Alfacalcidol modulates oxidative stress parameters in the peripheral blood of patients with active rheumatoid arthritis

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Abstract: Hormone D and its analogues display immunomodulatory activities providing a beneficial effect in immunoinflammatory diseases. The aim of this study was to assess the effect of alfacalcidol treatment on superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) activity and glutathione (GSH) and malondialdehyde (MDA) levels in patients with active RA. Sixteen patients with active RA and twenty controls were enrolled in the study. Blood samples were taken before and after 12 weeks of alfacalcidol therapy (2 μg/day). Oxidative stress parameters were determined spectrophotometrically and by flow cytometry assessment. Disease activity was assessed using DAS28 score. The results revealed that alfacalcidol treatment, significantly (p = 0.04) reduced SOD activity and CAT activity (p = 0.001) in RA patients. The activity of GPx was significantly lower in RA patients before treatment, compared to controls (p = 0.04). After therapy, GPx activity was restored to control levels, and GSH levels were significantly reduced (p = 0.01). MDA levels in patients at the beginning of the study protocol, remained significantly elevated compared to controls (p = 0.01). Alfacalcidol treatment decreased MDA levels in patients (p = 0.19). Furthermore, 12-weeks alfacalcidol therapy, changed the response of RA patients’ PBMC to stimulation preventing the O2− production and mitochondrial membrane depolarisation. After alfacalcidol treatment, significant clinical improvement was observed.

Keywords: antioxidant activity; disease activity; additional therapy; beneficial; PBMC.
INTRODUCTION

One of the most common autoimmune diseases, rheumatoid arthritis (RA) is characterized by the persistent synovitis and systemic inflammation. In the last decade, important advances have been made in the diagnosis, clinical assessment and treatment of RA patients. However, our understanding of the underlying cellular and molecular mechanisms involved in pathogenesis of RA is still incomplete. It was shown that low vitamin D intake is associated with an elevated risk of RA development and furthermore, vitamin D level is associated with RA activity.1–3 The main source of vitamin D is from dietary and supplement intake, with the majority coming from the skin exposure to sunlight, specifically ultraviolet B (UV-B) rays. Higher cumulative average UV-B exposure was associated with decreased RA risk in female nurses aged 30–55 years.4 Vitamin D from the skin and diet is metabolized in the liver to 25-hydroxyvitamin D (25(OH)D), which is used to determine a patients’ vitamin D status. 25-hydroxyvitamin D is metabolized in the kidneys to its active form 1,25-dihydroxycholecalciferol or calcitriol (vitamin D₃) solely biologically active form acting as a steroid hormone.5 Vitamin D₃, has been shown to be a pleiotropic steroid hormone involved not only in regulation of calcium homeostasis and bone turnover, but also in immunomodulation, having potent anti-inflammatory, antiproliferative, prodifferentiation and antibacterial properties in various cells and tissues.6,7 Furthermore, vitamin D₃ can be produced by monocytes behaving as a paracrine factor thus having potent actions on all the cellular components of the immune system.8 Vitamin D₃ exerts its effects via a specific vitamin D receptor (VDR), found in activated lymphocytes, thymocytes and other immunocompetent cells. The significant enrichment of vitamin D response elements at RA associated loci support the hypothesis that vitamin D₃ plays a role in the development of RA.9

The contribution of oxidative stress to chronic inflammation of tissues10,11 and its involvement in pathophysiological mechanism of autoimmune diseases12 have been recently recognized. Oxidative stress reflects an imbalance between the systemic production of reactive oxygen species (ROS) and the antioxidant ability of the organism to readily detoxify the reactive intermediates or to repair the resulting damage. There is a strong evidence supporting the role of oxidative stress in cartilage degradation in experimental arthritis13 as well as in contribution to disease activity in RA patients.14 Pro-oxidation environment in RA results in redox imbalance, evidenced by increased production of reactive oxygen, nitrogen and sulphur15 species that amplify the synovial inflammatory-proliferative response.14 However, scarce information is available about the antioxidant properties of vitamin D₃ and its analogues, and some data are even controversial.16–18 Therefore, the role of vitamin D₃ therapy in RA patients deserves to be further explored.
Alfacalcidol (1α(OH)D3) is a synthetic vitamin D3 analogue, exerting full biological activity of calcitriol. In a recent study by Radovic et al.,19 the alfacalcidol therapy showed a beneficial effect, without any side effects, on the disease activity in 93.3 % of the patients with juvenile idiopathic arthritis (JIA), also known as juvenile rheumatoid arthritis (JRA), which is the most common form of arthritis in children and adolescents. This study revealed that alfacalcidol therapy induced significant changes in antioxidative enzyme activities.

