

# An Investigation on Chemotaxis Activity, Respiratory Explosion and the Phagocytosis of Peripheral Blood Neutrophils in Patients Affected With EOP

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**Statement of Problem:** Patients affected with “Early Onset Periodontitis” (EOP), are not similar regarding their neutrophil deficiency and researches have yielded contradictory results in this field.

**Purpose:** The purpose of this study was to evaluate different aspects of neutrophils’ activities in peripheral blood of such patients, especially those affected with prepubertal periodontitis and juvenile periodontitis, in Iran.

**Materials and Methods:** twenty patients, of periodontology department of dental faculty of Tehran University of Medical sciences, associated with 16 healthy subjects as controls, were referred to department of Immunon & Allergy Childrens Hospital, Medical Center Tehran University, in order to investigate the relationship between the rate of chemotaxis, phagocytosis and respiratory explosion of peripheral blood neutrophils, with EOP disease. In order to measure chemotaxis rate, Boyden Chamber method was used. Also, for evaluation phagocytosis, the percentage of neutrophils that ingested yeast was investigated microscopically. To measure NADPH oxidase enzyme activity, which acts at the beginning of neutrophils respiratory explosion pathway, slide method was used. Measurements of phagocytosis, NBT and chemotaxis in control and case groups were compared using one-sample t test.

**Results:** Results showed that there was no significant difference between the mean of chemotaxis of patients (104.85)μ- and the control group (116.93) μ-. However, 15% of patients showed a significant decrease in chemotaxis that 33% of them were affected with prepubertal periodontitis and only one case was affected with juvenile periodontitis. The average rate of phagocytosis in patients and controls were 68.7% and 67.31%, respectively. No significant difference between the average of Nitro Blue Tetrazolium Test (NBT) positive neutrophils in patients (97.45%) and the controls (97.62%) was observed. This study showed that females affected 3 times more than males.

**Conclusion:** This study showed that there was not significant difference between the average of phagocytosis, NBT and chemotaxis in patients with EOP and controls and females.

**Key Words:** Early onset periodontitis - Neutrophil- Chemotaxis phagocytosis - Respiratory explosion

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Neutrophils are the first defensive line against dental plaque microorganisms and their reduction, both quantitatively and qualitatively, is effective on the incidence and development of periodontal diseases especially Early Onset Periodontitis (EOP) group.

For the first time, in 1977, Cianciola, stated that the chemotaxis of neutrophils is disturbed in Localized Juvenile Periodontitis (LJP).<sup>(1)</sup>

Lavin<sup>(2)</sup> in his investigation observed that, 86% of patients with juvenile periodontitis had a significant inhibition in the chemotaxis of neutrophils in comparison with the controls. Seventy five and 25% had cellular, and serum deficiencies respectively. Patients with rapidly progressive periodontitis (RPP) showed 48% deficiency in chemotaxis (33% cellular and 66% serum deficiency). He stated that cellular and serum deficiency has the most important role in the development of LJP and RPP diseases, respectively.

Van Dyke et al<sup>(3)</sup> observed that there is a relationship between the abnormality of neutrophil chemotaxis in patients with LJP with a superficial glycoprotein (110.000mr), called GP110, which is a part of the matrix of neutrophil membrane. They showed that there is a significant reduction in GP110, existing in the surface of neutrophils in patients with LJP and generalized juvenile periodontitis (GJP) in comparison with the control group which can be considered as an effective sign for diagnosis, and using this property, it is also probable to provide a special probe to study the functions of neutrophil chemotaxis.

Offenbacher et al<sup>(4)</sup> in an investigation observed that patients with LJP, whose chemotactic response against formil methionyl leucyl phenylalanin (FMLP) and ESA (Endotoxin Activated Serum) had a kind of deficiency, were also impaired in chemotaxis against LTB<sub>4</sub> (Leudoterin B<sub>4</sub>).

Agrawal et al<sup>(5)</sup>, in a study on patients with LJP stated that approximately 70-75% of such patients have disorder in PMN Leucocytes

chemotaxis. They believed that, these cells show an intercellular anomaly against mechanisms responsible for  $Ca^{++}$  movement that is related to neutrophils chemotaxis deficiency. Moreover, Agrawal and Suzuki<sup>(6)</sup> through experiments on serum and neutrophils of patients with LJP, in comparison with healthy subjects, concluded that serum of patients with LJP contains factors which influence on PMNS of healthy subjects through down regulation of FMLP receptor and reducing chemotaxis. However, this phenomenon can be neutralized using anti TNF (Tumor Necrotizing Factor) and anti IL<sub>1</sub> (Interlukin), because TNF and IL<sub>1</sub>, have an undeniable role in modulation of PMN cells function.

