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A CONTROLLED COMPARATIVE EVALUATION OF NEUTROPHIL FUNCTIONS IN TYPE II DIABETIC AND NON-DIABETIC INDIVIDUALS WITH CHRONIC GENERALIZED PERIODONTITIS: A BIOCHEMICAL & MICROBIOLOGICAL STUDY.

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Abstract

Background: The important role of PMNs in optimal functioning of the immune defence system has led to speculation that a partly compromised system could severely weaken the defence mounted against a bacterial insult and permit the occurrence and progression of infections, similarly, it has also been postulated that the compromised PMN function in patients with periodontitis & diabetes may lead to the greater destructive activity of the periodontium as compared to non-diabetic patients with periodontitis.

Aim: To evaluate the possible correlation of the severity of periodontal disease and neutrophil abnormalities in diabetics and non-diabetics

Methods and Materials: The study comprised of 60 subjects, inclusive of both sexes who were in the age group of 40-50 years and were divided into 2 groups of 30 diabetics and 30 non-diabetics with chronic periodontitis.Neutrophil functions which included chemotaxis, phagocytosis, microbicidal function, and superoxide production were estimated & compared between Type II diabetic and non-diabetic patients with chronic generalized periodontitis. The periodontal status was assessed by Periodontal Index & Gingival Index.

Results: In the present study the mean and standard deviation of chemotaxis of neutrophil between the two groups was calculated. By applying Student's Unpaired 't' test there is a highly significant difference between mean values of chemotaxis at positive (i.e. $p<0.01$) and no significant difference at negative and serum in diabetic and non-diabetic cases (i.e. $p>0.05$). And there is a highly significant decrease in the mean values of chemotaxis from positive to negative and serum diabetic and non-diabetic cases. (i.e. $p<0.01$).
Conclusion: Patients with defective neutrophil function often experience rapid and severe periodontal destruction which is more in the diabetic group compared to the non-diabetic groups, which might be one of the reasons for the increasing severity of periodontitis.

Keywords: Neutrophil functions, periodontitis, type II diabetes

INTRODUCTION:
Periodontal diseases are one of the most common microbe-induced chronic inflammatory conditions that lead to gingival inflammation, periodontal tissue destruction, and alveolar bone loss. However, as our understanding of the pathogenesis of periodontal diseases expands, it is becoming clear that most of the tissue damage that characterizes periodontal disease is caused indirectly by the host response to infection rather than directly by the infectious agents.[1]
The influence of diabetes on the periodontium has been thoroughly studied. The majority of clinical and epidemiological evidence demonstrates that individuals with diabetes tend to have a higher prevalence and more severe or rapidly progressing forms of periodontitis than non-diabetics.[2,3] The mechanisms of increased susceptibility may include the development of microangiopathy, formation of nonenzymatic advanced glycation end products (AGEs),[4,5,6] changes insubgingival microbiota, GCF glucose levels, periodontal vasculature, host response, and collagen metabolism and sialadenosis leading to xerostomia in diabetic patients.[7] A more important contribution may be a profound effect of diabetes on polymorphonuclear leukocyte functions.[8]
Diabetes mellitus comprises a heterogeneous group of disorders characterized by altered glucose tolerance, and impaired lipid and carbohydrate metabolism. It is associated with a number of complications directly resulting from hyperglycemia.[3] The 5 classic major complications of diabetes include microangiopathy, nephropathy, neuropathy, microvascular disease, and delayed wound healing. Periodontitis has been recognized as the sixth major complication of diabetes.[9]
Neutrophils are the first line of defence against bacteria that invade tissues and blood. These cells differentiate in the bone marrow, circulate in the blood for 8–12 h, and then enter the tissues where they function for 2–5 days before dying. They kill bacteria in blood or interstitial fluid by phagocytosing them, thereby exposing the ingested bacteria to a variety of potent bactericidal proteins and oxidizing agents.[10]
The important role of PMNs in optimal functioning of the immune defence system has led to speculation that a partly compromised system could severely weaken the defence mounted against a bacterial insult and permit the occurrence and progression of infections, similarly, it has also been postulated that the compromised PMN function in patients with periodontitis & diabetes may lead to the greater destructive activity of the periodontium as compared to non-diabetic patients with periodontitis.
Hence, in the present study, an attempt is made to evaluate the possible correlation of the severity of periodontal disease and neutrophil abnormalities in diabetics and non-diabetics & to assess the influence of diabetes mellitus on the neutrophil functions as estimated by chemotaxis, phagocytosis, killing of P.gingivalis and superoxide production (NBT).

