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Periodontal Disease Markers among Patients with Long COVID: A case-control study

ABSTRACT

Background: Long COVID affects around 32-87% of COVID-19 patients by causing persevering symptoms surpassing 4 weeks following the initial infection. Long COVID expresses a connection with a notable cytokine triad, namely IL-1β, IL-6, and TNF-α. Periodontal disease also involves proinflammatory cytokines production including IL-1β, IL-6, and TNF-α. Consequently, long COVID, which exerts an influence on proinflammatory cytokine release, could affect periodontal status.

Objective: This study aims to see whether long COVID affects periodontal status severity, based on proinflammatory cytokines levels involved in both diseases, namely IL-1β, IL-6, and TNF-α.

Methods: Patients were divided into periodontitis or gingivitis patients and then were further divided into two groups, previous COVID-19 patients and non-COVID-19 patients (controls). Gingival sulcus fluids were obtained from each patient using paper points inserted in patients' sulcus, and ELISA tests were carried out to measure IL-1β, IL-6, and TNF-α levels.

Results: Levene Test indicated that there were no substantial differences between IL-1β, IL-6, and TNF-α levels (0.057, 0.135, and 0.341 respectively) in COVID-19 patients with gingivitis in comparison to control group with gingivitis, with average IL-1β, IL-6 and TNF-α levels seen higher in control group compared to COVID-19 patients. There were also no substantial differences between IL-1β, IL-6, and TNF-α levels (1.00, 0.567, and 0.666 respectively) between COVID-19 patients with periodontitis and control group with periodontitis. Although higher levels of IL-6 and TNF-α were found higher in COVID-19 patients in comparison to control group.

Conclusion: Levels of IL-6 and TNF-α in periodontitis patients with long COVID were found higher in comparison to controls. But in spite of that, higher IL-1β, IL-6, and TNF-α levels were not found in long COVID subjects with gingivitis, as well as IL-β levels in the periodontitis group. Further studies with an increased number of subjects are needed to further determine the connection between these two diseases.

Keywords: Long COVID, IL-1β, IL-6, TNF-αLong COVID, IL-1β, IL-6, TNF-α

INTRODUCTION

Coronavirus disease 19 (COVID-19), which has had detrimental consequences over more than the preceding two years, was prompted by Severe Acute Respiratory Distress Syndrome Coronavirus-2 (SARS-CoV-2), causing the demise of more than 5 million people. Approximately 20% of those who were infected by SARS-CoV-2 would require intensive care unit (ICU) admission, where subsequently approximately 25% of these patients were deceased, mostly as a consequence of severe inflammation and thromboembolic complications.[1] COVID-19 also has an immense impact towards dentistry worldwide, including dentists, dental patients, and also dental practice. Dentists are in risk and prone to COVID-19 infection, due to exposure to pathogen, long working hours, psychological stress, and fatigue.[2] Moreover, COVID-19 has given rise to psychological and mental health problems, both for COVID-19 patients and non-COVID patients undergoing self-isolation, social distancing or quarantine; giving rise to problems such as loneliness, depression, and isolation.[3]

Periodontal disease is a multifactorial infectious disease, with plaque accumulation as the main cause, predisposed by a lot of factors, such as poor oral hygiene, age, sex, diabetes, HIV, menopause, pregnancy, smoking, along with others. Psychological stress affects periodontium by the way of neuro-immune endocrinological mechanisms. Psychological stress affects hypothalamus, which alters the adrenal, which then produces several substances (catecholamine, chromogranin A, neuropeptides), resulting in a decreased immune response. Stress also affects the autonomic nervous system, where the adrenal glands releases prostaglandins and other proteases, subsequently affecting the periodontium. These two factors both contribute to periodontal disease and poor periodontal treatment effectivity. [4] Patients with COVID-19 also commonly have systemic manifestations, giving rise complications affecting the respiratory, endocrine, metabolic, cardiovascular, hematological, vascular, gastrointestinal, neurological, renal, musculoskeletal and hepatic system. [5]

