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RESEARCH ARTICLE

Evaluation of Neutrophilic Receptors; CXCL8 and CXCR2 in Patients with Chronic Periodontitis Compared to Healthy Subjects by Real Time PCR Method

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Abstract:

Objective:

This study aimed to evaluate the levels of CXCL8 and its receptor (CXCR2) in gingival tissue neutrophils from patients with chronic periodontitis compared to periodontal healthy subjects using Real Time PCR method.

Methods:

The test group consisted of 21 patients with chronic periodontitis and the control group consisted of 18 healthy individuals. In both groups, gingival tissue samples were obtained during periodontal surgery. CXCL8 and CXCR2 RNA in tissue samples were examined by PCR method, and then the levels of genes expression were measured. Mann-Whitney U nonparametric test was used for statistical analysis.

Results:

CXCL8 gene expression in the gingival tissue of the test group with chronic periodontitis was significantly higher than the control group ($p=0.028$). CXCR2 gene expression in the gingival tissue of the test group with chronic periodontitis was significantly lower than the control group ($p=0.043$). In both test and control groups, there was a negative correlation between CXCL8 and CXCR2 gene expression. This correlation was statistically significant in the test group ($p=0.001$), but there was no significant correlation in the control group ($p=0.431$).

Conclusion:

The results of this present study suggested that the level of gene expression for CXCL8 was greater in patients with chronic periodontitis and CXCR2 was greater in healthy individuals. Although in people with chronic periodontitis, CXCR2 decreases slightly as CXCL8 levels increase.

Keywords: Chronic periodontitis, PCR, Gene expression, CXCL8, CXCR2, Neutrophils.

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1. INTRODUCTION

Periodontal disease is an inflammatory disease involving the tissues that support the teeth caused by specific microorganisms or a group of microorganisms. It is natural for the host immune system to defend itself against pathogens to prevent tissue damage. Chronic periodontitis is a chronic inflammatory reaction of the periodontium to a bacterial

infection that causes loss of tooth support and alveolar bone resorption resulting in tooth mobility and tooth loss [1, 2]. Periodontal destruction is mostly caused by cell inflammatory responses, mainly neutrophils [3].

Interleukin 8 (IL8 or chemokine (C-XC motif) ligand 8, CXCL8) is a chemokine produced by macrophages and other cell types such as epithelial cells, airway smooth muscle cells, and endothelial cells. In humans, the protein IL8 is encoded by the CXCL8 gene. There are many receptors on the membrane surface that can attach to IL8; the most frequently studied types are G protein-coupled serpentine receptors and CXCRs.

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Expression and correlation of IL8 vary between receptors (e.g., CXCR1 > CXCR2) [4].

In the first stage, the secretion of histamine at the site of infection dilates capillaries near the site of injury, which slows blood flow to the site and forces leukocytes such as neutrophils to come close to the endothelium and moves away from the center of the lumen, where blood circulation is at its highest rate. When this happens, there is a weak interaction between selectins expressed on neutrophils and endothelial cells (indicating which one increases between CXCL8 and other cytokines) [5].

When neutrophils circulate in the vessels, it connects to the CXCL8 molecule via a G-coupled protein mediator receptor that is in charge of cellular signaling. The binding of CXCL8 to CXCR1 /2 on the neutrophil surface induces neutrophils to increase the number of receptors and express integrin and leukocyte function-associated antigen-1 (LFA-1), which are involved in continuous binding to intracellular adhesion molecule1 (ICAM-1) receptors expressed on the epithelium. Another key function of CXCL8-stimulated cellular messengers is the initiation of oxidation. This process allows the production of proteolytic enzymes and reactive oxygen species required for the breakdown of extracellular matrix (ECM) and cell membranes [6].

Hiyari *et al.* identified a family of CXCLs as mediators in lipopolysaccharide (LPS)-induced periodontitis. In their study, they found a sixfold difference in people with genetic background in LPS-induced bone resorption. An increase in CXCL10 protein and C57BL / 6J proinflammatory immune cells and cytokines was also observed. Genetic deletion of CXCR3 (receptors 9 and 10 CXCL) showed a 50% reduction in bone resorption and osteoblast reduction after LPS injection [7].

