH)D. The levels of serum albumin, triglycerides (TG), total cholesterol, high density lipoprotein cholesterol (HDL-C) and LDL-C were measured using commercially available kits based on routine methods on Architect C 8000 System (Abbott Laboratories, Abbott Park, Illinois, USA). The fibrinogen levels were measured with colorimetric method (BCS XP autoanalyser SIEMENS Diagnostic System), and the reference ranges were between 180-400 g/L for fibrinogen. High- sensitivity C-reactive protein (hs-CRP) levels were measured with nephelometric method, ranging between 1-3 mg/L (BN2 autoanalyser SIEMENS Diagnostic System).

The serum TAS levels were determined using an automated measurement method based on the bleaching of characteristic color of a more stable 2,2'-azino-bis (3-ethylbenz-thiazoline 6-sulfonic acid) (ABTS) radical cation by anti-oxidants (5). In the measurement, the results are expressed in mmol Trolox equivalents/L (mmol Trolox equiv./L). Serum TOS was defined via a novel automated measurement method (9). Oxidants present in the sample oxidize the ferrous ion-o-dianisidine complex to ferric ion. The oxidation reaction is enhanced by glycerol molecules, which are abundantly present in the reaction medium. The ferric ion makes a colored complex with xylenol orange in an acidic medium. The color intensity, which can be measured spectrophotometrically, is related to the total amount of oxidant molecules present in the sample. The assay is calibrated with hydrogen peroxide (H_2O_2), and the results are expressed in terms of micromolar H_2O_2 equivalents per liter (μmol H_2O_2 equiv./L).

The IMA levels were measured using a colorimetric assay developed by Bar-Or and cols. (10) and based on the measurement of unbound cobalt after the incubation with sera from the patients. Increased amount of IMA results in less cobalt binding and more residual unbound cobalt available for complex with a chromogen [dithiothreitol (DTT)], which can be measured photometrically. The procedure was performed as follows: 50 μL of 0.1% cobalt chloride was added into

200 µL of serum, gently mixed and held for 10 min for adequate cobalt-albumin binding. DTT of 50 µL was added at a concentration of 1.5 mg/mL as a colorizing agent. The reaction was stopped 2 min after adding 1.0 mL of 0.9% NaCl. The colored product was measured at 470 nm, compared with a serum-cobalt blank without DTT and reported in absorbance units (ABSU). Adjusted IMA was calculated as (individual serum albumin concentration/median serum albumin concentration of the population) × IMA ABSU value. This formula was applied to correct the IMA values for serum albumin. The median serum albumin concentration of each group was used separately (11).

Analysis of ox-LDL was performed on serum samples using the Eastbiopharm ox-LDL ELISA Kit (Hangzhou Eastbiopharm Co. Ltd.) in accordance with the manufacturer's guidelines. Absorbance was measured at 450 nm on an ELx800 Absorbance Microplate Reader (BioTek, Winooski, VT, USA). This assay employs a quantitative sandwich enzyme immunoassay technique measuring ox-LDL. The resulting concentration values are reported in ng/L.

Statistical analysis

All statistical analyses were performed with SPSS 15.0 (SPSS Inc. Il. USA) statistical soft-ware. The Kolmogorov-Smirnov test was used for the compliance with the normal distribution. All parameters, except for ox-LDL and hs-CRP, were within the normal distribution ranges. The comparisons between groups were performed via the student's t test for parametric variables, and the Mann-Whitney U test for non-parametric variables. The paired student's t and Wilcoxon tests were used to compare the parameters in patient's group. Descriptive analyses were presented using mean±standard deviation (SD) for normally distributed variables, and median and range (min-max) for non-normally distributed variables. The chi-square test was used to investigate the differences between groups regarding the categorical variables. The Pearson's and Spearman's correlation analyses were performed in order to document possible associations between parametric and non-parametric variables, respectively. A p value less than 0.05 was accepted as statistically significant.