The goal of this study was to examine the effects of alfacalcidol therapy on the superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) activity, glutathione (GSH) levels in erythrocytes and malondialdehyde (MDA) in the plasma of patients with active RA in comparison to the healthy controls. We were also interested in the therapy effect on sensitivity of RA patients peripheral blood mononuclear cells (PBMC) to stimulation.

EXPERIMENTAL

Sixteen female RA patients (average age 53±13), in the Outpatient Clinic of the Institute of Rheumatology, Belgrade, Serbia, were consecutively enrolled in the study. Controls were twenty age- and sex-matched healthy volunteers selected from the staff. All subjects signed the information consent before participation in the study.

The study was conducted in compliance with the Declaration of Helsinki, International Conference on Harmonization and Good Clinical Practice Guidelines, and was approved by the Ethics committee of the Institute of Rheumatology in Belgrade, Ethic Committee of the University of Belgrade, School of Medicine, and by Medicines and medical Devices Agency of Serbia.

Inclusion criteria were as follows: established diagnosis of RA (ACR/EULAR criteria 2010),20 at least six months prior to enrolment; methotrexate treatment for 12 weeks or longer with stable dose of 10-25 mg/week for 8 weeks or longer; active disease defined as 28-joint disease activity score (DAS28) >3.2. If non-steroidal anti-inflammatory drugs (NSAIDs) are administered, patients have to be using stable dose of NSAID for at least four weeks. Glucocorticoids (systemic and/or local use) were not allowed for at least one month before entering the study.

The treatment protocol considered the continuation of previous therapy with the addition of alfacalcidol as oral commercial preparation at the dose of 2 μg daily during 12 weeks.

Clinical and laboratory assessment of disease activity (serum concentration of C-reactive protein (CRP), sedimentation rate (SE), number of swollen and tender joints) and safety (regular biochemistry analyses including serum total and ionized calcium concentration and urine calcium concentration as well) of the studied drug were evaluated during regular visits (before start of the therapy, in four-week intervals and after completion of the alfacalcidol therapy). Disease activity was assessed using the 28-joint DAS28.21

