

Oxi-Inflammatory Stress and Biomolecular Deterioration: A Mysterious Convergence in Post COVID Patients

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Abstract

Background: Despite several scientific efforts against COVID 19, conundrum of biomolecular deterioration in Post COVID syndrome patients are still in dark at an unprecedented scale globally and affected the patient's health multidimensionally. It is conceivable that patients recovered from COVID-19 after second wave are at enhanced risk of secondary complications.

Aim: The present study was carried out to estimate the serum vitamin D and total antioxidant activity (TAC) along with markers of oxi-inflammatory stress in post COVID patients diagnosed RT-PCR negative after second wave of COVID-19 and to determine their role in predicting secondary complications.

Methodology: 50 subjects (30-55 years) of Delhi-NCR region were recruited and categorized into two groups (n=25 in each group; on the basis of their history of COVID infection). By using standard methods, study group parameters were estimated in Post COVID patients and statistically compared it with that of 25 non affected healthy controls by using student's t-test.

Result: Serum CRP, TNF- α , MDA and uric acid levels were significantly high (p<0.05) in Post COVID patients as compared to healthy controls. Conversely, serum vitamin D and TAC levels along with SOD activities were found to be significantly low (P<0.001) in Post COVID patients as compared healthy controls. However, ceruloplasmin level was altered insignificantly (p<0.1) with respect to Group I subjects. Vitamin D levels were positively correlated with TAC and SOD activity (P<0.001) and negatively correlated with MDA, CRP, TNF- α and uric acid levels in post COVID patients.

Conclusion: Therefore, the present study emphasizes the dire need of special attention to Post COVID population by providing vitamin D supplementation, antioxidant and mineral rich diet along with adoption of regular aerobic exercise not only to rejuvenate the biomolecular homeostasis but also to reduce oxi-inflammatory stress mediated future complications.

Keywords: TNF-alpha, Malondialdehyde, total antioxidant activity, inflammation, vitamin D.

INTRODUCTION

Since its origin in the year 2019, COVID-19 has proved to be one of the major health disasters known to humankind taking millions of lives and affecting billions of lives. Although the mortality rates have come down drastically, the Post COVID effects are still of great concern to the medical community. As soon as the virus gets inside the host, the immune system is activated to act in response in opposition to the foreign organism with the help of phagocytic cells and dendritic cells that uses reactive oxygen species and cytokines, that can create an inflammatory response in the host further aggravating the fight against COVID-19 infection. It has been seen that COVID 19 infection causes acute inflammatory response ensuing in the pro-inflammatory cytokines storm that becomes a reason of acute lung damage.^[1] In various studies, it has been shown that there is a strong link between presence of pro-inflammatory components and reactive oxygen species [ROS].^[2] The oxidative stress occurs not only due to depletion of endogenous enzymic and non-enzymic antioxidants reserves (superoxide dismutase, catalase, vitamin C etc.) but also due to an unfettered generation of ROS which in turn upsetting the redox circuits characterized by biomolecular damage such as lipid peroxidation, DNA damage etc.^[3] The domino effect of oxidative stress also continues to irregular cell signalling.^[4,5] Thus, OS negatively affects a range of processes such as inflammation, apoptosis, immune cell activation, cardiovascular remodeling, renal dysfunction, and excitation of the sympathetic nervous system.^[6]

Recently, apart from role of vitamin D in calcium homeostasis and bone metabolism, beneficial effects of vitamin D as an immune modulator in COVID 19 have received much attention. Moreover, vitamin D enhances neutrophil activity and reduces the deteriorative effect of pro-inflammatory cytokines.^[7,8] At a molecular level, vitamin D appears to reduce oxidative stress.^[9] Although contradictory evidences have been documented in relation to

vitamin D supplementation and COVID severity, the assessment of vitamin D level along with markers of oxi-inflammatory stress in post COVID era has yet not been carried out.^[10,11]

It is conceivable that there is a close link between altered levels of vitamin D and markers of oxi-inflammatory stress in increasing the frequency and complexity of post COVID patients. Therefore, the present work aimed to identify the alterations in the levels of vitamin D and its association with the markers of oxi-inflammatory stress in Post COVID patients and to determine their negative impact on health as a long term affect of COVID19 in post COVID era.