MATERIALS AND METHODS:
The subjects for this study were selected from the outpatient's Department of Periodontology, VPDC&H & OPD of a Private Diabetic Clinic, Sangli.

The study comprised of 60 subjects, inclusive of both sexes who were in the age group of 40-50 years and were divided into 2 groups of 30 diabetics and 30 non-diabetics with chronic periodontitis.

INCLUSION CRITERIA:

- Number of teeth present: 20
- >30% of the site with chronic periodontitis with the presence of disease activity as recorded by the Gingival Index (Loe & Silness J).

EXCLUSION CRITERIA:

1. History of any systemic disease for the non-diabetic group.
2. History of any systemic disease other than diabetes for the diabetic group.
3. History of antibiotic therapy within 6 months prior to the study.
4. Alcoholic & smoker patient.
5. History of scaling and root planning within 6 months.

A standard proforma consisting of the following data: name, age, sex, medical and past dental history, personal history, oral hygiene habits, Russell's periodontal index & gingival index (Loe and Sillness J) for each patient was recorded. Each patient was examined using a mouth mirror and William's graduated periodontal probe under artificial light.

NEUTROPHIL FUNCTION TEST[11, 12]

The tests include chemotaxis, phagocytosis, the killing of P.gingivalis, and superoxide estimation. 5 ml of venous blood was drawn from the antecubital vein with a needle and a disposable syringe; 2.5 ml of this blood was transferred into a plain vial (for phagocytosis) and the remaining 2.5ml into the vial containing EDTA and transported to the laboratory.

PREPARATION OF PMNs CELLS [11]

After doing a total count and differential count of WBCs to confirm their presence in the normal range, the blood collected vials with EDTA were mixed with equal quantities of Minimum Essential Medium (MEM) and one-third volume of 6 % dextran and kept at room temperature for 1 hour. The supernatant was collected in test tubes and centrifuged at 3000 RPM for 5min. Three layers were formed of supernatant, white blood cells, platelets, and red blood cells. It is washed 3-4 times with Phosphate Buffered Saline (PBS) to remove traces of plasma and dextran and cells were maintained in Minimum Essential Medium (MEM).

SPECIFIC GRANULE RELEASE ASSAY [11]

Specific granule release from stimulated human neutrophils was done using a qualitative test by Nitroblue Tetrazolium (NBT) test.

METHOD: (Stimulated test)

0.3 % solution of NBT in 0.34% Sucrose was made. One part of this solution to one part of PBS was added and used fresh. The same concentration was used in the test and control samples.

	STIMULATED	UNSTIMULATED
NBT	**50 microliter**	**50 microliter**
MEM	**200 microliter**	**250 microliter**
Endotoxin	**50 microliter**	**-----**
Blood	**100 microliter**	**100 microliter**

The above-mentioned solution was incubated at 37^{0}C for 30 min and gentle spinning at 400 g for 3-4 min was done. The supernatant was discarded. A drop of PBS was added and the cells were gently resuspended in the small volume of fluid at the bottom of the tube. A film was made by allowing a drop of this fluid to dry on a microscope slide, fixed gently by heating, and counterstained with Giemsa stain for 15 min. It was washed in tap water, dried and mounted, and using a 100x oil-immersion objective, 200 neutrophils count were counted and the percentage of NBT-positive cells containing blue deposits was counted. FIGURE:1

Result: Less than 10% of normal unstimulated neutrophils contain blue granules. Stimulated cells may be over 90% positive.

PHAGOCYTOSIS ASSAY[11]

Preparation of P. gingivalis suspension

Commercially available P. gingivalis in blood agar is grown in Schaedler broth at 37º C, 48 hours prior to phagocytosis. The cultures are spun at 1500 x gyrations for 10 minutes in a centrifuge. The deposits were washed twice with phosphate buffer saline and filtered twice through sterile gauze and resuspended in Hanks balanced salt solution at 5 x10^7 CFU/ml.

	Test	Control
WBC's	250 microliter	250 microliters
BACTERIA	250 microliters	250 microliters
PLASMA	250 microliters	----
MEM	250 microliters	500 microliters

The above-mentioned contents were mixed and incubated at 37°C for 30 min. It was then centrifuged at 200g for 5 min and the supernatant was removed with a micropipette leaving a droplet into which the sediment was suspended. The smears were prepared, dried in the air, and were stained with methylene blue. At least 200 neutrophils were examined. A count of the number of ingested bacteria associated with each cell was counted. FIGURE:2 & 3

Results: Normal neutrophils may contain anything from no bacteria to five or more per cell. A normal control is tested at the same time as the patient's cells and any difference is noted.