Blood samples for vitamin D (25(OH)D) levels of our patients were obtained after at least 8 h overnight fasting, before (week 0 = W0) and after 12-week long alfacalcidol therapy (week 12 = W12). The level of vitamin D was determined by electrochemiluminiscence assay from Roche Diagnostics (Elecsys 2010). Level of vitamin D < 20 ng/ml indicates deficiency, levels 21–29 ng/ml indicate insufficiency and levels ≥ 30 ng/ml indicate sufficiency.
The activity of antioxidant enzymes SOD, CAT and GPx and the level of GSH were determined in isolated and lysed erythrocytes, while MDA was determined in plasma, of the controls and patients, before (W0) and in RA patients after 12-week long (W12) alphacalcidol therapy using manual spectrophotometric method. Hemoglobin (Hb) concentration in lysed erythrocytes was measured by hematology analyzer. SOD activity was assayed as the ability of supernatant obtained after Hb precipitation from lysed erythrocytes, to inhibit the radical-mediated autooxidation of epinephrine (using epinephrine hydrochloride, Sigma). Obtained values were expressed as the number of units (U) per gram of hemoglobin in erythrocyte haemolyzate (gHb). The intra-assay and inter-assay CVs were 5.2 and 6.7 %, respectively. CAT activity was determined based on the formation of stable complex of hydrogen peroxide with ammonium molybdate whose absorption was measured spectrophotometrically at 405 nm (using hydrogen peroxide solution 30 %, Zorka Pharma, ammonium molybdate, Sigma–Aldrich). Results were expressed as U/gHb, the intra-assay CV was 3.9 % and the inter-assay CV was 8.5 %. GPX activity in lysed erythrocytes was measured according to previously described spectrophotometric method (using 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid – HEPES, Sigma–Aldrich; ethylenediaminetetraacetic acid, disodium salt, Sigma; L-glutathione reduced, Sigma; β-nicotinamide adenine dinucleotide 2′-phosphate reduced tetrasodium salt hydrate, Sigma; tert-butyl hydroperoxide solution, Sigma; glutathione reductase, Sigma–Aldrich) and presented as U/gHb (the intra-assay CV was 5.7 % and the inter-assay CV was 7.2 %). GSH was determined according to the method by Sedlak and Lindsay (using the Ellman’s reagent 5,5′-dithiobis-(2-nitrobenzoic acid), DTNB, Sigma–Aldrich; tris(hydroxymethyl)aminomethane, Serva, Germany), and obtained values were expressed as μmol/gHb (the intra-assay CV was 3.6 % and the inter-assay CV was 7.3 %). Lipid peroxidation, measured as malondialdehyde (MDA) level, was determined spectrophotometrically in a reaction with thiobarbituric acid. Thiobarbituric acid reacts with MDA released from polyunsaturated fatty acids of plasma membrane phospholipids injured by ROS, forming a yellow complex whose absorbance was measured at 533 nm (trichloroacetic acid, Sigma–Aldrich; 2-thiobarbituric acid, Sigma). Results were expressed as nmol of MDA per mL (the intra-assay CV was 5.2 % and the inter-assay CV was 7.9 %).

Mitochondrial production of superoxide anion (O₂⁻), one of ROS, as well as mitochondrial membrane potential were assessed on peripheral blood mononuclear cells (PBMCs) of controls and RA patients (W0 and W12) using flow cytometry. Peripheral blood mononuclear cells (PBMCs) were obtained from venous blood of controls and RA patients (on W0 and W12). PBMCs were isolated from heparinized blood by density gradient centrifugation using LymphoPrep (Axis Shield, Norway). The PBMC were maintained at 37 °C in a humidified atmosphere with 5% CO₂ in HEPES (20 mM)-buffered RPMI 1640 cell culture medium supplemented with 10 % fetal bovine serum (FBS), 2 mM-glutamine, and 1 % of antibiotic/antimycotic mixture (all from PAA, Austria). Cells (2.5×10⁵ cells/well) were incubated in 24-well plates (Sarstedt, Nümbrecht, Germany). Cells were stimulated 30 min after seeding for 4 h, with phorbol 12-myristate 13-acetate (PMA) (Sigma–Aldrich), final concentration 10 ng/ml and ionomycin (Sigma–Aldrich), final concentration 1.25 μM. Each experiment contained untreated and stimulated (control, patients W0 and patients W12) cells.

Flow cytometry assessments were performed using FACSCalibur (BD Biosciences, Heidelberg, Germany) equipped with CellQuest Pro software for acquisition and analysis. The light-scatter channels were set on linear gains and the fluorescence channels on the logarithmic scale. A minimum of 10,000 cells was analyzed for each condition, adjusting the
threshold settings so that the cell debris was excluded from the data acquisition. The superoxide anion production was analyzed by measuring the increase in red fluorescence (FL2) intensity emitted by a superoxide-specific fluorochrome, dihydroethidium (DHE; Sigma–Aldrich), which was incubated with cells (20 mmol/l) for 30 min. Obtained results were presented as the increase of mean fluorescence intensity (compared to 1 in non-stimulated cells). Mitochondrial membrane potential was assessed using a lipophilic cation JC-1 (R&D Systems), which has the property of aggregating upon membrane polarization thus forming an orange-red fluorescent compound. The dye cannot access the transmembrane space if the potential is disturbed, and remains in or reverts to its green monomeric form. The cells were stained with JC-1 as described by the manufacturer, and the green monomer and the red aggregates were detected by flow cytometry. Obtained values were expressed as the green/red (FL1/FL2) fluorescence ratio, and the increase corresponded to the extent of mitochondrial depolarization. The results are presented as the increase of FL1/FL2 fluorescence ratio (compared to 1 in non-stimulated cells).