Material and Methods

50 healthy subjects of either sex belonged to age group 30-55 years and who were residents of Delhi-NCR region were included in the study. The study group subjects were divided into two groups on the basis of their history of COVID 19 infection. 25 healthy subjects (12 male and 13 female) who were not affected with COVID during COVID pandemic were included in Group I (Control group). In Group II, Post COVID 19 patients diagnosed RT-PCR negative after second wave of COVID and belonging to age 30 – 55 yrs of either sex (11 male and 14 female). A general information or pre-experimental questionnaire regarding demographic information, family history and limited physical examination including blood pressure measurement was completed from all the subjects after taking their informed consent and approval of protocol by ethics committee of college.

Inclusion criteria: Written informed consent was obtained from all the subjects included in the study. Subjects who don't under any medical treatment or taking antioxidant supplement for at least 1 month prior to blood collection were included. Height was measured by using wall mounted scale whereas weight was measured with subject barefoot and lightly dressed by using digital weighing machine. The Body Mass Index (BMI) was calculated as $[BMI = \text{Weight (kg)} / \text{Height (metre}^2\text{)}]$. Blood pressure was measured by mercury sphygmomanometer using auscultatory method. To diminish any confounders developed by other health complications, post COVID 19 patients diagnosed RT-PCR negative after second wave of COVID were recruited. In addition, post COVID subjects who had previously under any medical treatment including supplementation of antioxidants were not excluded from the study if the subject agreed that no supplements would be taken in the seven days before entry into the study.

Exclusion criteria: None of the subjects had family history of concomitant diseases, such as diabetes mellitus, hepatitis, renal failure and neurological disorder. In addition, patients with established cardiovascular complications, pregnancy, lactation, obesity (BMI > 30), Stage I and stage II hypertension (BP >129/89 mmHg), smoking habit, renal failure, liver disease, hypothyroidism or who did not follow study instructions were also excluded from the study.

Fasting blood samples were collected in plain vial from antecubital veins avoiding venostasis from each patients and healthy controls. Blood samples destined for measurement of study group parameters were centrifuged at 3500 rpm for 10 min within 1 h of collection and serum was stored at -80°C until analysis. The serum concentrations of TNF- α and CRP were measured with commercially available enzyme-linked immunosorbant assay (ELISA) kits (R&D Systems, USA) according to the manufacturer's instructions.

Serum MDA levels were estimated by thiobarbituric acid (TBA) reaction.^[12] Serum lipid peroxide was measured by precipitating lipoproteins with trichloroacetic acid (pH 2-3) and boiled with thiobarbituric acid which reacts with Malondialdehyde, forming a MDA-TBA to get pink color. The pink colored complex that occurred was refrigerated to room temperature and measured by using a spectrophotometer at 530 nm.

Estimation of serum 25 OH vitamin D level was done in VITROS EciQ immunodiagnostic system by chemiluminescence method which involve the release of the 25 (OH) vitamin D in the sample from the binding protein using a low pH denaturant and the subsequent competition of the free 25 (OH) vitamin D with horse radish peroxidase (HRP) labeled 25 (OH) vitamin D reagent for monoclonal anti-Vitamin D bound to the wells.^[13] Unbound materials were removed by washing. The bound HRP conjugate was measured by a luminescent reaction. A reagent containing luminogenic substrates (a luminal derivative and a peracid salt) and an electron transfer agent was added to the wells. The oxidation of luminol derivative was catalyzed by HRP bound conjugate and the produced light signals were read by the system. The amount of HRP conjugate bound was indirectly proportional to the 25 (OH) vitamin D concentration present in the sample.