Daniel et al<sup>(7)</sup> regarding the chemotaxis of patients with LJP, achieved some valuable results. They found out that, the first phase of calcium response which is related to releasing sequestered intercellular calcium is normal in the neutrophils of the abovementioned patients. But, the second phase of calcium response, which is associated with the activation of membrane canal and the flux of extracellular calcium, seems deficient.

Mouynet et al<sup>(8)</sup> compared the chemotaxis of peripheral blood neutrophils in patients with EOP, gingivitis and adult periodontitis, with healthy subjects. In this investigation Subagar method was used. Results showed that there was no significant difference, between the four groups, regarding directional immigration.

The purpose of this study was to compare the chemotaxis, phagocytosis and NBT of peripheral blood neutrophils in patients with EOP with healthy people.

### Materials and Methods:

This study was a case- control one and the subjects were the patients with EOP disease who referred to the periodontology department of dental faculty, Tehran medical sciences university.

In this research, 20 and 16 patients were

considered as experimental and control groups, respectively.

Patients affected with EOP, Preferably juvenile periodontitis or prepubertal periodontitis, were accepted.

#### **Exclusion criteria**

- Presence of systemic diseases which probably interfere with immunological system such as: diabetes, corton consumption etc.
- Taking antibiotics during the previous 6 months.
- Smoking and taking alcohol
- Taking immunosuppressive or immunosuppressive drugs.
- Pregnancy.
- Malnutrition
- Orthodontic treatment

The process of technique was consisted of clinical examination, observation and interview. After announcing their agreement and completing the questionnaire, the intended patients, associated with the controls who were their friends, were referred to immunological section of paediatrics centre in Imam- khomeini hospital. The relationship between the rate of chemotaxis and phagocytosis with peripheral blood neutrophils and the fresh blood sample (which mixed with heparin) were obtained.

Also for respiratory explosion test, 1<sup>cc</sup> EDTA added to blood sample to prevent clot formation.

Different methods used for each of the examinations, were as follows:

#### **Phagocytosis:**

This test evaluates neutrophils ingestion power. To do this, yeasts were used through a microscopic method. First, yeasts became inactive in boiling water bath. Then, they were washed by PBS buffer and after counting them, definite volume of yeasts was mixed with a definite volume of a normal person serum (for opsonizing yeasts). So as against each neutrophil, there were 5 yeasts, for a period of

15 minutes, the tube containing the above mixture, was placed in 37°C ben mari. Immediately, the tube was put in ice to stop phagocytosis.

Then, 1% methyleneblue color was added to it at a volume, the same as the cell and yeast mixture volume. Finally, the percentage of cells that had ingested yeast was determined microscopically.

#### **NBT (Nitro Blue Tetrazolium Test):**

Respiratory explosion in neutrophils were accompanied by increased oxygen consumption and superoxide production, in addition to light distribution (Chemi Luminescence).

In this experiment, the revival property of the color of Nitroblue tetrazs volume solution (yellow) to formazane insoluble crystals (dark blue) was used in order to assess the NADPH oxidase enzyme activity which acts in the beginning of respiratory explosion of phagocytes such as neutrophils.

In this way, conducted by slide method, in order to obtain PMA/NBT solution, NBT powder, Phorbol Myristate Acetate (PMA) 1mg/ml, Bovin Serum Albumin (BSA) 17.5mg were souled in 1ml Phosphate Buffer Solution (PBS) buffer and the NBT yellow solution was provided with PMA and then 50  $\mu^1$  of PMA/NBT solution was mixed with 50  $\mu^1$  blood in a tube (with the anti- coagulation EDTA), and were placed for 30 seconds in 37°C in ben mari.

The tube of blood mixture and PMA/NBT color, seconds, were sentrifuged in 1500 rpm for 3 seconds. Finally, a slide was obtained from the sediment, at the bottom of the tube, containing blood cells.

Blood slides were stained with gimsa color and studied microscopically. The percentage of neutrophils containing formazan crystals was calculated. This percentele is indicative of neutrophils which have the ability to revive NBT color. In other words, NADPH oxidase enzyme was active in them.

**Chemotaxis:**

There were different methods to examine the chemotaxis of leukocytes. One of them was using Boyden chamber, with filters with the thickness of 13mm and holes with the diameter of 3  $\mu\text{m}$ . Filters were made of Nitrocellulose. To determine the directional movement of neutrophils against LPS chemotactic factor, 14 patients with LJP and 6 patients with PPP, were examined by Boyden chamber method and compared with 16 healthy subjects. The normal range of chemotaxis was + chemotactic factor (CF) 77-125 & -cf 22-67.