Statistical analysis was performed using t-test for independent samples, t-test for related samples (for parametric data) and Wilcoxon and Mann-Whiney tests (for nonparametric data). For testing the normality of the distribution parameters, the Kolmogorov–Smirnov test was used. Correlation between parameters was determined using Spearman and Pearson test. The results are presented as mean±standard deviation (SD, data with normal Gaussian distribution) or Median (IQR, interquartile range) for non-Gaussian distribution data.

RESULTS

At the beginning of the study, vitamin D (25(OH)D) levels in patients were sufficient (W0, 30±11 ng/ml) and remained constant after 12-week treatment (W12, 31±13 ng/ml), indicating that alfacalcidol does not cause significant \( (p > 0.05) \) change in patients’ 25(OH)D level. Furthermore, during 12 weeks of alfacacidol treatment and follow-up period no side effects were noticed in patients with RA. Serum calcium and ionized calcium levels remained within physiological range of values. Although the increase in urine calcium levels was detected in some patients, obtained values remained within physiological range.

Parameters of antioxidant enzymes (SOD, CAT and GPx) activity and GSH levels in the erythrocytes and plasma MDA levels, of RA patients before and after 12 weeks of alfacalcidol treatment, as well as for control group are presented in Table I.

The alfacalcidol treatment significantly reduced both SOD (W0/W12; \( p = 0.04 \)) and CAT (W0/W12; \( p = 0.001 \)) activity in erythrocytes of RA patients. While SOD activity was reduced to the levels obtained for the healthy control group, CAT activity after alfacalcidol treatment was decreased to the levels significantly lower than in the controls (\( p = 0.0001 \)). However, no significant difference in CAT activity in erythrocytes of RA patients before (W0) therapy in comparison to the controls was observed (W0/Ctrl; \( p = 0.37 \)). The activity of GPx was significantly lower in erythrocytes of RA patients before treatment (W0) compared to the controls (\( p = 0.04 \)). In accordance with lower GPx values, GSH levels were significantly higher in erythrocytes of RA patients (W0) compared to
the controls ($p = 0.03$). Even though 12-weeks of alfacalcidol therapy restored erythrocytes’ GPx activity to control levels, no significant difference was detected between values obtained for W0 and W12 ($p > 0.05$). On the other hand, the 12-week long alfacalcidol therapy significantly reduced GSH levels ($p = 0.01$). In addition to glutathione levels and antioxidant enzyme activity, oxidative stress was further evaluated through measuring the MDA levels produced in lipid peroxidation initiated by ROS. In the present study, although alfacalcidol treatment decreased MDA levels in patients, the difference did not reach a statistical significance (W0/W12; $p = 0.19$). However, MDA levels in patients at the beginning of the study protocol (W0), remained significantly elevated compared to the controls (W0/Ctrl; $p = 0.01$), suggesting the presence of oxidative stress in patients with active disease (Table I).

