Status of Serum Vitamin D Levels In Patients of Rheumatoid Arthritis: A Pilot Study

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Abstract
Rheumatoid arthritis (RA) is an autoimmune disease characterized by periods of remission and flares of symmetrical joint inflammation leading to joint deformities. Previous studies regarding association of vitamin D deficiency with RA have suggested controversial findings. Moreover, limited studies have been conducted using serum levels of vitamin D measurement in Indian Population. Hence we planned this study with objective to evaluate the status of serum vitamin D levels in patients of rheumatoid arthritis and to compare it with healthy volunteers. We included patients of RA between age group of 18-65 years after getting permission from IEC of GMC, Nagpur. Control group were either patients attending to general medicine OPD or healthy volunteers. Serum levels of 25-OH-Vitamin D3 were determined at Clinical Pharmacology Laboratory of GMC, Nagpur using HPLC instrument and < 20 ng/ml were considered as vitamin D deficiency. In statistical analysis, the serum 25-OHD levels between patients and controls were compared using Mann-Whitney test with Graph Pad prism software version 5.01. Six female patients and 26 controls including 15 males and 11 females were enrolled. The mean age of patients (47.33± 7.28 years) and controls (45.04±10.24 years) did not differ significantly. Slightly higher mean levels of vitamin D were observed in patients of rheumatoid arthritis (21.36±13.03 vs 18.41±13.66). The statistical significance between serum levels of vitamin D of patients and controls could not be established. Our study showed no statistical association between low vitamin D level and incident RA however the findings should be looked into giving a due consideration to low power of the study. Further larger studies might throw light on the association between low vitamin D level and incident RA.

Key Words
25-Hydroxycalciferol, Disease Activity, Arthritis

Introduction and background
Rheumatoid arthritis is an autoimmune disease is characterized by periods of remission and flares of symmetrical joint inflammation which slowly progresses to joint and cartilage destruction leading to deformities. Though the exact cause is not known, both genetic and environmental factors have been implicated in the etiopathogenesis. The disease commonly affects women of age group 30-50 years although prevalence increase with increasing age in both men and women. The prevalence of rheumatoid arthritis in Indian population have been estimated to vary between 0.3% to 0.75% and based upon 2011 census, more than 36 million patients are expected to be suffering from rheumatoid arthritis in India.
Vitamin D is essential for bone mineral metabolism and is obtained from either dietary sources or sun exposure. Dietary sources fulfill only 20% of the body's daily requirement of vitamin D while synthesis in the skin after sun exposure is the most important for maintenance of adequate levels in body.\(^3\)

In recent years, there is growing interest in exploring extra-osseous actions of vitamin D because of its involvement in the regulation of immune functions and it has been implicated in several inflammatory autoimmune disorders such as rheumatoid arthritis, type 1 diabetes mellitus and multiple sclerosis etc.\(^4\) Several studies regarding role of vitamin D in pathogenesis and progression of rheumatoid arthritis have reported controversial findings. In a study on 62 patients of rheumatoid arthritis, negative correlation between serum level of vitamin D and measured by DAS 28 (-0.38), pain assessed by visual analog scale (-0.49) and disability assessed by the Health assessment questionnaire disability index (-0.54) (p<0.01) was observed.\(^5\) However, in a study involving 79 patients of rheumatoid arthritis, estimation of serum vitamin D levels at 1 year, 2 years and 5 years before the onset of symptoms showed no difference between vitamin D serum levels in patients who later developed rheumatoid arthritis and healthy controls suggesting that vitamin D might not be a factor in pathogenesis of rheumatoid arthritis.\(^6\)

Such controversial reports might be due to several difficulties in establishing a concrete relationship between vitamin D deficiency and autoimmune rheumatologic disorders including different cut-off levels used by researchers, presence of several confounding factors associated with rheumatoid arthritis including use of glucocorticoids, sunscreen use etc. and use of dietary intake reports for analysis instead of measuring the serum concentration of vitamin D.\(^7\) As we found limited studies using serum levels of vitamin D measurement to establish its role in rheumatoid arthritis and moreover extremely limited amount of data is available regarding the serum vitamin D levels and its relationship with the rheumatoid arthritis in Indian population, we planned the current study with the objective to evaluate the status of serum vitamin D levels in patients of rheumatoid arthritis and to compare it with healthy volunteers.

**Material and Methods**

Study was started only after getting permission from the Institutional Ethics Committee of Government Medical College, Nagpur. We included patients of rheumatoid arthritis between age group of 18-65 years, who presented for routine medical follow-up examination to an orthopedic outpatient department and diagnosis was confirmed by an orthopaedician using the American College of Rheumatology 2010 revised criteria.\(^8\) All patients were taking appropriated treatment with respect to initial diagnosis to achieve remission. Control group were selected either from patients attending to a general medicine outpatient department or from healthy volunteers. They had no rheumatic diseases or other skeletal symptoms based on history and clinical examination.