Serum total antioxidant activity was estimated spectrophotometrically by the method involving reaction of standardized solution of iron EDTA complex with hydrogen peroxide, i.e. Fenton type reaction, leading to the formation of hydroxyl radicals. This reactive oxygen species degrades benzoate, resulting in the release of TBARS. Antioxidants from the added plasma cause the suppression of production of TBARS. The reaction was measured spectrophotometrically at 532 nm.^[14]

Serum ceruloplasmin levels were estimated by Ravins's method (1961).^[15] Ceruloplasmin due to its oxidase activity, catalyses the oxidation of substrate p- phenylenediamine chloride into purple coloured oxidation product, measured spectrophotometrically at 530 nm. Serum uric acid levels were estimated by Caraway's method²¹ in which uric acid react with phosphotungstic acid in alkaline medium forming a blue color complex which is

measured at 700 nm.^[16] Erythrocyte SOD activity was measured by Marklund and Marklund's method (1974).^[17] The enzyme SOD inhibits the auto-oxidation of pyrogallol by catalyzing the breakdown of superoxide. The inhibition of pyrogallol oxidation by SOD is monitored at 420 nm and the amount of enzyme producing 50 % inhibition is defined as one unit of enzyme activity.

Statistical analysis: After estimating study group parameters, data were entered manually in Microsoft Excel sheet of windows 2007 and result were processed using online Graphpad software. Values were expressed as Mean \pm SD and Student's t test was used to compare the significance of mean difference between study group subjects. Pearson co-relation coefficient was used to determine the relationship among the markers P value <0.05 and <0.001 were considered as significant and highly significant respectively.

Result

Table 1 represented the demographic indices and clinical profile of the study group subjects, which include mean age, Body mass index (BMI) and blood pressure. BMI measurement and blood pressure were elevated insignificantly (P<0.1) in Group II as compared to Group I subjects. There was a significant decrease (p < 0.05; 25.1% low) in the level of Vitamin D in Group II as compared to Group I subjects, as depicted in Figure 1. Marked occurrence of systemic inflammation was noticed by the increase in the levels of CRP and TNF- α in the Group II patients (p < 0.05; 35.42% and 48.42% high respectively) as compared to Group I subjects. (Figure 2). Although the increase in the levels of serum Ceruloplasmin were noticed, the change was not significant (p < 0.1; 17.43% high). Serum MDA and Uric acid were significantly increased in Group II as compared to Group I (p < 0.05; 37.5% and 28.72% high respectively) as documented in Table 2. On the other hand, serum TAC levels (p < 0.05; 27.69% low) and erythrocyte SOD activity were markedly decreased (p < 0.05; 11.68% low) as represented in Table 2. The overall alteration in the levels of oxi-inflammatory stress markers, as characterized by marked elevation in systemic inflammation and MDA along with decrease in the antioxidant reserves, reflect the diagnostic importance of these markers in post COVID patients.

Interestingly, correlation studies revealed that the level of Vitamin D was inversely proportional to the level of lipid peroxidation i.e. MDA and Uric acid; and directly proportional with the levels of TAC and SOD activity (p<0.05), as presented in Table 3. Vitamin D was also found to be inversely correlated with the increase in inflammatory markers (p<0.05) which indicates the association of Vitamin D with oxi-inflammatory stress and elevated biomolecular deterioration in Post COVID patients.

