Salivary Cortisol as a Stress Biomarker and Total Viable Count of Salivary Bacterial Microbiome among COVID-19 Patients

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ABSTRACT

Background: The COVID-19 virus outbreak had a massive effect on many parts of people's lives, as they were advised to quarantine and lockdown to prevent the virus from spreading, which had a big impact on people's mental health, anxiety, and stress. Many internal and external factors lead to stress. This negatively influences the body's homeostasis. As a result, stress may affect the body's capacity to use energy to defend against pathogens. Many recent investigations have found substantial links between human mental stress and the production of hormones, prohormones, and/or immunological chemicals. some of these researches have verified the link between stress and salivary cortisol levels. The aim of this study is to measure salivary cortisol as a stress biomarker as well as a total viable count of salivary bacterial microbiome among COVID-19 patients.

Materials and methods: a sample of 84 adults patients was collected who were divided into two groups: the COVID-19 group consists of 42 patients and the COVID-19 free group which consists of 42 subjects. All subjects undergo a PCR test to confirm their health status. The collection of Un-stimulated saliva was done. Laboratory investigations were carried out to measure the total viable count of the salivary bacterial microbiome by culturing on Brain Heart Infusion Agar and to evaluate the salivary cortisol level using cortisol kit (Elecsys Cortisol II).

Results: SPSS version 21 was used for statistical analysis. According to the statistical analysis, the salivary cortisol and total viable count of salivary bacterial microbiome values were substantially greater in the COVID-19 group than in the COVID-19 free group.

Conclusion: A positive association was found between salivary cortisol and the total viable count of the salivary bacterial microbiome. So, when the concentration of salivary cortisol is elevated in the COVID-19 group, the level of the total viable count of the salivary bacterial microbiome is also elevated.

Keywords: Cortisol, COVID-19, Stress, Biomarker, Oral bacteria. **(Received: 22/7/2021, Accepted: 11/9/2021)**

INTRODUCTION

Stress is described as a set of physiological and psychological responses that activate a person's defense mechanism in response to a range of threats. In reaction to stress, the hypothalamus-pituitary-adrenal (HPA) axis is predominantly stimulated. [(1)] There are several techniques to stress management in humans and animals, many of which rely on the quick identification of damage induced primarily by stress and are referred to as biomarkers. [(2)] The stress indicators show that the individual is disturbed and exerts a lot of energy to maintain homeostasis. [(3)] A biomarker is an indicator used to assess the pathologic and physiologic condition of the individual. [(4)]

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According to the FDA, an ideal biomarker should be easily tested, capable of distinguishing between different disorders, safe and harmless, and specific for certain pathological and physiological conditions. [(5)] Stress biomarkers might be hormones such as adrenaline and cortisol, enzymes such as alpha-amylase and lysozyme, proteins such as secretory IgA, or prohormones such as Chromogranin A. [(6)] Many studies have suggested that there is a substantial link between stress and various saliva components such as cortisol, SIgA, a-amylase, and CgA. [(7)] Cortisol is a steroid hormone synthesized by the zona fasciculata of the adrenal cortex, and it is the most investigated component in saliva as a stress indicator. [(8)] Salivary cortisol is found in a free, unbound form and correlates with unbound blood plasma cortisol. It is lipophilic and requires a carrier which is usually a protein for traveling around the body. The protein binds the majority of cortisol (65%) in blood plasma. Approximately 70% of unbound blood cortisol is a salivary