TABLE I. The activity of antioxidant enzymes (SOD, CAT and GPx) and glutathione (GSH) levels in erythrocytes and MDA in plasma of RA patients’ before and after twelve weeks of alfacalcidol therapy; SOD – superoxide dismutase; CAT – catalase; GPx – glutathion peroxidase; GSH – glutathion; MDA – malondialdehyde; U – international unit of enzyme activity; Hb – hemoglobin; RA – rheumatoid arthritis; W0 – before therapy; W12 – after 12 weeks of therapy; values presented are means ± SD or median (IQR); *$p < 0.05$ compared to W12; **$p < 0.01$ compared to W12; $^p < 0.05$ compared to control; $^{**}p < 0.01$ compared to control

<table>
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<th>Therapy</th>
<th>SOD</th>
<th>CAT</th>
<th>GPx</th>
<th>GSH</th>
<th>MDA</th>
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<tr>
<td>Control</td>
<td>450±110</td>
<td>1300±600</td>
<td>5.2±1.8</td>
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<td>106±57</td>
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<td></td>
</tr>
<tr>
<td>W0</td>
<td>510±100*</td>
<td>1500±800**</td>
<td>3.7±1.6*</td>
<td>6.0±2.1**#</td>
<td>163±30##</td>
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<tr>
<td>W12</td>
<td>470±130</td>
<td>258 (302)#</td>
<td>3.9 (0.8)</td>
<td>3.7±1.2</td>
<td>149±42</td>
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In order to get further insight into the effect of 12-week long alfacalcidol treatment, we measured the intensity of superoxide anion ($O_2^-$) production and mitochondrial membrane potential in controls and patients (W0 and W12) stimulated PBMC. Obtained results showed that stimulation caused the increase in DHE fluorescence intensity in control PMBC indicating the $O_2^-$ production (Table II). Furthermore, in patients’ PBMC at the beginning of the study (W0), superoxide production was higher in comparison to the healthy controls, but this difference failed to reach a statistical significance (W0/Ctrl; $p = 0.09$). Also, FL1/FL2 ratio increased in stimulated controls as well as in patients (W0), suggesting the ability of stimulation to cause depolarization of the inner mitochondrial membrane (Table II). On the other hand, alfacalcidol treatment changed the response of patients (W12) PBMC to stimulation as evidenced by the reduced superoxide production (W0/W12; $p = 0.10$) and the absence of the inner mitochondrial membrane depolarization (W0/W12; $p = 0.001$). Therefore, these findings suggest the protective influence of alfacalcidol on PBMC sensitivity to oxidative stress induced by stimulation.
TABLE II. Production of superoxide anion (O$_2^-$) and change of mitochondrial membrane potential in controls and patients’ stimulated PBMCs before and, in patients, after twelve weeks of alfacalcidol therapy; DHE – dihydroethidium; JC1 – lipophilic cation; PBMC – peripheral blood mononuclear cells; W0 – before therapy; W12 – after 12 weeks of therapy; values presented are means ± SD; *p < 0.05 compared to W12; **p < 0.01 compared to W12; p < 0.05 compared to control; #p < 0.05 compared to control

<table>
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<th>Therapy</th>
<th>DHE</th>
<th>JC1</th>
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<td>Control</td>
<td>1.7±0.5</td>
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<tr>
<td>W0</td>
<td>2.2±0.6</td>
<td>1.3±0.2**</td>
</tr>
<tr>
<td>W12</td>
<td>1.5±0.5</td>
<td>0.8±0.2</td>
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After 12-weeks of alfacalcidol treatment, significant clinical improvement was observed. Disease activity score (DAS28) significantly decreased from 5.8±0.9, before treatment (W0) to 4.3±1.0 (p < 0.01) after (W12) therapy. At the beginning of the study (W0), most of the patients (84 %) were in the group of high-active disease (DAS28 > 5.1) and 16 % of the patients were with the moderate-active disease (3.2 < DAS28 < 5.1). There were no patients with the low-active disease (DAS28 < 3.2). After treatment (W12), only 16 % of the patients remained in the group of high-active disease, 68 % of them were in moderate-active disease, and 16 % of the patients had low-active disease (DAS28 < 3.2), Fig. 1.