Table 1: Demographic and clinical profile of study group subjects (Mean \pm SD)

S No	Particulars	Group I (n=25)	Group II (n=25)
1)	Age (years)	40 \pm 6.2	42 \pm 6.0
2)	M:F ratio	13:12	11:14
3)	Height (meter)	1.59 \pm 0.030	1.60 \pm 0.032
4)	Weight (Kg)	58.2 \pm 2.1	65.8 \pm 2.7
5)	BMI (Kg/m ²)	24.7 \pm 1.3	25.8 \pm 1.2*
6)	Systolic blood pressure (mmHg)	110.8 \pm 3.42	114.5 \pm 3.40
7)	Diastolic blood pressure (mmHg)	76.5 \pm 2.42	78.1 \pm 2.50

where,

* p<0.1 : Non-significant; ** p<0.05 : Significant;

Table 2: Biomarkers of Oxidative Stress in study group subjects (Mean \pm SD)

Sl. No	Parameter	Group 1	Group 2
1	TAC (mmol/l)	1.30 \pm 0.28	0.94 \pm 0.20**
2	Uric Acid (mg/dl)	4.70 \pm 1.18	6.05 \pm 1.45**
3	SOD (U/gmHb)	1562.80 \pm 80.4	1380.2 \pm 110.6**

4	Ceruloplasmin (mg%)	26.84 ± 1.72	31.52 ± 1.77*
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where,

* p<0.1 : Non-significant; ** p<0.05 : Significant;

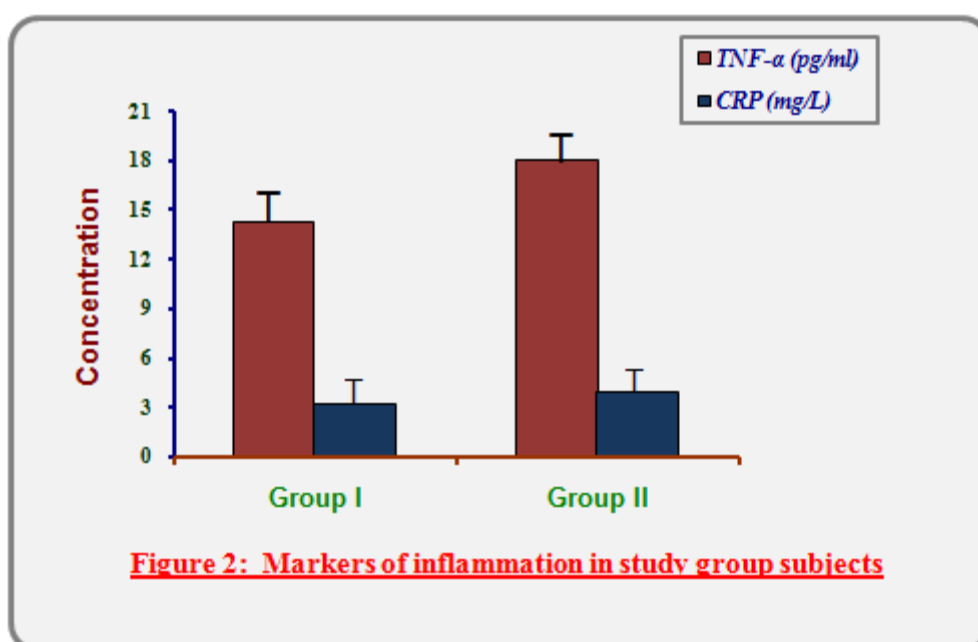
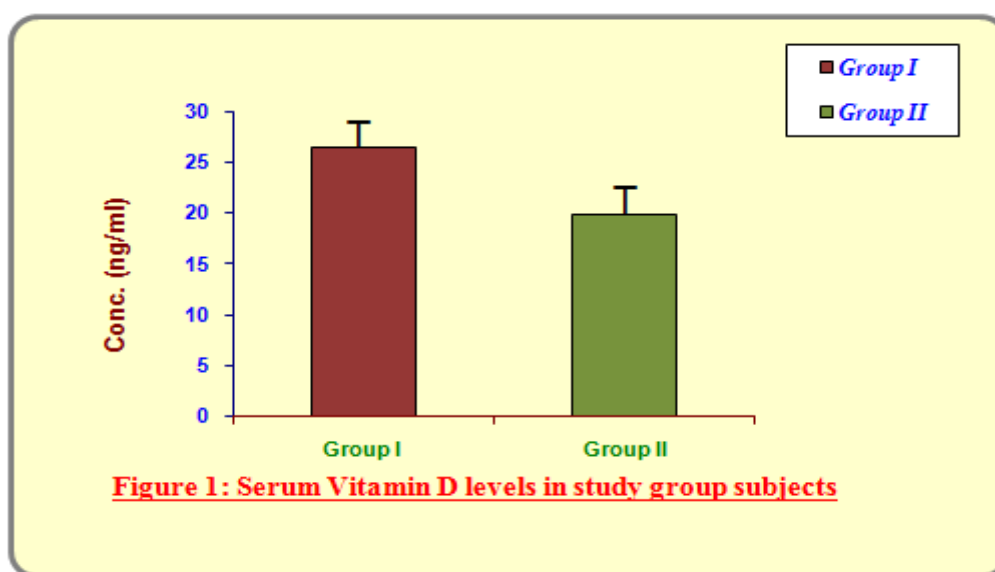
Table 3. Correlation coefficient (r) between vitamin D level and marker of oxi-inflammatory stress in Post COVID patients.