RESULTS

Forty-one patients (33 female, 8 male) with vitamin D deficiency and mean ages of 40.2 ± 9.4 years were

present in the study. The control group consisted of 30 healty subjects (24 female, 6 male) with mean age of 41.29 ± 8.6 years. No significant difference was detected between the patients and controls in respect to age and gender (p > 0.05 for all parameters). The levels of TAS, TOS, IMA, ox-LDL, hs-CRP and fibrinogen were measured in the patient and control groups. Additionally, these measurements were repeated in 17 patients after the replacement of vitamin D. The replacement of Vitamin D was orally administered to vitamin D deficient group as 50.000 IU once a week for 8 weeks. The demographic and laboratory data of the groups are presented in table 1.

Table 1. Demographic and laboratory data of the study groups

	Patients (n = 41)	Controls (n = 30)	p
Age (yrs)	40.2 ± 9.4	41.29 ± 8.6	0.666
Female/Male	33/8	24/6	0.959
25(OH)D (ng/mL)	10.72 ± 5.31	32.09 ± 3.61	< 0.001
Fasting plasma glucose (mg/dL)	87.02 ± 9.34	92.21 ± 11.71	0.056
Total cholesterol (mg/dL)	190.82 ± 41.69	189.46 ± 33.99	0.887
LDL-C (mg/dL)	113.46 ± 37.00	119.91 ± 31.78	0.457
HDL-C (mg/dL)	51.29 ± 10.12	46.00 ± 10.04	0.094
Triglycerides (mg/dL)	115.97 ± 53.94	127.85 ± 48.68	0.356
Serum Ca (mg/dL)	9.11 ± 0.37	9.11 ± 0.26	0.915
Serum P (mg/dL)	3.41 ± 0.38	3.30 ± 0.36	0.227
Serum ALP (U/L)	67.36 ± 19.76	73.96 ± 17.82	0.172
TAS (mmol Trolox equiv./L)	1.28 ± 0.11	1.42 ± 0.15	< 0.001
TOS (µmol H_2O_2 equiv./L)	4.98 ± 4.00	3.48 ± 1.43	0.035
IMA (ABSU)	0.54 ± 0.10	0.38 ± 0.16	< 0.001
Ox-LDL (ng/L)	1451.30 (703-6776.30)	1618 (669.70-3823.0)	0.895
Hs-CRP (mg/L)	1.14 (0.16-11.70)	1.26 (0.27-9.85)	0.780
Fibrinogen (g/L)	392.99 ± 69.06	329.93 ± 97.74	0.003

25(OH)D: 25-hydroxyvitamin D; LDL-C: low density lipoprotein cholesterol; HDL-C: high density lipoprotein cholesterol; Ca: calcium; P: phosphorus; ALP: alkaline phosphatase; TAS: total antioxidant status; TOS: total oxidant status; IMA: ischemia-modified albumin; ox-LDL: oxidized-LDL; Hs-CRP: high-sensitivity C-reactive protein.

Baseline plasma 25(OH)D levels were lower in vitamin D deficient group, compared with controls (10.72 ± 5.31 ng/mL *vs.* 32.09 ± 3.61 ng/mL, p < 0.001). No significant difference was found in the levels of fasting plasma glucose, total cholesterol, LDL-C and HDL-C

between the groups. Furthermore, no significant difference was found between the groups regarding to the levels of serum Ca, P and alkaline phosphatase (ALP) (p > 0.05 for all parameters).

The IMA and TOS levels in the patients were significantly higher than those of controls (p < 0.001 and p = 0.035, respectively). The TAS levels were significantly lower in patients, compared to controls (p < 0.001). In addition, fibrinogen was significantly higher in patients than controls (p = 0.003) while ox-LDL and hs-CRP levels were similar between the two groups (p > 0.05 for all parameters).

In 17 patients whose oxidative stress parameters were measured again after the replacement of vitamin D, it was observed that the TAS level was significantly increased (p = 0.037), and the levels of TOS and fibrinogen were significantly decreased (p = 0.043 and p = 0.010, respectively). No alterations were seen in the IMA, ox-LDL and hs-CRP levels after the replacement (p > 0.05 for all parameters) (Table 2).