Souto *et al.* examined the association between chemokines and dendritic cells in chronic periodontitis. It was found that most CD1a + immature dendritic cells are associated with the chemokine (C-C motif) ligand 2 (CCL2), CCL20, CCL3, and CXCL8 chemokines are also associated with high severity in patients with periodontitis [8].

Fredriksson *et al.* investigated the effect of gene purification on peripheral neutrophil subtypes in patients with chronic periodontitis. The results of this study showed that the subgroup with high intensity of fluorescence (IFL) production in patients with chronic periodontitis had a higher response to IL8 than the same subgroup in the control group. No other differences in ROS production or priming effects were observed between patients with chronic periodontitis and the control group ($p < 0.05$). Finally, they concluded that patients with chronic periodontitis had a subset of peripheral neutrophils that showed a greater response to priming IL8 than the control group [9].

Shelburne *et al.* stated that CXCL8 encompasses a wide range of biological activities. The authors of this study suggested that *Porphyromonas gingivalis* might play a role in CX-mediated immunopathogenicity [4].

The review of the literature indicates that IL8 is a key mediator of inflammation, which plays an important role in

neutrophil recall and neutrophil degranulation. For example, it has been suggested as a pro-inflammatory mediator in gingivitis and psoriasis. Due to the importance of CXCL8 and its receptor (CXCR2), and the fact that very limited research has been done to investigate the association of these factors with periodontitis, in the present case-control study, we aimed to evaluate the levels of CXCL8 and its receptor (CXCR2) in gingival tissue of patients with chronic periodontitis.

2. MATERIALS AND METHODS

A total of 39 patients who were candidates for the periodontal flap and crown lengthening surgery in the Periodontology Department of Mashhad Dental School were selected for this study. The study's protocol obtained ethical approval from Mashhad University of Medical Sciences (IR.MUMS.DENTISTRY.REC.1398.021). All participants signed a consent document based on the principles of the Helsinki Declaration and were assured of the confidentiality of data. This study was conducted in 2018 and 2019 in Mashhad Dental School.

The criteria for chronic periodontitis were attachment loss and a minimum probing depth of 6mm or higher in at least eight sites, along with bleeding on probing (BOP) and radiographic bone loss. The data were collected in a field-based manner, and among the 39 patients, 21 patients had chronic periodontitis and the remaining 18 patients were healthy. Exclusion criteria were as follows: having any systemic diseases or any condition that might interact with periodontal disease, such as diabetes, HIV infection, history of topical or systemic usage of inflammatory drugs in 6-month period, pregnancy or using contraceptive drugs, existence of endodontic lesions, having aggressive periodontitis, and smoking.

In patients with periodontitis, a biopsy was obtained from the buccal area of the deepest proximal pocket and through a horizontal cut with a 3mm distance from the palatal gingiva. In patients with normal gingiva and without periodontitis, a similar biopsy of gingival tissue was obtained during crown lengthening surgery for prosthetic treatment.

TaqMan method was used to evaluate the expression of CXCL8 and CXCR2.

2.1. RNA Extraction and Real-Time PCR

Tissue samples were harvested and transferred to a tube containing RNAlater solution (Qiagen, Germany) kept at -20°C to prevent RNA degradation. Total RNA was extracted from samples using an RN easy mini-kit (Qiagen, Germany) according to the manufacturer's instructions. The RNA was then reverse transcribed to complementary DNA (cDNA) with oligo-dT primers on 1 μg of total RNA using First Strand cDNA Synthesis Kit (Fermentase, Germany). Real-time PCR was performed on the cDNA samples using Premix Ex taq (TAKARA, Japan) and Rotor-Gene 6000 system (Corbett, Australia). The sequences of primer pairs for real-time PCR were:

CXCL8: Forward

(5'→3')- CGGAAGGAACCATCTCACTGTG

Reverse

(5'→3')- AGAAATCAGGAAGGCTGCCAAG

CXCR2: Forward

(5'→3')- TCTTCTGGAGGTGCCTACAGG

Reverse

(5'→3')- GAAATCTTCAAAGCTGTCACTCTCC

CXCR2: Forward

(5'→3')- TCTTCTGGAGGTGCCTACAGG

Reverse

(5'→3')- GAAATCTTCAAAGCTGTCACTCTCC

β2M: Forward

(5'→3')- TTGTCTTTCAGCAAGGACTGG

Reverse

(5'→3')- CCACTTAACTATCTTGGGCTGTG

The amplification of a single product for each primer set was confirmed by electrophoresis analysis on a 2% agarose gel and melting curve in real-time PCR. Serial dilution standard curves were developed for target and reference genes to relatively quantify the copy number of every single gene. The Rotor-Gene 6000 machine and its software were used to analyze the standards and the unknown mRNA copy number. The relative quantity of each mRNA was normalized to the relative quantity of β2M mRNA. Then the relative CXCL8 and CXCR2 expression levels for each sample were calculated by an equation of:

$CXCL8 \text{ or } CXCR2 \text{ Normalized Index} = \frac{\text{copy number of genes of interest (CXCL8 or CXCR2)}}{\text{copy number of reference gene (}\beta 2M\text{)}}$

2.2. Statistical Analysis

For statistical analysis, Kolmogorov–Smirnov, T-test, Chi-square, Mann-Whitney U, and Spearman tests were used. The difference was considered significant if $p < 0.05$.

3. RESULTS

3.1. Descriptive Analysis

In this study, a total of 39 individuals, including 13 men (33.3%) and 26 women (66.7%) with a mean age of 33±6 years and an age range of 25 to 46 years, were enrolled and the target group was divided into two groups; test and control.

3.2. Normality check

Kolmogorov–Smirnov test was used to examine the normal distribution of the studied variables. The results showed that only the age variable has a normal distribution, so a parametric test should be used for it. Other variables lacked a normal distribution, necessitating the use of appropriate nonparametric tests.

3.3. Age Variable

We used the t-test parametric test. The age range was 21

years in the test group and 19 years in the control group. The mean age in the test group (34.1) was higher than the mean age in the control group (33.6), but the difference was not significant ($P= 0.783$).

3.4. Gender Variable

We used Chi-square nonparametric test to examine the gender variables. The number of men in the test and control groups was 7 (33.3%) and 6 (33.3%), respectively, and the number of women in the test and control groups was 14 (66.7%) and 12 (66.7%), in turn. In general, the gender distribution in the study groups did not differ significantly from each other ($P= 0.632$).

3.5. CXCL8 and CXCR2 Variables

Given the fact that the distribution of data was not normal in CXCL8 and CXCR2 variables, a nonparametric Mann-Whitney U test was used in the data analysis.

As it is observed in Table 1, the difference in the amount of both genes in the test and control groups was statistically significant. CXCL8 gene expression in the gingival tissue of the test group with chronic periodontitis was significantly higher than the control group ($P=0.028$). CXCR2 gene expression in the gingival tissue of the test group with chronic periodontitis was significantly lower than the control group ($P= 0.043$).

Table 1. Comparison of Mean CXCL8 and CXCR2 between Test and control groups based on Periodontal Status (Healthy and Periodontitis).

Variables (Genes)	Group	N	Mean (GEI)	SD*	Median	Test Result
CXCL8	Test	21	7.14	10.34	3.5	Z= -2.197
	Control	18	2.26	1.23	1.9	P= 0.028
CXCR2	Test	21	1.33	0.7	1.3	Z= -2.028
	Control	18	2.06	1.2	2.2	P= 0.043

*SD: Standard deviation

We used the nonparametric Spearman test to examine the correlation between CXCL8 and CXCR2 gene expression.

As it is observed in Table 2, in both test and control groups, there was a negative correlation between CXCL8 and CXCR2 gene expression. In other words, as CXCL8 values increase, CXCR2 decreases slightly. This correlation was statistically significant in the test group ($P= 0/001$), but there was no significant correlation in the control group ($P= 0/431$).

Table 2. Correlation between CXCL8 and CXCR2 in study groups.