cortisol concentration, only 1 to 15% of cortisol in the blood is in its unbound or biologically active form. The cortisol that remains is bound to serum proteins. [9] Cortisol levels rise in response to physical or mental stress, and they are influenced by workload and sleep deprivation. The highest cortisol concentrations were found 45-60 minutes after waking up, and the lowest concentrations were found in the evening hours, which corresponds to variations in plasma cortisol. [10] The salivary analysis is less invasive than blood sampling, which can be uncomfortable and artificially elevate cortisol levels. [11] Stress-induced elevations in cortisol have two main effects: they release glucose, which is required for giving energy to organs, and they mediate changes in immunity, which are crucial for restoring physiological equilibrium. [12] Microorganisms live on the surface tissues of all humans, including the skin, oral cavity, respiratory system, gastrointestinal tract, and urogenital tract. The amount and kind of microorganisms a person possesses vary according to age, diet, and personal hygiene level. They are known as the human body's normal microbiome. [13] Bacteria have been thought to be the most relevant component of the human microbiome. [14] the Human Oral Microbiome had about 600 predominant bacterial taxa, with various subsets predominating at multiple surfaces such as teeth, gingival sulcus, tongue, cheeks, hard/soft palate, and tonsils. [15] The two types of tissues in the oral cavity that bacteria colonize are soft and hard tissues. It is also clear that the oral cavity has a number of different bacterial growth niches, each with its unique bacterial profile that varies by location and individual, based on lifestyle and physiological variations, each individual has a specific microbiome. [16] *Streptococcus*, *Veillonella*, *Porphyromonas*, *Rothia*, *Actinomyces*, *Corynebacterium*, *Treponema*, *Fusobacterium*, *Prevotella*, *Neisseria*, *Eikenella*, *Haemophilus*, *Lactobacterium*, *Eikenella*, *Leptotrichia*, *Peptostreptococcus*, *Staphylococcus*, *Eubacteria* and *Propionibacterium*, according to a research findings done by Zarco et al. (2012), are the main genera with the biggest presence in the oral cavity. The primary aim of the present study was to evaluate cortisol value in saliva to examine the presence of stress among COVID-19 patients in comparison with healthy adults in the same period of time and under the same circumstances, measure the total viable count of salivary bacterial microbiome of COVID-19 patients in comparison with COVID-19 free group and evaluate the correlation between salivary cortisol and total viable count of salivary bacterial microbiome among COVID-19 patients.

MATERIALS AND METHODS

After the proposal and the revision of the study protocol, approval was obtained from the Scientific and the Ethics Committee in College of Dentistry/University of Baghdad/Iraq, the study lasted for 4 months from January until the end of April 2021. The samples were collected in Dar AL-Tamreedh hospital in Baghdad Medical City in the morning from 9-11 o'clock. The inclusion criteria for this study were medically fit patients with no systemic diseases and a positive PCR test for COVID-19 and recently infected (not more than 2 days) to ensure the good health of the patient who took no antibiotics that could alter the bacterial count. Patients were excluded from this study if they had signs and symptoms of COVID-19 but negative result on PCR tests also patients with positive PCR test on antibiotic treatment or on medications due to history of chronic disease. The patients were informed not drink or eat anything (excluding water) two hours before collecting the samples, Saliva has been collected at the same time of day, 9-11 a.m. and fixed collection time (5 minutes). The amount of unstimulated saliva obtained was between 1-3 ml in test tube, and samples were collected under similar conditions to collect 84 samples from COVID-19 patients and COVID-19 free subjects. COVID-19 free subjects have been used as a control group including 42 healthy adults of both genders, samples were collected from Dar AL-Tamreedh hospital / PCR test center for travelers. After collecting the salivary sample from each patient, the tubes were placed in a cool box with ice to transfer them to the lab to be cultured within less than an hour. Part of the saliva was centrifuged for 10 min at 3000 rpm, and the clear supernatant was collected and kept in the freezer at -20°C until use. The salivary cortisol level was determined by using the cortisol kit (Elecsys Cortisol II) by cobas e 411 system and according to the manufacturer's instructions and the results were expressed in µg/dl. The other part is used for culturing bacteria in brain heart infusion agar. Tenfold serial dilution was prepared using

phosphate buffer solution, 0.1 ml was withdrawn by micropipette from the dilution (10^{-4}, 10^{-6}) then a microbiological spreader was used to inoculate the 0.1 ml on the Brain Heart Infusion agar from each dilution after that an incubator was used to incubate the agars; the agars were incubated for 48 hours, at 37◦c and finally after incubation the total bacterial count cfu/ml was determined by the use of colony counter. These were the first calculated values after 48 hours from collecting each sample, while the cortisol levels were examined after completing all the samples collection to calculate all the values at the same circumstances.