Particulars	CRP	TNF- α	MDA	TAC	SOD	Cerulo- plasmin	Uric acid
Vitamin D	-0.607 **	-0.618 **	-0.563**	0.612 **	0.584 **	-0.218 *	-0.360**

Where,

* p < 0.05 : Significant,

** p < 0.001 : Highly significant



Discussion

Since last couple of years, COVID 19 pandemic has affected human health in multiple ways. It has been documented that COVID 19 exert deteriorative effect on human health and responsible for decreased potential and physical activity of patients even after confirmation of RT-PCR report negative.^[18,19,20] Moreover, persistence of oxi-inflammatory stress in post COVID era is characterized by excessive production of pro-inflammatory cytokines, biomolecular deterioration and alteration in antioxidant reserve status which in turn increases the susceptibility to develop variety of health related complications in the coming years.^[21,22]

Amongst various factors, alteration in vitamin D level in post COVID subjects has now been receiving much attention towards solving the unanswered question related to the development of future risk of health related complications and, thus, can help to prevent and reduce the economic burden in post COVID subjects. Vitamin D has been found to be effective in maintaining calcium homeostasis, in improving immunity, endothelial dysfunction and nitric oxide availability, and reducing atherosclerotic parameters. Vitamin D modulates contraction, inflammation and remodeling tissue. Deficiency of vitamin D have also been found to be associated with age related diseases, hypertension and in pregnancy related complications.^[7,8,23] However, the relationship between altered vitamin D status and effect of COVID 19 in post COVID population has yet not been fully elucidated.

Post COVID group subjects revealed a significant depletion in vitamin D levels ($p < 0.05$; Figure 1) as compared to healthy control which could be explained as a long term impact of COVID19 on healthy population. It has been implicated to the least exposure to sunlight or limited time outdoors due to home quarantine, latitude, season or lack of dietary sources also. In addition, we also observed negative correlation of vitamin D deficiency with markers of inflammation and lipid peroxidation; and positively correlation with antioxidant reserve system (Table 3) in post COVID subjects which reflects its protective role against oxi-inflammatory stress and depletion in vitamin D may ingress the risk of disease development. Marked depletion in vitamin D status has been documented in COVID patients and in various pathological conditions such as diabetes and cardiovascular complications as well.^[8,13]

Interestingly, progressive enhancement of pro-inflammatory cytokines (IL-6, TNF- α), facilitates the recruitment and attachment of circulating leukocytes to the vessel wall and thereby plays a key role in cardiovascular complications. Moreover, angiotensin-converting enzyme 2 (ACE2), the receptor for SARS-CoV-2, is expressed by endothelial cells. The interaction of SARS-CoV-2 and ACE2, leads to endothelial activation which may result in loss of vascular integrity; expression of leukocyte adhesion molecules; change in phenotype from antithrombotic to prothrombotic; cytokine production; platelet activation, thrombosis and inflammation.^[24,25]