Group	N	Spearman Correlation Coefficient	P-Value
Test	21	-0.625	0.001
Control	18	-0.044	0.431

4. DISCUSSION

In this study, the levels of CXCL8 and CXCR2 in normal and gingival tissues with periodontitis were determined through the Real-time PCR method.

The results of this present study suggested that the level of gene expression for CXCL8 was greater in patients with chronic periodontitis and CXCR2 was greater in healthy individuals. Although in people with chronic periodontitis, CXCR2 decreases slightly as CXCL8 levels increase.

It was also observed that the CXCL8 gene has an indirect correlation with CXCR2, which was higher in patients with chronic periodontitis than in healthy individuals, and this relationship was statistically significant only in the test group but not in the control group therefore we can conclude that in people with chronic periodontitis, CXCR2 decreases slightly as CXCL8 levels increase.

Therefore, in general, this study suggests that the CXCL8 and CXCR2 genes can be used to diagnose people with chronic periodontitis.

Chronic periodontitis is an inflammatory response of periodontal tissues to dental plaque microorganisms that can cause tissue damage. It is a multifactorial disease caused by several species of bacteria. These bacteria interact with host tissues and cells, causing a wide range of cytokines, chemokines, and mediators, some of which destroy periodontal structures such as tooth-supporting tissues, alveolar bone, and periodontal ligament.

Due to the cost and even time-consuming nature of periodontal treatment and the need for long follow-up periods, as well as the recurrent and destructive nature of the periodontal disease, it is important to know more about disease prevention and treatment in the early stages.

The first step in preventing and treating the disease is to better understand the biological occurrence of the disease. The pathogenesis of periodontal disease has been studied by many researchers. Current evidence shows specific and destructive immune and inflammatory responses at different stages of periodontal disease [10, 11].

Due to the inflammatory nature of chronic periodontitis, chemokines are secreted following the response of inflammatory cells to regulate migration and activate leukocytes [12]. Interleukin 8 (IL8 or chemokine (C-XC motif) ligand 8, CXCL8) is a chemokine produced by macrophages and other cell types such as epithelial cells, airway smooth muscle cells, and endothelial cells. In humans, the protein interleukin 8 is encoded by the CXCL8 gene [4].

Several methods for identifying immune factors in tissue samples with periodontitis include immunofluorescence, ELISA, biochemical tests, and molecular methods (PCR). PCR is now widely used due to its high sensitivity and specificity, as well as its ability to save time, money, and manpower.

In this study, gingival biopsy specimens were analyzed and examined in detail by Real Time PCR using the Taqman method. With this technique, it is possible to quantitatively measure the expression of genes with low expression in tissues. Thus, this study made it possible to accurately measure the expression of CXCR2 and CXCL8 genes.

In general, according to the present study, the expression of CXCR2 and CXCL8 genes in patients with chronic periodontitis is significantly altered; As CXCL8 increases,

CXCR2 decreases slightly.

Many studies have shown an increase in CXCL8 in patients with chronic periodontitis. In agreement with this study, Souto *et al.* reported an increased expression of CCL3, CCL5, CXCL8, and CCL19 in patients with chronic periodontitis. The authors of this study also suggested that CXCL8 was significantly associated with bleeding during probing, pocket depth, and loss of clinical connections, all of which are symptoms of periodontitis [8]. Blengio *et al.* observed that dendritic cell maturation leads to the encoding of genes for various neutrophil-absorbing members of the CXC family of chemokines, including CXCL2, CXCL3, CXCL5, CXCL6, and CXCL8 [13].

The reason for this finding is that CXCL8 increases with the invocation of neutrophils in inflammatory sites and plays an important role in the immune response [14]. In this regard, subtypes of peripheral neutrophils are more responsive to CXCL8 in patients with chronic periodontitis; therefore, the reduction of CXCR2 may be justified in this way [9].

The result of Puellman *et al.*'s study was also consistent with this present study. They suggested that T cell receptors (TCRs) increased by 5 to 8% in human neutrophil subtypes, followed by known agonists and increased IL8 secretion [15]. Bauer *et al.* also showed the parallel arrangement of markers in relation to neutrophils in their study [16].