Serum 25-OHD was measured by HPLC method, and serum concentrations less than 20 ng/ml were considered as vitamin D deficiency. Individuals with gastrointestinal, kidney, endocrine and pulmonary diseases, systemic debilitating diseases, patient older than 65 years of age and those taking anticonvulsant drugs or those on vitamin D supplements were excluded. Informed consent was obtained from all the participants of this study by using informed consent form. All participants were informed regarding their laboratory results by communicating directly with them on their cell phones.

Serum levels of 25-OH-Vitamin D3 were determined at Clinical Pharmacology Laboratory of Government Medical College, Nagpur using Shimadzu High Performance Liquid Chromatography instrument. For determination of serum 25-OH-Vitamin D3 we used ClinRep®HPLC Complete Kit with order no. 35000 containing mobile phase, internal standard, lyophilized serum calibrator, precipitation reagent and analytical column. Blood samples were collected from controls and cases using all aseptic precautions. 3 ml of blood was withdrawn from anterior cubital vein, allowed to clot and then serum separated using centrifuge machine. Afterwards, 400 µl of serum sample mixed with 500 µl of...
precipitant and subsequently 400 µl of internal standard was added to this mixture. After mixing the sample for 30 sec on a vortex mixer, centrifugation done and 50 µl of upper liquid phase of the supernatant was used for analysis by injected into Shimadzu HPLC system. Chromatograms of the measured samples (controls and patients) were compared with serum calibrators for stability of peaks and retention times. 25-OH-Vitamin D2/D3 calculation done by using internal standard method via peak area.

Keeping in view of our primary aim of the study, to evaluate the status of serum vitamin D levels in patients of rheumatoid arthritis, the sample size was calculated using PS (Power and Sample Size Calculation version 3.1.2, 2014) software. In a previous study the response within each subject group was normally distributed with standard deviation 29nmol/l. Assuming true difference in the experimental and control means as 22 nmol/l, sample size was calculated as 28 per group in order to reject null hypothesis so that the population means of the experimental and control groups are equal with probability (power) 0.8. The Type I error probability associated with this test of null hypothesis was 0.05. In statistical analysis, the serum 25-OHD levels between patients and controls were compared using Mann-Whitney U test. Graph Pad prism software version 5.01 was used for statistical analysis.

**Results**

<table>
<thead>
<tr>
<th>S.N.</th>
<th>Groups</th>
<th>No. of cases</th>
<th>Age± SD</th>
<th>Serum 25-hydroxyvitamin D(25-OHD) levels</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Mean (ng/ml)</td>
</tr>
<tr>
<td>1</td>
<td>Patients</td>
<td>6</td>
<td>47.33±7.28</td>
<td>21.36±13.03</td>
</tr>
<tr>
<td>2</td>
<td>Controls</td>
<td>26</td>
<td>45.04±10.24</td>
<td>18.41±13.66</td>
</tr>
</tbody>
</table>

Table no. 1- Table Showing levels of Vitamin D in Patients and Controls
Although total 28 patients were initially screened, only 6 (all females) patients could meet the required inclusion criteria, hence only six patients with rheumatoid arthritis and 26 controls (15 males and 11 females) were included in this study. The mean age of patients and controls did not differ significantly (47.33± 7.28 of patients vs 45.04±10.24 of controls; the 95% confidence intervals for mean age of patients and controls were 39.69-54.98 and 40.9-49.18 respectively). Slightly higher mean levels of vitamin D were observed in patients of rheumatoid arthritis (21.36±13.03 vs 18.41±13.66). The 95% confidence intervals for serum levels of Vitamin for patients and controls were 7.678 -35.03 and 12.9- 23.93 respectively. The level of vitamin D deficiency was almost same in both groups (65% vs 66%). Because of low power of this study (7%), the statistical significance between serum levels of vitamin D of patients and controls could not be established, though the Mann Whitney test has shown non-significant result.

Discussion
The study was conducted with the objective to evaluate the status of serum vitamin D levels in patients of rheumatoid arthritis and to compare it with healthy volunteers. The findings of this study implies that neither the serum level nor the serum vitamin D deficiency in patients of rheumatoid arthritis were significantly different from controls. Although level of vitamin D deficiency was almost equal in both groups and both groups were having comparable serum levels of vitamin D, slightly lower levels of serum vitamin D in controls seen in this study might be attributed to less sunlight exposure.