Initially, SARS-CoV-2 gains entry by the binding of ACE2 protein to alveolar epithelial cells which subsequently act upon COVID-19 progress by initiating the production of chemokines, interferons, interleukins (IL), and tumor necrosis factor α (TNF-α), which then provoke the innate and adaptive immune system.[6] Evidence found of late shows that a range of manifestations can

still persevere after the resolution of acute infection, which is referred to as long COVID, that exerts an influence on multiple organs.[7] National Health Institute for Health and Care Excellence defines this condition as manifestations that continue or develop after acute COVID-19 infection and cannot be justified by an alternative diagnosis, which entails ongoing symptomatic COVID-19 during 4-12 weeks following initial infection, and post-COvid-19 syndrome surpassing 12 weeks following the initial infection. The National Institutes of Health (NIH) also defines long COVID as a sequelae that persists surpassing 4 weeks following the initial infection.[8] Long COVID is clinically characterized by the presence of fatigue, exercise intolerance, brain fog, shortness of breath, joint pain, fever, sleep, anxiety disorders, gastrointestinal symptoms, and palpitations that could persist for months. Around 21.7-87% COVID-19 patients undergo long COVID. [9]

The immune response is a compelling factor that determines pathogen and host interaction, in which if this process is dysregulated, it would grow into a 'cytokine storm,' causing rapid production of cytokines that are in control of immunopathological reactions. Altered serum levels of cytokines such as IFN-α, IFN-γ, IL-1β, IL-6, IL-12, IL-18, IL-33, TNF-α and TGF-β are found correlated to COVID-19.[10] In spite of the fact that a lot of other risk factors (age, comorbidities) could affect COVID-19 severity, one study found a connection between long COVID with the elevation of notable cytokine triads, IL-1β, IL-6, and TNF-α, in which three are all involved in pain perception, anxiety, depression, and inflammation[11].

On the other hand, periodontal disease has a high prevalence of 50% in the adult population all over the world. The periodontal tissues, even in healthy circumstances, has immune cells continuously present in the gingiva, maintaining balance between the host and the oral biofilm and keeping the immune response active.[12] Periodontal disease is a multifactorial infectious disease that is mediated by immune responses. Higher cytokine levels in periodontal tissue and serum were found in patients with periodontal disease, which is similar to the cytokine storm occurring in COVID-19 patients.[13]

Periodontal infection is also associated with systemic diseases, including bacteremia and systemic release of inflammatory mediators. Cytokines are produced by fibroblasts, epithelial cells, neutrophils, and macrophages in the acute phase of periodontal disease; and produced by lymphocytes in advanced periodontal diseases. During an inflammatory response, the first stage of periodontal disease is characterized by the secretion of proinflammatory cytokines such as IL-

1, IL-6, and TNF-α in opposition to periodontal bacteria. [14] This disease is initiated by an increase in blood flow, amplified vascular permeability, and the ingress of neutrophils, monocyte macrophages, with ensuing T cells and B cells appearing, producing cytokines (e.g. IL-1β, IL-6, and TNF-α), degrading epithelial cell tissues, and consequently creating bone resorption.[15]

In this regard, it is possible that long COVID, which exerts an influence on proinflammatory cytokine release, could affect periodontal status. This is based on the fact that periodontal disease could occur on account of increased host inflammatory responses as a COVID-19 consequent.[16] This study aims to see whether long COVID affects proinflammatory cytokines, namely IL-1β, IL-6 and TNF-α in periodontal disease. Therefore, in this study, we aim to compare inflammatory mediator levels of long COVID patients (patients who have experienced COVID-19 previously) with inflammatory periodontal diseases, compared to non-COVID 19 patients (controls).

MATERIALS AND METHODS

The research done was a pilot study, in which we divided the subjects into two groups, namely the gingivitis and the periodontitis group. In the gingivitis group, a sum of twenty samples were used, involving 10 COVID-19 patients and 10 controls. A sum of four samples in the gingivitis group were subtracted due to low levels of protein exhibited after Bradford protein assay test was done. Therefore, these four samples were unable to be continued with ELISA test.

Whereas in the periodontitis group, 20 samples were used, including 10 COVID-19 patients and 10 controls. The results of this clinical study suggested that there was a correlation between periodontal disease and long COVID, delineated by altered levels of proinflammatory cytokines. This hypothesis was substantiated by higher levels of IL-6 and TNF-α that were seen higher in previous COVID-19 patients with periodontitis in this study.

Obtaining Samples

Samples were obtained in dental hospital of Trisakti dental faculty and research was done in biomedical laboratory of Trisakti. Subjects were determined based on inclusion criteria (gingivitis patients with a probing depth of ≤3 mm and bleeding on probing / BOP score ≥10%; periodontitis patients with clinical attachment loss / CAL on ≥2 non-adjacent teeth or ≥3 mm on

buccal/oral surfaces on ≥2 teeth; patients with a minimum amount of 20 teeth; COVID-19 patients who were tested positive with PCR test and experiencing symptoms 4 weeks after the initial symptoms appeared, long COVID-19 patients discerned by online questionnaire regarding long COVID symptoms that they might experience) and exclusion criteria (active smokers, type II diabetes mellitus patients, pregnant and breastfeeding patients, patients who has undergone periodontal treatment during the pandemic, aggressive periodontitis patients).