The impact of salivary cortisol and total viable count of salivary bacterial microbiome were determined using a statistical test (student's t- test, simple linear regression, Descriptive analysis included percentages or mean ± standard deviation SD) relied on SPSS program version 21, if the p-value was <0.05 it was considered as statistically significant.

RESULTS

A total of 84 patients participated in this study, they were 52 males (62%) and 32 females (38%), they ranged in age from 20 to 55 years with a mean age of 34.6 years. According to the findings of the present study, salivary cortisol levels were significantly different between the two groups, the lower mean was found among COVID-19 free group (0.62) compared to COVID-19 group (0.68). also total viable counts of salivary bacterial microbiome levels were also significantly different between the patient and control group (p= 0.029), the lower mean was among COVID-19 group free (42.28 x10^6) while COVID-19 group mean value was (78.88 x 10^6) as shown in (Table 1).

Table 1: Salivary cortisol level (µg /dl) and total viable count of salivary bacterial microbiome (CFU/ml) between COVID-19 group and COVID-19 free group.

Variables	COVID-19 group N=42		COVID-19 free group N=42		Statistical analysis	
	mean	SD	mean	SD	t-test	p-value*
cortisol	0.68	0.016	0.62	0.013	18.12	0.007
Total viable count of salivary bacterial microbiome x10^6	78.88	46.54	42.28	41.99	3.78	0.029

[*] P>0.05 Non significant

[**] P<0.05 Significant

The statistical analysis showed that there was a positive correlation between salivary cortisol and total viable count of salivary bacterial microbiome levels in COVID-19 group, the r value (0.214) while there is a negative correlation between salivary cortisol and total viable count of salivary bacterial microbiome levels, the r value (-0.21) as shown in (Table 2).

Table 2: the correlation coefficient between salivary cortisol and total viable count of salivary bacterial microbiome in COVID-19 and COVID-19 free group.

Variables	COVID-19 group N=42 r-value	COVID-19 free group N=42 r-value
cortisol X total viable count of salivary bacterial microbiome	0.214	-0.210

By using simple linear regression, a positive association was found between salivary cortisol and the total viable count of the salivary bacterial microbiome in COVID-19 group. A negative association was found between cortisol and total viable count of the salivary bacterial microbiome in COVID-19 free group.

DISCUSSION

Recent technical advances in the processing and evaluation of salivary components have produced reliable results that increase the possibility of using this biological source, which is comparatively safer, cheaper, and less invasive than its traditional alternatives, such as blood and urine. [16] Saliva provides an optimum and non-invasive biological source for the quantitative and qualitative assessment of chemical and physiological mediators associated with various conditions, such as stresses, diseases. The major findings of the study were that there was a significant increase in cortisol concentration, which has been developed as a method to assess psychological stress, associated with an increase in the total viable count of salivary bacterial microbiome which is mainly due to drop in the immunity of the body. However, the relation between salivary cortisol level and total viable count of the salivary bacterial

microbiome in the COVID-19 group could be explained through several possible reasons like; an increase in salivary cortisol may affect the microbial colonization processes such as adhesion and coadhesion. [17] Higher cortisol level might suppress the oral immunity and induce the proliferation of the bacteria. [18]

During stress, the body's cortisol level increases the production of ―acid which provides a suitable medium for these bacteria, also an atrophic change of the major salivary glands caused by corticosteroids may lead to a decrease in the quantity (volume) and the composition of the saliva, meanwhile, decreasing the salivary secretion will reduce the clearance of cariogenic bacteria. [19] - Due the limitations of the current study, thereis the small number of cases in comparison to the cases of COVID-19 in Iraq, some patients were uncooperative and refused to participate in the study, many patients complained of dry mouth and difficulty in collecting 3 ml of saliva and many samples eliminated from the study due to containing sputum due to cough.