As mentioned earlier, vitamin D might play a role in modulation of immune function and inflammatory responses has been suggested because vitamin D is known to be synthesized normally by antigen presenting cells like macrophages and dendritic cells while activated lymphocytes synthesize it during inflammatory process only. It is believed that vitamin D suppresses the production of IFN-y and IL-2 while increase the production of IL-4, IL-5 and IL-10 cytokines. Because of this, an association of vitamin D deficiency and autoimmunity has been suggested.([10])

Although we found no overall correlation between vitamin D levels and rheumatoid arthritis, our data cannot provide evidence against such an effect. Because many previous studies have suggested association of vitamin D deficiency in pathogenesis of rheumatoid arthritis. In a study from Morocco, total 170 patients with mean age of 50±12.1 years were enrolled with the objective to evaluate the prevalence of vitamin D insufficiency in patients with rheumatoid arthritis (RA) and its association with disease activity, severity and physical disability, reduced levels of vitamin D were observed in all patients, with deficiency in 35.5 % of cases and insufficiency in 65.5 % of cases. Authors attributed the above findings to the deficiency of vitamin D leading to decrease stimulation of Th2 (T helper cell) response leading to increased production of pro-inflammatory cytokines such as IL6, IL17, Interferon gamma, TNF-alpha, etc.[11] Similarly in an open-labeled randomized trial comparing triple disease-modifying anti-rheumatic drug (DMARD) therapy and 500 IU 1,25 dihydroxy vitamin D3 + calcium combination versus triple DMARD and calcium alone by Gopinath et al., low levels of serum vitamin D were related to moderate-high disease activity in and supplementation of 500 IU of 1,25 dihydroxyvitamin D daily was given to previously DMARD-naïve patients with early rheumatoid arthritis along with triple DMARD therapy showed significantly higher pain relief (50%vs. 30%, P=0.006) in patient group than healthy controls.[12]

Heidari B. et al. conducted a study in which the levels of vitamin D in 108 established rheumatoid arthritis patients and 39 undifferentiated inflammatory arthritis patients were compared with 239 healthy controls. They observed no significant differences in mean serum vitamin D between rheumatoid arthritis and healthy controls(37 ± 37.7 vs. 33.2 ± 28.6 ng/ml, P = 0.96) however the mean serum vitamin D levels in patients with undifferentiated inflammatory arthritis were significantly lower than in the controls (25.1 ± 23.9 vs. 33.2 ± 28.6 ng/ml, P =0.04). Authors concluded that vitamin D deficiency in undifferentiated inflammatory arthritis may lead to activation of B cells and production of auto-antibodies like anti-cyclic citrullinated antibodies, antinuclear
antibodies and rheumatoid factor due to which the disease may ultimately progress to early rheumatoid arthritis. Also such deleterious effect of vitamin D deficiency might be due to increased bone resorption.[13] Although the low number of patients in our study might have been insufficient to obtain statistically significant values, finding in our study about non-association of vitamin D deficiency and rheumatoid arthritis is consistent with many previous studies.

In one study, large cohort of 186,389 women followed from 1980-2002 in the Nurses' Health Study and Nurses' Health Study II and information about vitamin D intake and medical history related to systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) was prospectively collected. The diagnosis of SLE and RA was confirmed by conducting medical record review of charts for the American College of Rheumatology (ACR) diagnostic criteria for SLE and RA. No strong evidence of association between vitamin D intake and the risks of SLE or RA was observed. However authors failed to explain the discrepancies of findings in this study with earlier studies.[14] Also no associations between reported dietary intake of vitamin D during adolescence and risk of RA or SLE in adulthood was reported from same cohorts.[15] In another study involving 32,435 women without the history of RA enrolled in the Women's Health Initiative Calcium plus Vitamin D trial were randomized to receive either placebo or 1000 mg of calcium carbonate plus 400 IU of vitamin D3, of which 163 incident RA cases were identified over an average period of 5.1 years. When compared with placebo, calcium plus vitamin D supplementation did not demonstrate a statistically significant effect on RA incidence, rather the study suggested that multiple high vitamin D exposures may increase the risk of developing RA.[16] In a study by Turhanoflu AD et al. involving sixty-five patients with RA with the objective to evaluate the relationship between serum vitamin D levels and disease activity and health status in rheumatoid arthritis, no difference in serum levels of vitamin D seen between patients with RA and healthy controls although lower levels were observed in patients having high disease activity. Authors asserted that because extremely limited amount of studies have been conducted involving measurement of serum levels of vitamin D in RA patients, deficiency of vitamin D cannot be attributed to the onset of RA, rather higher vitamin D levels might be observed in patient with RA than in healthy controls.[17] This might also explain higher levels of vitamin D in RA patients observed in our study.

The limitations of this study include small sample size for RA group which was not adequate to reach statistical significance and as all patients were on their routine treatment with disease modifying anti-rheumatic drugs, we did not assessed the disease activity scores using suitable disease activity score questionnaire (e.g. DAS-28) so association between serum 25-OHD and disease activity could be established. Additionally, this study only included women with RA, so the results cannot be extrapolated to men. On the other hand, the strengths of this study are that unlike many previously conducted studies, we included a healthy control group and also we actually measured serum levels of vitamin D using the most sophisticated technique available today rather than relying on dietary intake to determine levels of vitamin D.

Conclusion

We found no statistical difference between the serum levels of vitamin D of the rheumatoid arthritis and healthy controls. Due to the less sample size and less power of the present pilot study the convincing statement on association between Rheumatoid arthritis and vitamin D levels cannot be commented. Further carefully constructed clinical intervention studies with adequate power are required to address key questions pertaining to role of vitamin D in RA disease onset and activity.

References