BACTERICIDAL ASSAY[11]

The above-mentioned contents are incubated at 37º for 30 min. then to that is added 250 µl of 2.5% sodium deoxycholate, 1ml of 0.01% methylene blue, and centrifuged at 1500 x at 4ºc for 10mins. Remove supernatant with Pasteur pipette leaving a droplet to resuspend the organisms. Put the suspension in an ice bath until ready for counting. Counting should be done within 3 hours with a Neubauer chamber. Count at least 300 bacteria. Dead bacteria appear blue.

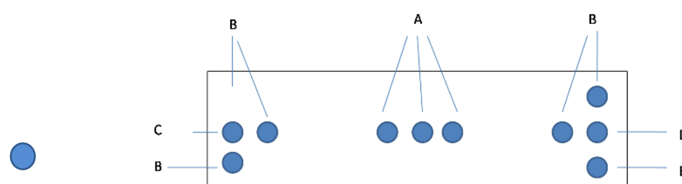
Result: The normal range of killing may vary from laboratory to laboratory but should be between 20-35% of organisms killed in one hour. The control tube should give less than 10% of the dead organism. FIGURE:4

NEUTROPHIL CHEMOTAXIS ASSAY[12]

UNDER AGAROSE TECHNIQUE

PREPARATION OF AGAROSE WELLS:

1) Series of three wells 3mm in diameter were cut and spaced 3mm apart.
   For this purpose, an 11 gauge punch such as Pasteur pipette attached to a vacuum was used.
2) Arrangement of wells for agarose chemotaxis assay.



A) FMLP chemoattractant.
B) White blood cells.
C) MEM.
D) Serum.

PROCEDURE FOR CHEMOTAXIS:

A) 0.01 ml FMLP chemoattractant was added to A well.
B) 0.01ml 2.5×10^7 cells/ml were added to B well.
C) 0.01ml MEM was added to C well.
D) 0.01ml serum was added to Dwell.

PMN's were incubated for 37^0C for 2 hours.

After incubation cells were fixed and stained which are as follows:

a) Culture plates were flooded with 3-5 ml methanol for 30 min and removed.
b) 3-5 ml formalin was flooded for 30 min.
c) Agarose was removed.
d) WBC's were stained on the bottom of culture plates with Wright's stain for 15 min and washed with distilled water.

CALCULATIONS:

1) Culture plates were projected onto the white background so that well is magnified to 5 cm.
2) Chemotaxis was measured as linear distance (in cm) that WBC cells (from B) have migrated from the margin of well toward FMLP chemoattractant (A).

Results: The normal range for spontaneous migration is 0.2 to 0.4 mm and for chemotaxis to FMLP is 0.6 to 1.8 mm.

RESULTS:

Neutrophil functions which included chemotaxis, phagocytosis, microbicidal function, and superoxide production were estimated & compared between Type II diabetic and non-diabetic patients with chronic generalized periodontitis. The periodontal status was assessed by Periodontal Index & Gingival Index.

1. Comparison of the periodontal index in diabetic and non-diabetic groups. After applying the Chi-square test there is a highly significant association between the periodontal index in both diabetic and non-diabetic groups (i.e. $p<0.01$). Incidence of beginning destructive periodontal disease is more (26.66%) in non-diabetics as compared to diabetics (6.66%), established destructive periodontal disease is more in diabetics (56.66 as compared to non-diabetics (56.00%) and terminal disease is more in diabetics (36.66%) as compared to non-diabetics (23.33%).

2. In the present study, Gingival Index between diabetic and non-diabetic groups was compared. After applying the Z test, there is no significant difference between the proportions of the gingival index in diabetic and non-diabetic cases (i.e. $p>0.05$). i.e, both groups showed a similar incidence of mild, moderate, and severe gingivitis.

3. Estimation of superoxide production by the neutrophils of diabetic and non-diabetic groups in the presence of Nitroblue Tetrazolium (NBT) dye, the amount of superoxide production is directly proportional to the number of neutrophils containing blue color formazan crystal. By applying Student's Unpaired 't' test there is a significant difference between mean values of unstimulated and stimulated NBT assay in diabetic and non-diabetic cases. (i.e. $p<0.05$). Also, there is a highly significant difference between mean values of NBT assay in unstimulated and stimulated (i.e. $p<0.01$).