CONCLUSION

The results of the current study reveal a significant difference in the cortisol values and total viable count of salivary bacterial microbiome between COVID-19 and COVID-19 free groups. The increase in cortisol values was in COVID-19 group, cortisol considers as one of the markers to assess the stress, also the evaluation of the total viable count of salivary bacterial microbiome revealed a rise in its value in COVID-19 patients, this may be due to a decrease in patient's immunity, or due to neglecting for the oral hygiene standards due to the sickness.

Also there is a positive correlation between cortisol and the total viable count of the salivary bacterial microbiome in the COVID-19 group, so when Salivary Cortisol level increased, there is an increase in the total viable count of the salivary bacterial microbiome in the same group, in comparison with the COVID-19 free group which has a negative correlation between cortisol and total viable count of salivary bacterial microbiome.

Conflicts of interest: None to declare.

Ethical clearance: All experimental protocols were approved under the Department of Basic Science, College of Dentistry, University of Baghdad, and all experiments were carried out in accordance with approved guidelines.

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الخلاصة

الخلفية: كان لتفشي فيروس كوفيد -١٩تأثير هائل على أجزاء كثيرة من حياة الناس ، حيث نُصحوا بالحجر الصحي وحظر التجول لمنع انتشار الفيروس ، مما كان له تأثير كبير على الصحة العقلية للناس والقلق والتوتر. تؤدي العديد من العوامل الداخلية والخارجية إلى الإجهاد ، مما يؤثر سلبًا على توازن الجسم. نتيجة لذلك ، قد يؤثر الإجهاد على قدرة الجسم على استخدام الطاقة للدفاع ضد مسببات الأمراض. لقد وجدت العديد من التحقيقات الحديثة روابط جوهرية بين الإجهاد العقلي البشري وإنتاج الهرمونات و / أو المواد المناعية. أثبتت بعض هذه الأبحاث الصلة بين الإجهاد ومستويات الكورتيزول اللعابي. الهدف من هذه الدراسة هو قياس الكورتيزول اللعابي كمؤشر حيوي للضغط بالإضافة إلى العدد الإجمالي للنباتات البكتيرية اللعابية بين مرضى كوفيد -١٩.

المواد والطرق: تم جمع ٨٤ عينة من البالغين الذين تم تقسيمهم إلى مجموعتين: تتكون مجموعة كوفيد -١٩من ٤٢مريضًا والمجموعة الخالية من كوفيد -١٩والتي تتكون من ٤٢ شخصًا. يخضع جميع الأشخاص لاختبار بي سي ار لتأكيد حالتهم الصحية. تم جمع اللعاب غير المحفز. تم إجراء الفحوصات المخبرية لقياس العدد الكلي للبكتيريا اللعابية عن طريق الزراعة على وسط مناسب وكذلك قياس مستوى الكورتيزول اللعابي باستخدام مجموعة الكورتيزول .

النتائج: تم استخدام الإصدار ٢١ من برنامج للتحليل الإحصائي. وفقًا للتحليل الإحصائي ، كان الكورتيزول اللعابي والعدد الكلي القابل للبكتيريا اللعابية أكبر بشكل كبير في مجموعة كوفيد -١٩ مقارنة بالمجموعة الخالية من كوفيد -١٩.

الاستنتاجات: تم العثور على ارتباط إيجابي بين الكورتيزول اللعابي والعدد الكلي الحي للنباتات البكتيرية اللعابية. لذلك ، عندما يرتفع تركيز الكورتيزول اللعابي في مجموعة كوفيد -١٩، يرتفع أيضًا مستوى العدد الإجمالي للبكتيريا اللعابية.