Throughout the course of the study, there have not been a study done on periodontal biomarkers observed on previous COVID-19 patients / patients with a COVID-19 history. Hence, this pilot study was commenced.

Informed consents were then obtained from all subjects after an explanation regarding the research was given, in conjunction with periodontal status checking (probing depth, Oral Hygiene Index-simplified/OHI-s, and CAL) to discern gingivitis and periodontitis patients. Subjects were then divided into two groups: previous COVID-19 patients and non-COVID-19 patients (controls), distinguished by a history of COVID-19 (tested positive by PCR test).

Gingival sulcus fluids were obtained to determine IL-1β, IL-6, and TNF-α levels. One tablet of Phosphate-buffered saline (PBS) was dissolved in 100 ml, then 200µl of PBS solution was transferred into an Eppendorf tube, which was then inserted in a cooler box containing ice cubes. Subjects were given cheek retractors, and the area was isolated using cotton rolls. Plaques were then removed and tooth surfaces were dried. Consequently, samples were collected using paper points on every tooth exhibiting positive BOP. Paper points were inserted in the sulcus to a depth of 1 mm for 30 seconds. Samples with saliva and blood contamination were excluded. The collected gingival sulcus fluids were inserted in Eppendorf tubes containing 200µl of PBS solution and stored at -80^{0}C degrees.

Samples Preparation

Samples stored at -80^{0}C degrees were prepared, by thawing them at room temperature and then vortexed to obtain even concentrations. Tubes were centrifuged at 2000 g, 4°C for 5 minutes. Concentration of extract obtained from centrifugation were then measured with 9 standards at different concentrations, namely, 2000 µg/ml, 1500 µg/ml, 1000 µg/ml, 750 µg/ml, 500 µg/ml, 250µg/ml, 125µg/ml, 25µg/ml, and 0 using Bradford method. Calculations were done for the

samples with the highest total protein concentrations, and consequently protein concentrations are equated into 250µg/ml by adding PBS to gather stock volume samples to the tune of 250µl.

ELISA test

IL-1β, IL-6, and TNF-α levels were measured using ELISA test. Samples were obtained and thawed at room temperature. Twenty ml of wash buffer concentrate was diluted 25 times with deionized or distilled water to yield 500ml of 1x Wash Buffer. Reagents, standard solutions, and samples are also brought to room temperature. Strips used in the research were inserted in frames (unused frames were stored at 2-8°C). As much as 50µl standard was added to the standard wells. In sample wells, 40µl samples are added. In addition, in sample wells, according to the cytokine being tested, add 10µl of anti-IL-1β antibody for IL-1β test, 10µl anti-IL-6 antibody for IL-6 test, or 10µl anti-TNF-α antibody for TNF-α test.

An amount of 50µl streptavidin-HRP were then added to the sample and standard wells, mixed, and covered with a sealer, and incubated for 60 minutes at 37^0C. Afterwards, the sealers were removed and the plates were washed 5 times with a wash buffer (soaking the wells with at least 0,35 ml wash buffer for as long as 30 seconds to 1 minute for each wash), and blotted with paper towels. Each well was then added 50µl each of substrate solution A and solution B, covered with new sealers, and incubated for 10 minutes at 37^0C in the dark. Consequently, 50µl of stop solution is added to each well, which will change the blue color into yellow color. Within ten minutes following the addition of a stop solution, the optical density value for each well is then read by a microplate reader set to 450 nm. Data analysis is then done.

RESULTS

In this study, there was a sum of 40 patients who were divided into two groups (20 COVID-19 patients and 20 controls), in which data collection was carried out randomly according to the predetermined inclusion and exclusion criteria. The 20 subjects in the control group consisted of 50% (10 subjects) of gingivitis where 50% (5 subjects) were aged between 21-30 years, 20% (2 subjects) were aged 31-40 years and 30% (3 subjects) were aged 41-50 years. The periodontitis subjects in the control group (10 subjects) consisted of 30% (3 subjects) aged 21-30 years, 20% (2 subjects) aged 31-40 years, 40% (4 subjects) aged 41-50 years, and 10% (1 subject) aged 51-60 years.