Table 2. Pre- and post-treatment oxidative stress parameters of patients with vitamin D deficiency

	Before (n = 17)	After (n = 17)	*p*
25 (OH) D (ng/mL)	8.33 ± 1.47	41.75 ± 14.28	< 0.001
Serum Ca (mg/dL)	9.13 ± 0.37	9.25 ± 0.28	0.286
Serum P (mg/dL)	3.41 ± 0.40	3.47 ± 0.35	0.594
Serum ALP (U/L)	65.23 ± 22.15	60.47 ± 16.65	0.057
TAS (µmol Trolox equiv./L)	1.25 ± 0.09	1.30 ± 0.07	0.037
TOS (µmol H_2O_2 equiv./L)	5.12 ± 4.70	2.78 ± 1.17	0.043
IMA (ABSU)	0.54 ± 0.10	0.47 ± 0.13	0.098
Ox-LDL (ng/L)	1391.35 (1059.7-6776.3)	1179.65 (736.3-6076.3)	0.394
Hs-CRP (mg/L)	0.94 (0.16-11.70)	0.96 (0.15-6.24)	0.112
Fibrinogen (g/L)	390.41 ± 63.04	334.90 ± 89.58	0.010

25(OH)D: 25-hydroxyvitamin D; Ca: calcium; P: phosphorus; ALP: alkaline phosphatase; TAS: total anti-oxidant status; TOS: total oxidant status; IMA: ischemia-modified albumin; ox-LDL: oxidized-LDL; Hs-CRP: high-sensitivity C-reactive protein.

A negative correlation was observed between vitamin D levels, and IMA and fibrinogen levels (r = -0.500, p < 0.001 and r = -0.391, p = 0.002, respectively) (Figures 1 and 2). In addition, vitamin D was positively correlated with TAS levels (r = 0.430, p < 0.001) (Figure 3). No significant correlation was found between vitamin D levels, and TOS, ox-LDL

and hs-CRP levels (p > 0.05 for all parameters). While a positive correlation was present between fibrinogen and hs-CRP (r = 0.374, p = 0.002), no correlation was observed between fibrinogen and other oxidative stress parameters (TAS, TOS, IMA and ox-LDL).

Serum Ca levels were negatively correlated with IMA levels (r = -0.406, p = 0.001), and no significant correlation was found between serum Ca, and TAS, TOS and ox-LDL (p > 0.05 for all parameters). In addition, serum P levels were not significantly correlated with the IMA, TAS, TOS and ox-LDL levels (p > 0.05 for all parameters).
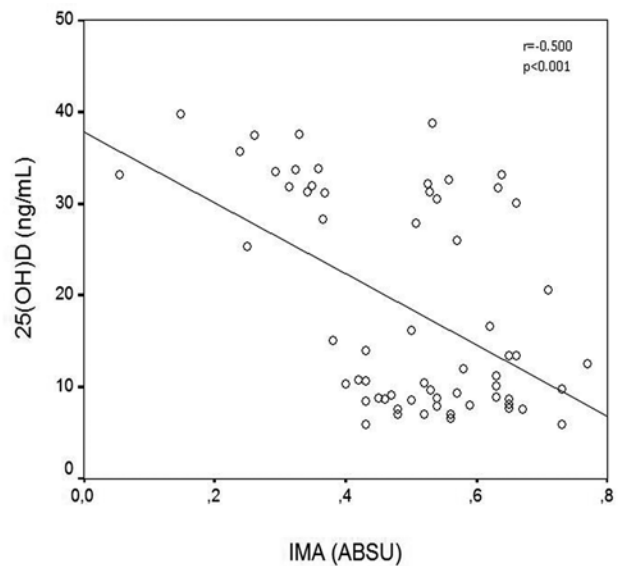

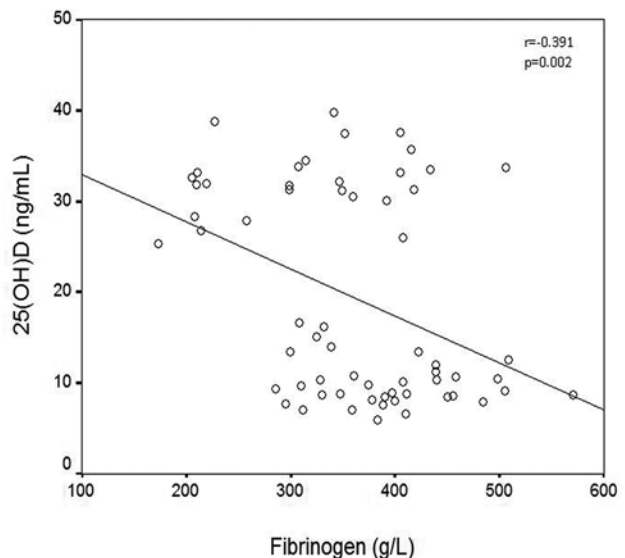**Figure 1.** The correlation between vitamin D and IMA levels.