Other similar studies have shown that CCL2, CCL3, CCL5, and CXCL8 levels increase in chronic periodontitis [17 - 19].

Similarly, Gainet *et al.* suggested that IL 8 increases in patients with chronic periodontitis but decreases again after successful treatment [20]. Both Fredriksson and Van Dervort's study showed that IL8 was significantly increased in patients with periodontitis [9, 21].

In contrast, HazemKhalaf *et al.* stated in their study that not only does CXCL8 expression increase in periodontitis, but it also decreases. This finding was also expressed in relation to IL-6 and IL-2 [22].

The reason for these differences may be due to the multifactorial expression of cytokines. For example, some studies, such as those of Nicu and Nibali, have suggested that IL-8 sensitivity and neutrophil function may be linked to genetic factors [23, 24].

Some studies reported that neutrophils express CXCR1 and CXCR2, followed by binding to CXCL8/IL8, GCP-2/CXCL6, and GRO α /CXCL1; therefore, the expression and secretion of these agents and cytokines are related to each other [25, 26].

In general, similar to previous studies, Charles E. Shelburne *et al.* stated in their paper that CXCL8 (interleukin 8, IL-8) encompasses a wide range of biological activities, including T cell, neutrophil, and basophil chemotactic properties. Interleukin-8 is also produced by a wide range of cell types and plays a significant role in the prevalence of acute inflammatory response in such a way that CXCL8 absorbs and activates leukocytes in the area of infection during the inflammatory process, leading to leukocyte secretion, which can lead to tissue damage [4]. The authors in this study showed

that *Porphyromonas gingivalis* may be involved in CXCL8-mediated immunopathogenicity; therefore, it is better to conduct more studies in the future to investigate the role of microorganisms in the development and expression of factors associated with chronic periodontitis such as cytokines and chemokines; For example, C. Damgaard has acknowledged in his study that *Porphyromonas gingivalis* binds to red blood cells to protect against reactive oxygen species (ROS); But at the same time, it enhances the release of inflammatory neutrophils and cytokines such as CXCL8, CCL2, and IL6 [27].

One of the noteworthy points in the expression of IL-8/CXCL8 in periodontitis is related to its activity and effectiveness. It directly affects the differentiation of osteoclasts and leads to bone resorption. It is suggested that more important studies should be performed on this cytokine in the future [28].

The difficulty of laboratory tests and providing a sufficient volume of tissue for laboratory tests was one of our limitations in this study.

CONCLUSION

The results of this present study suggested that the level of gene expression for CXCL8 was greater in patients with chronic periodontitis and CXCR2 was greater in healthy individuals. Although in people with chronic periodontitis, CXCR2 decreases slightly as CXCL8 levels increase.

Authors concluded that immunological examination of CXCL8 and CXCR2 expressed by inflammatory cells in tissue samples of people with chronic periodontitis may also be used as a method of prevention and even follow-up treatment in the early stages of the disease, but more research is needed to prove this in the future.

LIST OF ABBREVIATIONS

IL8	= Interleukin 8
CXCL8	= C-X-C Motif Chemokine Ligand 8
CXCR2	= C-X-C Motif Chemokine ligand 8 Receptor2
PCR	= Polymerase Chain Reaction
cdNA	= complementary DNA

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This study was approved by the Mashhad University of Medical Sciences Ethics Committee for research under the code IR.MUMS.DENTISTRY.REC.1398.021.

HUMAN AND ANIMAL RIGHTS

No animals were used for studies that are the base of this research. The reported experiments in accordance with the ethical standards of the committee responsible for human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2013.

CONSENT FOR PUBLICATION

All patients were given a written consent form and were

assured of the confidentiality of data.

AVAILABILITY OF DATA AND MATERIALS

The data that support the findings of this study are available from the corresponding author [F.S], on special request.

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The authors declare there is no funding source in this study

CONFLICT OF INTEREST

Dr. Amir Moeintaghavi is the Editorial Board Member of The Open Dentistry Journal.

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