In the previous COVID-19 patient group with a sum of 20 subjects, half of the subjects (10 people) experiencing gingivitis consisted of 90% (9 subjects) aged 21-30 years, and 10% (1 subject) aged 51-60 years. The other half of previous COVID-19 patient group (10 people) experiencing periodontitis consisted of 50% (5 subjects) aged 21-30 years, 20% (2 subjects) aged 31-40 years, 10% (1 subject) aged 41-50 years, and 10% (1 subject) aged 61-70 years. Comorbidities and of each subject were not recorded.

Based on long COVID-19 symptoms experienced by 20 COVID-19 patients, it was found that 45% (9 subjects) were patients with chronic COVID-19 because they had symptoms that persisted for more than 12 weeks after being declared negative for infection with the SARS-CoV-2 virus, while 55% (11 subjects) were post-acute COVID. Fatigue was the symptom with the highest percentage, namely 35% (7 subjects), 30% (6 subjects) experienced cough, 20% (4 subjects) experienced headaches, and 15% (5 subjects) experienced other symptoms such as sore throat, difficulty concentrating, flu, nausea, to shortness of breath.

OHI-s scores of each patient were tested statistically and were not significantly different. In the gingivitis group, 16 samples were used and were tested for normality using the Shapiro-Wilk test. Several samples in the gingivitis group were removed due to contamination, thus the protein levels were too low to be analyzed by ELISA test. Normality tests indicated that the data were distributed normally. The statistical test used for the gingivitis group was an independent T-test (Table 1). The periodontitis samples were used without any subtraction in the sample amount and tested for normality using Shapiro-Wilk test, which showed that the data were distributed normally. Statistical test used for the periodontitis group was also an independent T-test (Table 1).

Table 1. Independent Sample tested for gingivitis and periodontitis

		Mean (ng/L)	Std deviation	Sig
IL-1β	Control group with gingivitis	447.8143	47.38173	0.057
	COVID-19 patients with gingivitis	390.1222	60.49859	0.057

	Control group with periodontitis	182.8500	83.86997	1.000
	COVID-19 patients with periodontitis	182.8400	47.07463	1.000
IL-6	Control group with gingivitis	88.0857	12.04138	0.135
	COVID-19 patients with gingivitis	76.0111	17.02487	0.135
	Control group with periodontitis	33.1700	18.91243	0.567
	COVID-19 patients with periodontitis	37.2300	11.22884	0.567
TNF-α	Control group with gingivitis	48.9286	6.41293	0.341
	COVID-19 patients with gingivitis	43.7778	12.54370	0.341
	Control group with periodontitis	11.8600	7.15871	0.666
	COVID-19 patients with periodontitis	12.9600	3.27930	0.666

The Levene Test indicated that IL-1β, IL-6, and TNF-α significance levels in the gingivitis group were 0.057, 0.135, and 0.341, respectively, which implies that there were no substantial differences between IL-1β, IL-6 and TNF-α levels among COVID-19 patients with gingivitis and

controls with gingivitis. The average IL-1β, IL-6, and TNF-α levels were also seen higher in controls compared to COVID-19 patients.

The significance levels for IL-1β, IL-6, and TNF-α in the periodontitis group were 1.00, 0.567, and 0.666, respectively, which also implies that there were no substantial differences between IL-1β, IL-6 and TNF-α levels among COVID-19 patients with periodontitis and controls with periodontitis. However, higher levels of IL-6 and TNF-α were seen elevated in COVID-19 patients in comparison to controls.

DISCUSSION

In addition to systemic manifestations, COVID-19 is also correlated with local oral cavity signs, most commonly dysgeusia, taste loss, and mucosal lesions. Other periodontal tissues involving clinical manifestations still have an undecided relation to COVID-19.[17] These oral cavity manifestations, their plausible relationship with oral disease, and the probable mechanisms of hyperinflammation underlying these two diseases signify an association between COVID-19 and oral disease.[18] Long-term inflammation that happens in patients with COVID-19 may give rise to a pathological response in periodontal tissues, precipitating fibrinogen degradation, coagulation cascade, and altering bacterial flora which could prompt or aggravate periodontal disease.[19]