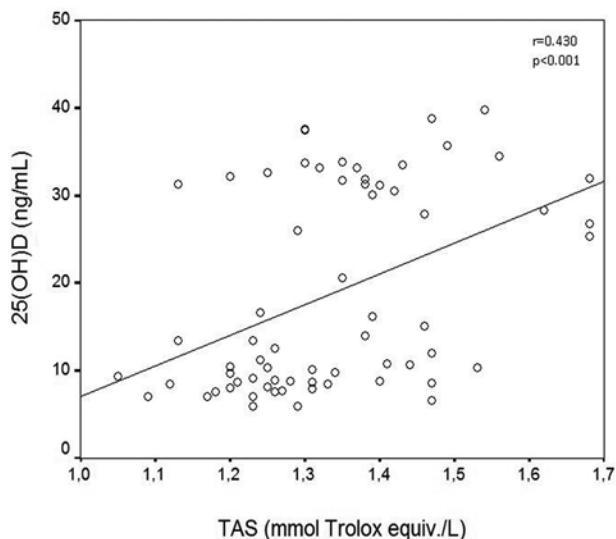**Figure 2.** The correlation between vitamin D and fibrinogen levels.

Figure 3. The correlation between vitamin D and TAS levels.

DISCUSSION

In the study, we assessed the oxidative status via the measurements of TAS, TOS, IMA and ox-LDL and observed that the TAS levels were decreased, while the TOS and IMA levels were increased in patients with vitamin D deficiency. In those with vitamin D deficiency, oxidative/anti-oxidative balance was observed to change in favour of oxidative stress. Even so, this balance was seen to shift to the opposite in patients whose vitamin D levels became normal rates.

Vitamin D plays an essential role in Ca homeostasis and bone metabolism. Suboptimal vitamin D status is associated with increased all causes and cardiovascular mortality (12). The effects of vitamin D on extra-skeletal pathologies may arise from the changes in inflammation, oxidative stress or parathyroid hormone (PTH) levels. In literature, there are studies related to the anti-oxidative feature of vitamin D. However, the exact mechanisms of how vitamin D supplementation may affect oxidant/anti-oxidant balance still remain unknown. Anti-cancer activity of vitamin D is also attributable to its anti-oxidant property (13). Sardar and cols. suggested that vitamin D was an anti-oxidant due to an increase in hepatic glutathione (GSH) levels in rats receiving cholecalciferol (14). In another study investigating the intracellular pathways activated by vitamin D in cultured human umbilical vein endothelial cells undergoing oxidative stress, Uberti and cols. reported that vitamin D may prevent endothelial cell death through the modulation of the interplay between apoptosis and autophagy, and that this effect is also

obtained by inhibiting superoxide anion generation, maintaining mitochondrial function and cell viability, activating survival kinases and inducing nitric oxide production (15).

The measurements of TAS and TOS are useful tests in the prediction of oxidative status. In a study by Salum and cols., cholecalciferol was shown to decrease liver-oxidative stress index significantly and to improve the serum total anti-oxidant capacity (TAC) in diabetic rats treated with cholecalciferol (500 IU/kg for 10 weeks) (16). In another study performed in diabetic patients, a significant positive correlation was observed between the 25(OH)D and TAC (17). We observed that the TAS level was decreased while the TOS level was increased in patients with vitamin D deficiency. Also, a positive correlation was determined between vitamin D and TAS levels.

IMA was initially proposed as a marker for the diagnosis of myocardial ischemia (10). However, IMA is not a tissue-specific marker of ischemia. The IMA levels are higher in very inflammatory and oxidative stress-associated diseases (18). Several authors have suggested that the generation of IMA from serum albumin is the consequence of contact with ROS (19,20). Our study is the first to investigate the IMA levels in patients with vitamin D deficiency. We observed that IMA levels were increased in those with vitamin D deficiency, and an inverse relationship was also present between vitamin D and IMA levels. The increase in the IMA levels may be another sign of oxidative stress in patients with vitamin D deficiency.