Also, CRP levels dropped from 29±28 mg/l (W0) to 10±12 mg/l (W12) (p = 0.02) supporting the clinical improvement evidenced by DAS28. Significant positive correlation ($r^2 = 0.64$, p = 0.02) was observed only between changes in CAT activity ($\Delta$CAT) and CRP level ($\Delta$CRP), Fig. 2.
Fig. 2. Alfacalcidol therapy causes significant clinical improvement of RA patients regarding disease activity score (DAS28). After twelve weeks of treatment (W12), 16% of the patients were in the group of high-active disease (DAS28 > 5.1), 68% were in moderate-active disease (3.2 < DAS28 < 5.1) while 16% had low-active disease (DAS28 < 3.2).

DISCUSSION

Herein we report no significant change in patients’ 25(OH)D levels before and after 12-weeks of alfacalcidol treatment. In accordance with this, it is of great importance to emphasize that measurement of 25(OH)D levels, as the only standardized tool to estimate vitamin D status in organism, is actually only the reflection of the balance between food and/or supplement vitamin D diet intake and its utilization in the local tissues as the active D hormone.27

Superoxide anion (O$_2^-$) is believed to be one of the initiators of free radical production reactions. SOD is an enzyme responsible for the conversion of O$_2^-$, an important ROS, to hydrogen peroxide. In this study, alfacalcidol treatment (12-weeks), significantly (W0/W12; $p = 0.04$) reduced SOD activity in erythrocytes of RA patients to the levels obtained in the healthy control group. The results of the present study are similar to that published by Radovic et al.,19 which showed that 12-weeks of alfacalcidol treatment in patient with juvenile idiopathic arthritis (JIA), reduced SOD activity to the levels even lower than in healthy control group, confirming role of vitamin D in regulation of cell redox system. One explanation could be the fact that SOD production is stimulated through Th1 cellular and humoral immunity that are reduced by the alfacalcidol treatment. Similarly, Cimen et al.28 observed higher SOD levels in RA patients than in control group, suggesting that excessive free radical production might be through xanthine–xanthine oxidase system rather than an impaired antioxidant system. Likewise, Vijaykumar et al.26 reported increased levels of SOD and GPx in their study, which could dismutate the excess superoxide radicals that are generated and diffused from the inflammatory sites due to over expression of antioxidant defense system of RA patients. However Akyol et al.30 and Ozkan et al.31 reported no change in SOD levels between RA patients and controls. However our findings are contra-
dictory to the findings of Chandankhede et al. and Desai et al., that showed decreased activity of antioxidant enzyme SOD in RA patients compared to the controls, but the patients included in their studies were not in the active stage.

Furthermore, our results showed that 12-weeks long alfacalcidol treatment changed the response of RA patients’ PBMC to stimulation by preventing the O2– production and mitochondrial membrane depolarisation thus supporting the result of reduced SOD activity in RA patients erythrocytes after this prolonged treatment. Considering that vitamin D is a membrane antioxidant able to inhibit iron-dependent liposomal lipid peroxidation by stabilizing the cell membranes, this result indicates possible protective influence of alfacalcidol on PBMC sensitivity to oxidative stress induction upon stimulation. This could be significant since in our experiments stimulation caused increase in O2– production and depolarisation of mitochondria membrane, not only in patients before treatment (W0), in accordance with the results from Bulua et al., but also in healthy control PMBC as well (Table II).

CAT and GPx are enzymes involved in the neutralization of H2O2. While CAT reaction produces oxygen and H2O2, GPx uses GSH oxidized during the reaction that produces water from H2O2. In the present study the activity of CAT was higher in erythrocytes of RA patients before treatment compared to the controls. Beyond the cell protection against damage caused by ROS it was shown that expression of CAT in vitro and in vivo affects the expression of genes influencing inflammation. Interestingly, only for CAT we have shown that 12-week therapy with alfacalcidol significantly decreased activity of this enzyme to the levels much lower compared to healthy control. This finding strongly supports alfacalcidol as additional therapy in RA, because CAT demonstrated leading role in detoxifying H2O2 within erythrocytes, since this enzyme was strongly elevated in RA synovial fluid. Significant correlation between changes of CAT activity and CRP level suggests important role of CAT activity in RA pathogenesis.