By assessing the mean and standard deviation of phagocytosis of P. gingivalis as evaluated by MPN (mean particle number)& applying Student's Unpaired 't' test there is a statistically significant difference between mean values of phagocytosis in diabetic and non-diabetic cases attest. (i.e. $p<0.05$). And there is a highly significant increase in the mean values of phagocytosis from control to test in diabetic and non-diabetic cases. (i.e. $p<0.01$).

4. By applying Student's Unpaired 't' test there is no significant difference between mean values of bacterial killing at control (i.e. $p>0.05$) and highly significant difference at test in diabetic and non-diabetic cases (i.e. $p<0.01$) and there is a highly significant increase in the mean values of bacterial killing from control to test in diabetic and non-diabetic cases. (i.e. $p<0.01$).

5. In the present study the mean and standard deviation of chemotaxis of neutrophil between the two groups. By applying Student's Unpaired 't' test there is a highly significant difference between mean values of chemotaxis at positive (i.e. $p<0.01$) and no significant difference at negative and serum in diabetic and non-diabetic cases (i.e. $p>0.05$). And there is a highly significant decrease in the mean values of chemotaxis from positive to negative and serum diabetic and non-diabetic cases. (i.e. $p<0.01$).

Table 1. Comparison of mean values of NBT assay in Diabetic and Non-diabetic cases:

NBT assay	Diabetic (n=30)	Non-diabetic (n=30)	Student Unpaired 't' test value	'p' value	Significance
	Mean ± SD	Mean ± SD			
Unstimulated	38.07±13.50	26.50±7.46	4.12	p<0.05	Significant
Stimulated	72.77±11.02	69.53±11.9	1.98	p<0.05	Significant

Table 2. Comparison of mean values of Phagocytosis in Diabetic and Non-diabetic cases:

Phagocytosis	Diabetic (n=30)	Non-diabetic (n=30)	Student Unpaired 't' test value	'p' value	Significance
	Mean ± SD	Mean ± SD			
Control	3.23±0.43	3.23±0.43	0	p>0.05	Not significant
Test	4.20±0.76	4.43±0.62	1.78	P<0.05	significant

Table 3. Comparison of mean values of Bacterial killing in Diabetic and Non-diabetic cases:

Bacterial killing	Diabetic (n=30)	Non-diabetic (n=30)	Student Unpaired 't' test value	'p' value	Significance
	Mean ± SD	Mean ± SD			
Control	18.4±1.03	18.23±1.27	0.57	p>0.05	Not significant
Test	32.50±2.20	34.16±2.60	2.66	p<0.01	Highly significant

Table 4. Comparison of mean values of Chemotaxis in Diabetic and Non-diabetic cases:

Chemotaxis	Diabetic (n=30)	Non-diabetic (n=30)	Student Unpaired 't' test value	'p' value	Significance

	Mean ± SD	Mean ± SD			
Positive	**1.67 ± 0.38**	**2.12 ± 0.10**	**6.52**	**p<0.01**	**Highly significant**
Negative	**0.62 ± 0.06**	**0.606 ± 0.06**	**0.93**	**p>0.05**	**Not significant**
Serum	**0.66 ± 0.10**	**0.63 ± 0.06**	**1.42**	**p>0.05**	**Not significant**

DISCUSSION:

Periodontitis is a disease attributable to multiple infectious agents and interconnected cellular and humoral host responses. It is widely accepted that bacteria or bacterial substances of dental plaque are the primary etiologic factors in the initiation of inflammatory processes and periodontal disease. The specific microflora composition in the periodontal pocket is important in determining the severity and extent of destruction to the periodontium. However, an individual's susceptibility may serve as an important factor in the pathogenesis of periodontitis.

According to a few investigators, the primary factor responsible for the development of diabetic complications is prolonged tissue response due to hyperglycemia. Hyperglycemia results in various changes like the production of advanced glycated end products, changes in the subgingival microbiota, increase GCF glucose levels, alteration in periodontal vasculature, decreased host response, reduced collagen metabolism & sialadenosis. Also, there may be an increase in the local production of cytokines leading to connective tissue damage, bone resorption, and delayed wound repair.[13]

The periodontal index was found to be much higher in diabetic patients when compared to non-diabetic patients. In the beginning, destructive periodontal disease was recorded as 6.66%, established destructive periodontal disease to be 56.66%, and terminal disease in 36.66% in diabetic patients. Group II although having had shown 26.66% of patients suffering from beginning destructive periodontal disease which is significantly higher than that in Group I. 50% of patients of non-diabetic were suffering from having established destructive periodontal disease and 23.33% were having a terminal disease, these values were significantly lower than that of a diabetic group. The findings from the present were in agreement with studies done by Belting et al [14], Sandholm L et al [15], Hugoson et al [16] in which similar results were obtained.

