Innovations

Relationship of nerve growth factor in saliva and chronic periodontitis – an analytical cross-sectional study

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Abstract

Issues : *The complex interactions between the immune and the nervous systems has instilled a keen sense of interest among the humans which led to the discovery of nerve growth factor. The study aimed to determine the relationship of salivary nerve growth factor and chronic periodontitis comparing it with healthy individuals.* ***Methods:*** *An analytical cross sectional pilot study was conducted with 200 subjects from the out-patient department. Unstimulated salivary samples were collected and processed, for the analysis of NGF using sandwich ELISA technique.* ***Findings:*** *The data were then statistically analyzed which showed that the difference between cases and controls with regards to NGF concentration was found to be statistically significant (p=0.045) using Mann Whitney test. Spearman's correlation analysis of the gingival index scores with NGF concentration was very weak and negative, with no statistical significance (p=0.296).* ***Conclusion:*** *The study stands novel- being the first in-vivo study to ascertain the relationship between salivary NGF and chronic periodontitis. The findings of the study show a statistically significant association of nerve growth factor with chronic periodontitis which can help in better understanding of the immune mediated disease pathogenesis. NGF has been in use in various fields like ophthalmology, endocrinology, oncology etc. Understanding the role of NGF in chronic period ontitis marks the beginning of further research in using it as an adjunct in the control of inflammation. This study paves way for future research on the clinical application of NGF in dentistry as an adjunct to the other conventional modalities.*

Keywords: *1. Salivary NGF 2. Nerve Growth Factor 3. Chronic periodontitis 4. Bi-directional signals*

1.Introduction

Since the discovery of "nerve growth factor" (NGF) in the 1950s by Rita Levi Montalcini et al, its role in immune regulation and inflammation has been the most sought research until date. NGF is ubiquitous, also in non- neural tissues like skin, mucosa, saliva and other body fluids. The neuropeptide is known to produce bi-directional signals in immune regulation. The marked increase of NGF in inflamed tissues controls the release of inflammatory mediators like cytokines thereby suppressing the inflammation. Although micro-organisms stay as the primary etiologic agents, chemical mediators of inflammation are responsible for the destruction of periodontal tissues in a generalized dental condition like Periodontitis. Hence, it might have an influence in the levels of salivary nerve growth factor which is an inflammatory mediator.NGF has been in use in various fields like ophthalmology, endocrinology, oncology etc. This study marks the beginning and throws light on further research on the clinical application of NGF in dentistry as an adjunct to the other conventional modalities.

2. Literature Review:

Studies have shown the increase in NGF at the local site of inflammation, closely following the course of the disease.[2] The neuropeptide has a vital role in mediating the signals between the immune system and the nerve complex. This was initially observed in the CSF of patients with multiple sclerosis, in the synovial fluid of arthritic patients and the serum of patients with lupus erythematosus.Recent studies have also observed the association of NGF in temporo-mandibular joint disorders. The standard concentration of NGF in normal saliva is around 901.4±75.6 pg $^{ml-1}$ as per JW Nam et al in 2007 using ELISA.Literature evidences show the expression of nerve growth factor to be increased in stimulated saliva, in inflammatory and painful conditions. Studies also tried to correlate the degree of inflammation / pain with the concentration of NGF in tissues and other body secretions.A study done by Zane Laurina et al in 2009 revealed the expression of basic nerve growth factor in two types of epithelium – sulcular and gingival.The expression was numerous to abundant. An immunohistochemical study done by Verisheh Rastin et al in 2017 revealed the concentration of NGF marker in the dental pulp of chronic periodontitis patients to be 0.349 cells when compared to healthy individuals which was 0.052 cells.In 2014, Priscila Dias Nascimento Sander, correlated the salivary NGF concentration with the difference in pain status in temporomandibular joint disorder patients. Results revealed the mean sNGF levels in painful TMD patients to be 1114.39pg/ml when compared to controls which was 1163.89pg/ml. Thus the chance of having painful temporomandibular disorder was not related to the increase in the levels of NGF.There is no reported evidence of research, analyzing the relationship between nerve growth factor in saliva and chronic periodontitis, which is the basis of our study.