It has been shown that under conditions of severe oxidative stress GPx could be inactivated as O2– can inhibit peroxide function. This could explain our finding of GPx decreased activity in erythrocytes of active RA patients before treatment, compared to the controls. Also Cimen et al. found that GPx might not be playing an essential role in rheumatic events. The affinity of GPx for H2O2 is stronger than the affinity of catalase, which makes GPx more efficient at low levels of H2O2 concentrations.

Decreased activity of GPx reduced the utilization of GSH by erythrocytes and consequently increased levels of GSH. Observed decrease of GSH in erythrocytes after alfacalcidol therapy indicated that the patients were trying to recover normal GSH levels as disease improved.

In addition to glutathione and antioxidant enzymes, we have also evaluated the presence of lipid peroxidation by measuring the MDA levels. Our results
showed that, in patients, MDA levels were significantly elevated compared to the controls, but after alfacalcidol therapy patients’ MDA levels were lower, in accordance with other studies\textsuperscript{19,40} suggesting alfacalcidol protective role in pro-oxidative conditions.

After 12-weeks of alfacalcidol treatment, our patients experienced significant clinical improvement without side effects. Only one patient showed no improvement but also without disease worsening. Our results are in accordance to the study done by Andjelkovic \textit{et al.}\textsuperscript{8} who showed statistically significant clinical improvement in RA patients treated with alfacalcidol, in both the physician’s overall assessment and in the evaluated parameters of acute phase response.

It is well known that all cells of monocyte-macrophage lineage have capability to synthesize $1\alpha$-hydroxylase and locally produce D-hormone that acts as a potent immunomodulatory molecule, preventing T-cell over-stimulation and decreasing lymphocyte proliferation.\textsuperscript{41} D$_3$ hormone shifts T-cells from Th1 to Th2 phenotype, preventing tissue damage associated with Th1 responses.\textsuperscript{42} Th17, which produces the proinflammatory cytokine IL-17, is thought to play a role in the pathogenesis of autoimmune diseases, including RA. There is evidence suggesting that 1,25(OH)$_2$D$_3$ inhibits the secretion of Th17 cells via a number of pathways, including reduction of the expression of Th17 stimulatory factor IL-6.\textsuperscript{43} These results indicate that 12-week long alfacalcidol therapy is meaningful approach to improve RA patients’ clinical condition as evidenced by lowering DAS28 and CRP levels. The use of alfacalcidol instead of 1,25(OH)$_2$D$_3$ allows higher dosages to be used with lower risk of hypercalcemia, thus increasing its potential as a therapeutic agent.\textsuperscript{44} During treatment and follow-up period, in our study, serum and urine calcium as well as ionized calcium levels remained within physiological range.

The 25(OH)D form is the most plentiful and stable metabolite of vitamin D in human serum with high affinity to bind serum vitamin D-binding protein and other albumin superfamily in the blood. Therefore, the level of 25(OH)D in the serum is the best indicator of vitamin D entering the host, either by cutaneous synthesis or by ingestion in the diet. Nevertheless, this form is still not a hormone; rather, it is a prehormonal form of the natural hormone and does not exert almost any biologic activity in the body.\textsuperscript{45} As a result of the negative feedback regulating the final activation step of 25(OH)D into the active 1,25(OH)$_2$D$_3$ by the kidneys, the oral supplements of plain vitamin D will never lead to increase of hormone D$_3$ to the levels exerting immunomodulatory and/or antioxidative effects.\textsuperscript{44,46}

CONCLUSION

The present study demonstrates that twelve weeks of alfacalcidol treatment modulates the activity of antioxidant enzymes and intensity of lipid peroxidation.
that further correlates with significant reduction in disease activity of RA patients. We propose that hormone D could diminish the disease activity by either decreasing ROS production or expanding their neutralization by the action of antioxidant enzymes, suggesting the beneficial role of alfacalcidol as the additional therapy in RA. These data could contribute to better understanding and further research of the biological mechanisms of the disease and possibly provide new therapeutic approach.

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