Then, in both experimental and control groups, neutrophils were placed over Nitrocellulose and chemotactic factor, (which was obtained by a normal serum, simulated by LPS) was placed under Nitrocellulose filter. After that, cells were allowed to have a directional movement across the thickness of the filter for 3 hours.

Filters were fixed in Isopropanol alcohol and were stained by Hematoxylin and methylèneblue. Finally, by using of a 40 light microscope lens, the movement of neutrophils, through different filter layers, were investigated and reported according to micrometre.

**Results:**

The results from this study in each case, were as follows:

**Phagocytosis**

With regard to table I., the average percentage of phagocyte neutrophils for yeast, in case and control groups were 68.7% and 67.31%, respectively (The normal range was 50-85%) T test showed no significant difference between two groups.

**Respiratory explosion percentage (NBT)**

To investigate the percentage of NBT positive neutrophils, slide method was used.

According to table II, the average percentage of neutrophils containing formazan crystals, in case and control groups were 97.45% and

97.62%, respectively (Normal range is 90-100%).

Again, there was no significant difference between two groups (P-value: 0.757).

**Chemotaxis:**

According to the table III, the average rate of chemotaxis was estimated. In order to investigate the relationship between chemotaxis rate of neutrophils in peripheral blood of case and control groups, according to table III, single sample t test was used.

The estimated t, was less than critical P-value. In other words, there was no significant difference between patients with EOP and controls, regarding directional immigration of neutrophils (P-value = 0.136).

Two thirds ( $\frac{2}{3}$ ) of patients, with suppressed chemotaxis, were affected with prepubertal periodontitis. Therefore, in 33.3% of patients with PPP, a decrease in chemotaxis was observed, and half of them were females. While, only one case with juvenile periodontitis, its neutrophils' chemotaxis, had been significantly less than the control group (62.5%).

**Sexuality:**

With regard to table IV,  $\frac{3}{4}$  of all patients were female.

**Table I-** Descriptive statistics of phagocytosis of neutrophils in EOP and control group

Phagocytosis	E.O.P	Control
Mean	68.7000	67.3125
SD	8.8502	8.2843
Minimum	51.00	54.00
Maximum	80.00	79.00

P-value: 0.488

**Table II-** Descriptive statistics of NBT in EOP and control group

NBT	E.O.P	Control
Mean	97.4500	97.6250
S.D	2.1392	2.5788
Minimum	93.00	90.00
Maximum	100.00	100.00

P-value: 0.757

**Table III-** Descriptive statistics of chemotaxis in EOP and control group

Chemotaxis	E.O.P	Control
Mean	104.8500	116.9375
SD	34.5745	13.7233
Minimum	17.00	92.00
Maximum	141.00	136.00

P-value: 0.136

**Table IV-** Sex situation of mean and SD for chemotaxis and phagocytosis and NBT in EOP patients

Sex		NBT	Phagocytosis	Chemotaxis
Male	Mean	95/8000	67/4000	102/2000
	N	5	5	5
	SD	2/2804	11/1490	49/7464
Female	Mean	98/000	69/1333	105/7333
	N	15	15	15
	SD	1/8516	8/3655	30/1981
Total	Mean	97/4500	68/7000	104/8500
	N	20	20	20
	SD	2/1392	8/8502	34/5745

**Discussion:**

The discussion about the results of this study about phagocytosis, NBT and the chemotaxis of peripheral blood neutrophils, are as follows:

**Phagocytosis:**

The results of our study, regarding the percentage of phagocyte neutrophils, were similar to the study by Van Dyke et al<sup>(9)</sup>. In both studies, there was no significant difference between EOP patients and controls regarding the percentage of neutrophils containing micro organisms. Van Dyke et al<sup>(9)</sup> counted the number of ingested bacteria and found that, this finding, in patients with EOP is (10-20)% less than the controls, while, the percentage of neutrophils containing bacteria between patients and controls was similar. In fact, although neutrophils showed normal phagocytosis quantitatively, but their function was reduced qualitatively.

Considering that, there was no reduction in CB<sub>3</sub> receptors specific for phagocytosis, it was probable that the phagocytosis deficiency in neutrophils, was due to the difficulties in opsonization by different opsonins such as: Igs

and complements.

Wilson et al<sup>(10)</sup> stated that in patients with (LJP) RIIa FCY, is mostly of R<sub>137</sub> allele, which has less tendency to join the IgG<sub>2</sub>. On the other hand, in the serum of these patients', the produced IgG is of IgG<sub>2</sub> type. Therefore, opsonized bacteria are not ingested by neutrophils properly. Perhaps, in current research, one of the reasons that any kind of phagocytosis deficiency in patients with EOP, was not observed that we put the cells of an affected person with EOP, adjacent to the serum of a normal person who has a more suitable subclass of IgG to join CD<sub>32</sub> .(Allel R131).