3. Objective of the study:

The objective this study was to investigated the relationship of salivary nerve growth factor and chronic periodontitis

4. Methods of the study:

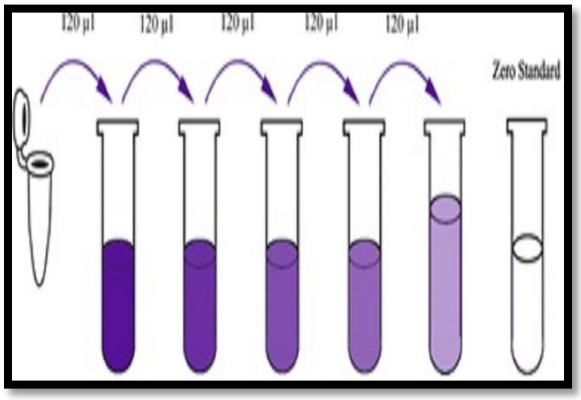
The study subjects were selected at random from the outpatient department. All the participants of the study underwent a complete systemic examination to evaluate their medical status. Data relating to age, gender and previous medical/surgical history were recorded. The participants were enrolled in the study from November 2018 to June 2019.The protocol for the study was given approval by the Institutional Review Board and the Ethical Clearance Board.

The study included 200 subjects divided into 2 groups: cases (patients with chronic periodontitis) and controls (healthy individuals). Patients who underwent periodontal therapy in past 6 months, existence of major systemic disease and /or immune system abnormality, aggressive periodontitis, clinically visible oral lesions, antibiotic use, immune modulation or anti-inflammatory drug usage in past 3months, smoking, pregnant and currently lactating females were excluded. All the eligible study participants were then selected and were informed about the study after which, a written consent was obtained. They were then subjected to a thorough periodontal examination based on Loe and Silness gingival index (1963) and the Probing pocket depth and clinical attachment level were recorded. Considering the periodontal inclusion criteria and the willingness of the patient to participate in the study, the final study group included 76 subjects – 38 cases and 38 controls.

The methodology includes collection of unstimulated whole saliva from the selected patients and preservation in cold storage. The procedure was scheduled within 8 am to 12 pm to avoid diurnal variation of the salivary levels. Prior to saliva collection, patient is asked to avoid consumption of alcohol, major meals, dairy products, high sugar content and caffeine, as they might lower the salivary pH and accentuate the bacterial growth, interfering with the assay values. Rinsing with clean water eliminates the food residues and reduces the chance of sample contamination.

After ensuring the preventive measures for saliva collection as stated above, the patient was asked to sit erect and expectorate the pooled saliva into the graduated, sterile saliva collecting disposable tube. The tubes were then centrifuged in a cooling centrifuge in batches with an rpm of 3000 for approximately 20 minutes. Following centrifugation, there was evidence of a discernible mass at the rear end of the saliva tube. Failure of this requires repetition of the centrifugation process following re-vortex of the sample tube. After this, the sample was pipetted out into aliquots to be stored in a deep freezer maintaining the temperature at -4ºC for further analysis using ELISA kit to determine the level of Human Nerve Growth Factor. BioAssay's Human Beta NGF kit was used for our study. Samples and the reagents were de-freezed to room temperature before starting the assay. The freshly prepared standards (Figure 1) and samples were loaded in the titre plate and incubated as per the manufacturer's instructions. Absorbance values were then measured using Labserv ELISA reader at 450nm, within 10mins of adding the stop solution.

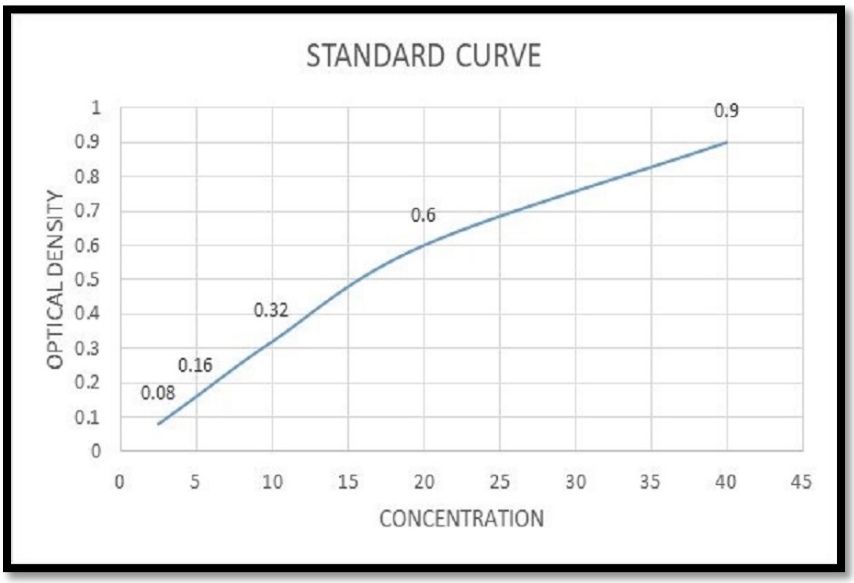
Figure 1 gives the preparation of the Standard solution



A standard curve is obtained by plotting the optical density values for each of the reference standard values against the salivary nerve growth factor concentration. The optical density values are marked along

the "Y-axis" and the NGF concentration along the "X-axis". A curve is drawn connecting the intersecting points of the two axes. Using the obtained absorbance values from the ELISA reader, the concentration of salivary NGF is determined from the standard curve (Figure 2) by interpolation.