Amplified systemic inflammation could result in periodontal disease, periodontal disease is a multifactorial infectious disease mediated by immune responses. Increased levels of cytokines in periodontal tissues and serum are found in periodontal disease patients juxtaposed to healthy controls.[20] This assertion is further supported by one case-control study by Anand et al., which ascertained that COVID-19 patients had higher average values of plaque scores, amount of mobile teeth, gingival bleeding score, probing depth, recession, and clinical attachment loss as opposed to controls, denoting a significant association between COVID-19 and periodontal disease.[21] In one review study, Kanchana Sukumar stated that the levels of serum proinflammatory cytokine levels in COVID-19 patients were also elevated, particularly IFN-c, IFNc-induced protein 10, IL-1b, IL-6, IL-12, and monocyte chemoattractant protein (MCP-1).[22] In two other meta-analyses studies, higher levels of IL-6 are detected in complicated COVID-19 cases compared with those with non-severe conditions.[23]

There are a lot of undetermined matters associated with long COVID, such as its etiology, prevention, and also treatment. It is known that older age, obesity, gender, hypertension, immunosuppression, asthma, and severe acute-phase disease are all associated with increased long COVID risk. Psychological stress is also linked to respiratory tract infection severity and duration. Stress may activate the hypothalamic-pituitary-adrenal axis, consequently dysregulate the immune system. Thus, psychological stress may be a risk factor for long COVID.[24]

Long COVID is denoted by persistent immune activation, comprising heightened levels of IL-1β, IL-6, and TNF-α, which reinforces the two hypotheses of the immune pathogenesis of long COVID, which are the unceasing immune response against unceasing viral antigens, and/or chronic immune cells reprogramming. A cohort by Schultheiß, et al. has proven these two hypotheses, substantiating the notion that long COVID is associated with heightened plasma levels of IL-1β, IL-6 and TNF-α.[11] It is then presumed that long COVID, which inflicts systemic inflammation by increasing cytokines related to periodontal diseases (e.g. IL-1β, IL-6, and TNF-α), would exert an influence on periodontal disease severity. Hence, this study is done to discern this presumption.

Interleukin-1β is a significant pro-inflammatory cytokine, which plays a part in infection and injury. They contribute in host reaction towards pathogens, and also aggravate damage in chronic diseases and tissue injuries.[25] The other two proinflammatory cytokines, IL-6 and TNF-α, both promote leukocyte recruitment, activation, and differentiation, along with B-cell maturation and T-helper cell subset expansion. Both of these cytokines are associated as an essential part of the acute COVID-19 immune response, although for the most part, IL-6 is more predictive for poor COVID-19 outcomes (e.g. respiratory failure, need of mechanical ventilation).[26]

In periodontal disease pathogenesis, IL-6 family is identified as a principal factor, in which several studies stated that SARS-Cov-2 infection in particular induces IL-6 production up to the extent of 1000-fold above standard range in several cases. Elevated IL-6 levels can precipitate adverse clinical outcomes and are associated with cytokine storm pathogenesis associated with both periodontitis as well as COVID-19, further denoting the plausible association between the two diseases.[23],[27] Interleukin-6 also play a part in innate and acquired immunity by the means of initiating the differentiation of B lymphocytes into antibody-producing cells. IL-8, on the other hand, acts as neutrophil chemoattractant.[28] Additionally, TNF-α is acknowledged to induce

tissue inflammation and endothelial activation. In uncontrolled circumstances, TNF-α could induce various inflammatory diseases involving multiple organ systems.[29]

But despite this finding, this study showed that the levels of proinflammatory cytokines were not seen higher in COVID-19 patients in comparison to controls in the gingivitis group. Interleukin-1β levels were not found higher either in the periodontitis group. This study also disclosed that there were no substantial differences between IL-1β, IL-6, and TNF-α between COVID-19 patients and controls in both gingivitis and periodontitis groups. Several samples in the gingivitis group were subtracted due to low levels of protein detected. This subtraction in the gingivitis group does provide a limitation in the study results.

CONCLUSION

Observations found in this study put forward a plausible connection linking periodontal disease and long COVID. Hypothetically, long COVID could exert an influence on periodontal disease, escalating systemic inflammation by increasing certain cytokines such as IL-1β, IL-6, and TNF-α, in which there are also correlated to periodontal diseases. In this study, we found heightened levels of IL-6 and TNF-α in periodontitis patients with long COVID compared to controls. However, higher IL-1β, IL-6, and TNF-α levels were not found in long COVID subjects with gingivitis, as well as IL-β levels in the periodontitis group. These could be on account of a small number of subjects or subtraction of some samples. Further clinical study with a considerable number of subjects is needed to further assess the relationship between these two diseases. Moreover, the discovery of long COVID impact on periodontal diseases clinically could raise awareness to clinicians to be more mindful of COVID-19 long-term impact on the dentistry field, particularly on periodontal health.

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