Several studies demonstrated that vitamin D may reduce lipid peroxidation (21,22). Kuzmenko and cols. investigated the effects of vitamin D on oxidative stress and lipid peroxidation in animals by determining the changes in lipid peroxidation before and after the replacement in vitamin D-deficient animals (23). They demonstrated that high levels of thiobarbituric acid reactive substances (TBARS) indicating that lipid peroxidation in vitamin D deficient animals were decreased significantly after vitamin D3 replacement, but still remained higher than controls (24,25). Tarcin and cols. also showed that TBARS were significantly lower after vitamin D replacement (26).

Oxidative stress was suggested to be effective in the pathogenesis of atherosclerosis (27). In particular, the oxidation of LDL-C by free radicals plays a central role in the formation, progression, and rupture of atherosclerotic plaques (27). A large body of evidence shows

that ox-LDL is involved in the very early, yet critical steps of atherogenesis, such as endothelial injury, expression of adhesion molecules, and leukocyte recruitment and retention, as well as foam cell and thrombus formation (28). LDL-C is the main factor for the increase in ox-LDL levels. We found that LDL-C and ox-LDL levels were similar in patients and controls. Additionally, no significant correlation was present between vitamin D and ox-LDL levels. The absence of such a difference regarding ox-LDL between our groups may be due to the fact that LDL-C levels were similar.

The relations between vitamin D concentrations and inflammatory markers were investigated in several studies, and controversial results were reported in terms of the association between 25(OH)D, hs-CRP and fibrinogen. In a study performed in 218 bedridden patients aged 65 or older from Helsinki, hs-CRP concentrations were reported not to be associated with 25(OH)D concentrations, and there were no major effects of vitamin D supplementation on hs-CRP or fibrinogen concentrations (29). No association was found between 25(OH)D and CRP concentrations in 1,381 participants in Framingham Offspring study (29-31). In a recent study including 2,723 adult men and women from the general population, an inverse association between 25(OH)D and fibrinogen, and a U-shaped association between 25(OH)D and hs-CRP concentrations with a nadir of 21–25 ng/mL were determined (32). In our study, it was observed that the fibrinogen levels were increased in those with vitamin D deficiency and became significantly decreased after the treatment. Also, an inverse association was seen between vitamin D and the fibrinogen levels. On the other hand, the hs-CRP levels of both groups were found to be similar.

The method used to measure the oxidative stress is very important. At present, no single method that can accurately measure the oxidative stress or its subsequent damage is present (33). The measurement of TAC provides the detection of cumulative action of all the anti-oxidants present in plasma and body fluids. The most commonly used colorimetric method used to measure TAS is 2,2'-azino-bis (3-ethylbenz-thiazoline 6-sulfonic acid) (ABTS) based methods. In our study, we determined TAS using an automated measurement method based on the bleaching of characteristic color of a more stable ABTS radical cation by anti-oxidants (5). For the determination of TOS, we used a novel method whose main components are H_2O_2 and lipid hydroperoxide (9).

In literature, an association is reported to exist between the oxidative stress and PTH (34,35). Tanaka and cols. reported that oxidative stress markers decreased in a patient exposed to parathyroidectomy due to primary hyperparathyroidism (34). Another study reported a positive correlaton between PTH and oxidative stress index in patients with chronic heart valve disease (35). One of limitations in our study was that PTH level could not be measured, and their relationships with oxidative stress parameters could not be investigated. Another was that oxidative stress parameters could be measured again in only 17 of the patients with vitamin D deficiency after the treatment.

In conclusion, the TOS and IMA levels were observed to increase while the TAS level decreased in the patients with vitamin D deficiency. One of cardiovascular risk parameters in these patients, fibrinogen level was also seen to increase. In light of these findings, it may be suggested that oxidative/anti-oxidative balance shifts in favour of oxidative side in vitamin D deficiency. So, further studies with larger sample size and investigating multiple oxidative stress parameters are needed to evaluate oxidative status in patients with vitamin D deficiency.

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