Figure 2 shows the Standard curve



5. Data Analysis:

Preliminary determination of the salivary NGF concentration in the cases and controls. The mean NGF concentration was found to be higher among the cases (154.52±39.99) compared to controls (139.30± 32.22). This difference between cases and controls with regard to NGF concentration was found to be statistically significant (p=0.045) using Mann Whitney U test.

Group	**NGF (mean±SD)**	**P value***
Cases	154.52±39.99	0.045
Controls	139.30±32.22	

Table 1. Comparison of NGF concentrations between cases and controls

Spearman's correlation analysis revealed the gingival index scores having a very weak, negative correlation with NGF concentrations (r= -0.121) which was not found to be statistically significant (p=0.296) . The negative correlation explains that there might be a decline in the NGF concentrations with a progression

in the disease.

Characteristic	Spearman's correlation coefficient	NGF	P value
Gingival index score	r value	-0.121	0.296

Table 2: Correlation between gingival index scores and NGF concentration among study subjects

The influence of age and gender on NGF concentrations among the study subjects were also analyzed using Mann-Whitney U test. The mean concentration of NGF in males was 142.44±10.82 and in females was 148.34±40.60. The difference between the values was statistically not significant (p=0.724). The mean concentration of NGF for group 1(age 15-19years) and group 2(20-45 years) were 100.00±10.82 and 148.84±36.30 respectively. The differences between the groups was statistically significant (p=0.01).

The mean concentration of NGF in cases (154.52± 39.99) was found to be higher than the controls (139.30±32.22). Statistical analysis of the obtained results revealed the difference between the cases and the controls with regard to NGF concentration to be statistically significant (p=0.045) using the Mann-Whitney U test. This proves the association of salivary NGF with chronic periodontitis.

6. Conclusions:

Nerve growth factor, a prototypical growth factor, a Nobel discovery by Rita Levi Montalcini and Stanley Cohen is a polypeptide belonging to the neurotrophin family essential for the survival and development of cells of the peripheral nervous system.NGF being a pleuripotent mediator, is widespread in distribution. It is present also in non- neuronal tissues such as skin, mucosa and saliva. It is initially exhibited in a 7s, 130-kDa complex of 3proteins - α, β, γ in a ratio of 2:1:2.

Besides being a distinctive neurotropic factor, numerous shreds of evidence state the varied biological activities of NGF on non-neuronal cells including the inflammatory cell population like neutrophils, mast cells, and macrophages that potentiate the healing of wounds and tissue repair. Neuropeptides like substance P and CGRP (Calcitonin gene related peptide) from the nerve endings cause dilatation of the blood vessels and extravasation of the plasma, promoting the migration of leucocytes and phagocytosis. It also affects the liberation of inflammatory mediators like cytokines from the immune cells.The immunoregulatory action of NGF was initially described by Aloe and Levi Montalcini et al in 1977 by injecting NGF in rats which resulted in increase in the size and count of the mast cells. NGF influences various activities like chemotaxis, phagocytosis, survival of the mast cells, lymphocytes and the granulocytes. The role of NGF and its precursor forms in wound healing was studied by Karl Schenck et al in 2016. In the cascade of oral wound healing, different forms of NGF in saliva act as luminal surveillance peptides and NGF plays a dynamic role in all the stages.

The cognizance about the regulation of the immune mechanisms and inflammatory responses is critical to comprehend the pathogenesis of oral diseases like periodontitis. Although the etiology is bacteria, the pathogenesis is mediated by the inflammatory response to the bacterial biofilm. When the identification of true pathogens has been indefinite, evidences show strong association of specific microbes with the disease

progression. In cases when the microbial presence shows no response in the progression of the disease, studies state that the periodontitis is the net effect of immune response and inflammatory process and not the mere presence of bacteria.

Periodontal diseases manifest in 2 ways: gingivitis and periodontitis. In both, there is an inclined colonization of bacteria at the dento-gingival margin. The host responds to the bacteria by regulating the inflammatory infiltrate in the sub-epithelial tissue beneath the periodontal pocket. The initial inflammation is physiologic with migration of the cells to the site of infection through the synthesis of cytokines and other inflammatory mediators. During this, the neuronal system also gets activated. They generate impulses that cause secretion of neurotransmitters, neuropeptides into ECF where they initiate the local response on other immune cells or other neurons. These neuropeptides have significant immunomodulatory action. Hence the focus of research shifts towards identification of these neuropeptides that can help understand the complex communication between the immune and nervous system resulting in alteration of the inflammatory response.NGF directly has a role in synthesis of inflammatory mediators like calcitonin gene related peptide as in the sensory neurons.