In the present study, serum CRP and TNF- α levels were increased significantly ($p < 0.05$; Figure 2) in Post COVID patients which reflect the persistence of systemic inflammation even after second wave, and thus, making the post COVID patients more susceptible to develop the disease early in later stages of life. Recently, role of pro-inflammatory cytokines in the activation of dysfunctional endothelial cells which contribute to the pathogenesis of thrombosis by altering the expression of pro- and antithrombotic factors has been documented.^[22,26]

Apart from inflammation, oxidative stress due to uncontrolled ROS production plays a crucial role in increasing the chances to develop health issues in post COVID population. Increased ROS production can ingress atherosclerotic event not only by endothelial activation and dysfunction but also by inducing cell membrane damage via lipid peroxidation in post COVID population.^[27] In the present study, serum malondialdehyde levels (marker of lipid peroxidation) were also found to be significantly high in Group II subjects ($p < 0.05$, Table 2) and positively correlated pro-inflammatory cytokines levels. These findings indicate that excessive ROS generation takes place in post COVID subjects which clarify the role of oxi-inflammatory stress mediated biomolecular deterioration and altered redox signalling in shaping the post COVID subjects more susceptible to develop health issues in future. Recently, increased levels of MDA were also reported in post COVID active rheumatoid arthritis patients.^[22,28] Moreover, Saxena et al reported that lipid peroxidation mediated electrolyte imbalance and production of protein radical in lipid membranes affects the normal ion transport, and thereby enhances the risk of cardiovascular complication.^[29]

In the present study, plasma TAC levels were decreased significantly in Group II as compared to Group I along with altered SOD, ceruloplasmin and uric acid levels (Table 2), which explain the combined effect of reduced antioxidant defense system and altered antioxidant enzyme activity due to amplified oxidative stress. Similarly, marked reduction in antioxidant defense system in post COVID as well as in subjects with other inflammatory diseases have been well documented in earlier studies.^[22,30] In addition, diminution of antioxidant defense system leads to enhanced lipid peroxidation which is well characterized by attack of ROS on lipid bilayer of intracellular organelles (lysosomes) and epithelial cells and thereby lead to various health issues such as cardiovascular complications, renal diseases and cancer etc.^[30,31,32] In the present study, MDA levels were found to be significantly high ($p < 0.001$) in post COVID patients with respect to healthy controls which authenticate the fact that lipid peroxidation plays a key important role in the degenerative health issues. Bindal et al and Sabitha et al,

in separate studies also showed that enhanced production of MDA reacts with nucleic acid and contribute significantly in mutagenesis and carcinogenesis.^[30,33]

Conclusion

The present study reveals that depletion in vitamin D levels along with elevated levels of oxi-inflammatory stress, has a role in altered cellular homeostasis in the subjects suffered from COVID-19 in pandemic. Thus, regular monitoring of vitamin D level may be an effective “treat to target” approach from a lens of therapeutic intervention strategy in regulating deteriorative events and its associated complications. Moreover, regular assessment of markers of oxi-inflammatory stress such as erythrocyte SOD activity, TAC and MDA along with TNF- α are additional approach to provide clear clinical picture with advancing of age in post COVID subjects. Furthermore, our study concludes that COVID19 deteriorates the human health and assessment of vitamin D levels and oxi-inflammatory markers incorporation with conventional health checkup parameters can be included to the battery of routine analysis in order to prevent the development of disease in early stage in the era Post COVID 19.

Limitation: The main drawback of this study was the sample size, which was less and it is recommended to perform a multicenter study in future, with a larger population. Long term follow up and inclusion of more investigations even at molecular basis also needed to get accurate result.

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Conflict of interest: There is no conflict of interests. All authors are equally contributed.

Ethical approval: Approved

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