The results of the present study demonstrated that diabetic patients with periodontitis had defects in neutrophil functions when compared to healthy subjects with periodontitis. The mean chemotaxis between FMLP of the diabetic group was 1.67 ± 0.38, the non-diabetic group was 2.12 ± 0.10 with p<0.01, which is in agreement with the study done by M. Manouchehr-Pour et al [17], Spagnuolo P et al [18] who showed significantly less chemotaxis with diabetes mellitus. They suggested that the defect in chemotaxis of diabetic leukocytes could contribute to increased infections in these patients.

When phagocytosis of P. gingivalis was evaluated by mean particle number (MPN), that is the number of P. gingivalis phagocytosed by the neutrophils, it was seen that there is no statistically significant difference in MPN between diabetic & non-diabetic group. The MPN of the diabetic group was 4.03±0.76, the non-diabetic group was 4.97±0.62 with p<0.05. Martha Walter et al [19] have performed a study to evaluate phagocytosis of neutrophil in diabetic & non-diabetic patients with periodontitis and found impaired phagocytosis in diabetic patients. The finding in this study shows similar results. In the present study, P.gingivalis was used as a model organism because it is one of the primary pathogens causing periodontitis.

Comparing the intracellular killing capacity of the neutrophils in both the groups of the patient; the diabetic group of the patient has demonstrated significantly low intracellular microbicidal function.

This abnormality was pronounced in the diabetics but was also present to some extent in healthy subjects with periodontitis. The mean values of the bacterial killing of the diabetic group were 32.50±2.20, the non-diabetic group was 34.16±2.60 with $p<0.01$. This finding is in agreement with the study by T.C. Alba-Loureiro[8] who stated that the local polysaccharides may alter oxidative burst capacity to impair killing.

When specific granule release was assayed using stimulated and unstimulated cells in the presence of Nitroblue Tetrazolium (NBT) dye which is an indirect method to estimate superoxide production, it was found that the amount of the blue color formazan crystal containing neutrophil was less in unstimulated cells in non-diabetic compared to diabetic subjects. Stimulated cells of both the groups showed that the dye was taken up into phagosomes and intracellular reduction of dye converted it to an insoluble blue color formazan crystal form which was visible in the light microscope. The NBT stimulated for the non-diabetic group was 69.53±11.9 and for the diabetic group was 72.77±11.02 with $p< 0.05$. The NBT unstimulated for the non-diabetic group was 26.50±7.46, and the diabetic group was 38.07±13.50 with $p<0.05$. This result was in agreement with the study performed by Martha Walters et al[19]who found that the superoxide production by neutrophils in diabetics was more than that in healthy subjects with periodontitis.

In diabetic patients, there is a definite correlation between neutrophil function like NBT assay, phagocytosis, bacterial killing, and chemotaxis with periodontal index. The increase in values of NBT assay (56.76 to 73.88) and a simultaneous decrease in values of phagocytosis (4.23 to 3.76), bacterial killing (33.5 to 30.64) and chemotaxis (1.90 to 1.63) as the periodontal status of the diabetic patient progress from beginning destructive periodontal disease to terminal disease has been reported in the present study. On the other hand, there is no significant correlation between neutrophil function and periodontal index in a non-diabetic group of patients.

CONCLUSION:

The following conclusions were derived from this study:

- In the diabetic group, periodontal destruction was more compared to the non-diabetic group.
- There is no significant correlation between the Gingival Index between the diabetic and non-diabetic groups.

Patients with defective neutrophil function often experience rapid and severe periodontal destruction which is more in the diabetic group compared to the non-diabetic groups, which might be one of the reasons for the increasing severity of periodontitis.

Future consideration:

Nevertheless, despite the complexity in determining the most important inflammatory and immune pathways in these diseases, an understanding of neutrophil function can aid in the development of new diagnostic and treatment approaches. In particular, the potential to reverse acquired neutrophil defects using a variety of approaches from basic mechanical debridement to the strategic therapeutic use of newly identified anti-inflammatory agents may lead to more cost-effective approaches in combating periodontal disease in diabetes.

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