**NBT Percentage:**

In this investigation there was no difference in percentage of NBT positive neutrophils between patients and control group. In other words, in both groups, NADPH oxidase enzyme activity was the same. In this regard, the results of this study was the same as Van Dyke's et al<sup>(9)</sup>. In his research, the rate of superoxide anion production and Lactoferrin release in patients and controls didn't have significant difference. Although, Suzuki et al<sup>(11)</sup>, reported superoxide production deficiency in stimulated cells by FMLP, but Van Dyke et al couldn't prove such finding by opsonized Zymosan as a stimulator. They believed that the only justification for such difference was that different receptors were stimulated and consequently different results were obtained. It should be noted that LJP neutrophils degranulation, as far as it was measured by enzyme release, was normal.

**Chemotaxis:**

The results of the researches to evaluate the rate of chemotaxis in PMN cells in patients with EOP have not been the same, and in some cases have yielded contradictory results. All patients in Van Dyke's<sup>(9)</sup> investigation, 86% of patients with JP in Lavin's study<sup>(2)</sup>, and 75% of subjects with LJP in Suzuki<sup>(11)</sup> study, comparing to the control group, had a significant chemotaxis

deficiency. On the other hand, the findings of Larjava's<sup>(12)</sup> investigation showed that (JP) patients' neutrophils, didn't have any chemotaxis role. Michael et al<sup>(13)</sup> couldn't find any significant difference among neutrophils' chemotaxis in four groups. Mouynet et al<sup>(8)</sup>, also didn't find any meaningful deficiency in the chemotaxis of peripheral blood neutrophils in patients with LJP, RPP, AP, gingivitis and healthy subjects.

In this research, although, there was no significant difference between the average of chemotaxis in patients with EOP, comparing to healthy subject.

Kinane et al<sup>(14)</sup>, using Boyden chamber among patients with EOP found increasing of chemotactic and chemokinetics behaviour of neutrophils. When they investigated the same parameters in the same group, but this time by subagar method, they couldn't find any difference in Locomotore capability, between patients cells and controls. Thus, it seems that, differences between two techniques, Justify different results of similar investigations. The problems accompanied by such techniques have been known for more than 30 years, but no acceptable method for evaluating Exvivo chemotaxis has been presented yet. One of the reasons for the presence of different results, is the unsimilarity of the type and concentration of chemical agent, applied as chemotactic factor which can affect on the results.

Page et al<sup>(15)</sup> studied Locomotore behaviour of neutrophils in patients with EOP which were chosen accidentally, in relation to the range of FMLP doses ( $10M^{-9}$  to  $10M^{-6}$ ). He stated that the cells of a person in a certain concentration, may show an inhibited chemotaxis, and in another concentration, a normal chemotaxis and finally, in the third concentration, high chemotaxis. The other fact is, daily changes which happen in patients, because, patient's cells may show different Locomotore behaviour every day.<sup>(8)</sup> Another reason is the differential and clinical diagnosis of the disease and the

special index applied for such diagnosis.

Another interesting point is that, although a relationship between PMNS abnormality and EOP development has been recognized, but, no persuasive explanation about PMN chemotaxis deficiency, in some patients with EOP, has been presented yet. Recently, it has been determined that LJP patients, regarding neutrophil deficiency, are not a homogenous group. Some laboratories believe that neutrophile chemotaxis has been suppressed up to (65-75)% and also there are documents, indicative of populations of LJP patients without neutrophil deficiency. The most probable explanation is the presence of differences among different populations and races.

As a result, according to Pederson<sup>(16)</sup> EOP is not always related to leukocyte dysfunction but, they are a group of interrelated diseases with a similar clinical view. They are different in etiology and host biologic defensive mechanisms against pathogenic bacteria.

Although the explanation of rapid tissue destruction in EOP, through PMNS dysfunction is tempting, but it is dangerous because decrease in neutrophils' Locomotore function is not the constant view of EOP patients.

It is obvious that neutrophils' dysfunction, makes the patient susceptible to increased sensitivity to EOP and other recurrent infections. However, decreasing in chemotaxis, in some cases will be the benefit of patients because through reducing neutrophils' speed toward the infected area, the evacuation of the contents of granules and other products such as oxygen radicals to the environment will be decreased. So, the rate of tissue destruction will be reduced. In other words, decrease in chemotaxis can be considered as a defensive mechanism to the benefit of the host.

It seems that chemotaxis deficiency in EOP patients is not so strong to result in a systemic disease and probably the increased activity of immune system, in such people, is more important.

Finally, as a new theory, regarding neutrophil deficiency in EOP patients, is their different HLA genetically which should be discussed especially about patients affected with prepubertal periodontitis with precision.

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