The current analytical study was done to determine the relationship between the salivary nerve growth factor and chronic periodontitis. The main objective of the study was to compare the levels of salivary NGF in chronic periodontitis with healthy individuals. Also, to see if there is any dysregulation in the levels of salivary NGF in the patients with chronic periodontitis.

The neuropeptide can commonly be isolated from neuronal, extra neuronal tissues and body secretions. Saliva is known to lack the drama of blood, the sincerity of sweat and the emotional appeal of tears. Thus, it has been considered as the diagnostic medium for our study for the following reasons: virtually unlimited supply, non – invasive sample collection, patient comfort, collection does not require trained professionals, eliminates risk of infection, cost effectiveness, allows repeated sample collection for serial analysis and rapid screening of large populations. Although saliva is a reliable diagnostic tool, it has certain limitations like: the influence of Circadian rhythm on the dynamics and kinetics of salivary biomarkers, the salivary composition is significantly lower compared to their concentration in serum.

The concentration of salivary NGF in our study was inconsistent with the previous results, due to the following reasons. There could be probable variation in the estimated levels of NGF with respect to the sensitivity and specificity of the immunoassay kit used in the study. Variations in the concentrations may be related to ethnicity. This is the first study to be carried out estimating the levels of sNGF in Indian population. Also the prolonged storage of the salivary samples could have affected the stability of the neuropeptides.

Spearman's correlation analysis of the gingival index scores with NGF concentration negative with a coefficient (r-value) of -0.121. Negative correlation indicates that there is a decline in the salivary NGF with the progression in the disease. This could be because of the immunoregulatory role of NGF to control the inflammatory process. The current study was done in a limited population and hence was found to be statistically insignificant (p=0.296). The negative correlation could also be because of the relatively smaller sample size. A larger population with varying grades of periodontitis should be considered in further research. This could help in further validation of the current study and also in understanding the role of NGF in chronic periodontitis.

The influence of age and gender on NGF concentrations among the study subjects were also analyzed using Mann-Whitney U test. The mean concentration of NGF in males was 142.44±10.82 and in females was

148.34±40.60. The difference between the values was statistically not significant (p=0.724) consistent with the results of the study done by Priscila Dias Nascimento Sander in patients with TMD.

The study samples were segregated into age groups based on the WHO guidelines. Most of them came under group 1(15-19 yrs) and group 2(20-45 yrs). The mean concentration of NGF for group 1 and group 2 were 100.00±10.82 and 148.84±36.30 respectively. The differences between the groups was statistically significant (p=0.01). The above data is contradictory to the fact that NGF concentration decreases with the increase in age which was proved in previous studies (JW Nam et al 2007).Considering a known fact that there might be a progression of periodontitis with age, the concentration of inflammatory growth factors like NGF also may be markedly increased. But to validate the above fact, it is necessary to correlate the NGF concentration with varying age groups in a large population. Our study did not focus on the correlation of age and NGF because the principle interest was to correlate it with the disease.

The current study thus has several strengths and limitations. Firstly, the control group was also recruited during the same time period as the cases. This ensures a consistency in the assessment and decreases the chance of information bias and misclassification. Another advantage is that the salivary samples were collected by trained professionals and the researcher who performed the analysis was blinded to the study group and outcome.

Considering the limitations of the study, misclassification among the cases and controls is unavoidable. Underestimation of the clinical data could also be a reason for misclassification. To overcome this, 2 or more observers could have been included in the study, validating the inter-observer reliability during examination. ELISA is a reliable technique for analysis unless proper standardization of the samples is done to avoid measurement errors. Equal amounts of sufficient quantity of the samples have to be collected, well enough for the standardization and the analytical procedure to be carried out. Prolonged period of storage of the samples may interfere with the stability of the neuropeptide.

NGF has already been used in the management of various inflammatory conditions in different forms- topical and systemic.Keiko Kawamoto and Hiroshi Matsuda in 2004, reviewed the role of NGF in wound healing. It has been used recently, to treat the corneal ulcers and bronchial asthma. Further studies can find its application in the therapeutics of oral inflammatory lesions like periodontitis, lichen planus and precancerous lesions like leukoplakia. To be used as a treatment adjunct, it is essential to understand its role in the etiopathogenesis of that particular disease.

To our knowledge, this is the first study that investigated the relationship of salivary nerve growth factor and chronic periodontitis. The findings of the study show a statistically significant association of nerve growth factor with chronic periodontitis. This might help in better understanding of the immune mediated disease pathogenesis. The relatively smaller sample size could not elucidate the correlation of salivary NGF with the severity of the disease which is a limitation of the current study. A study with a larger population can help to overcome this shortcoming. Understanding the role of NGF in chronic periodontitis marks the beginning of further research in using it as an adjunct in the control of inflammation.

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