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ORIGINAL RESEARCH

Potential Effect of Neutrophil Functional Disorders on Pathogenesis of Aggressive Periodontitis

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ABSTRACT

Introduction: Leukocytes play a key role in maintaining the balance between an effective host defence response to microorganisms and periodontal tissue destruction. Neutrophil dysfunction has been associated with increased susceptibility to periodontal diseases. We undertook this study to determine to what extent neutrophil dysfunction constitutes to the pathogenesis of aggressive periodontitis (AgP) in tropical country like ours.

Materials and methods: Age- and sex-matched groups consisting of 20 subjects each of generalized aggressive periodontitis (GAP)–cases and nonperiodontitis (NP)–controls. diabetes mellitus, HIV infection, prolonged antibiotic use and smoking were excluded.

Each neutrophil function was assessed using the chemotactic assay using case in, phagocytosis assay, candidacidal assay (for intracellular killing) and NBT assay (for respiratory burst failure).

Statistical analysis used: Student's t-test, Fisher's exact test and Chi-square test.

Results: In the study 17 out of 20 subjects (85%) had at least one abnormal neutrophil assay either hypofunctional or hyperfunctional of which 16 (80%) had hypofunctional assays and 8 (40%) had hyperfunctional assays. Defective phagocytosis was the commonest (50%) followed by chemotactic defect (45%), defective respiratory burst (40%) and defective intracellular killing (30%). Mean of chemotaxis assay was significantly less in AgP when compared to controls (103 *vs* 129 µm, $p = 0.002$), similarly for phagocytic defect (3.45 *vs* 4.65, $p \leq 0.001$) and with candidacidal assay (26.80 *vs* 37.35, $p < 0.001$).

Conclusion: The prevalence of neutrophil dysfunction, predominantly hypofunctional, was significantly very high in GAP patients with few even having hyperactive respiratory burst function. Multiple level neutrophil defects could account for the aggressive nature of AgP even in apparently healthy subjects.

Keywords: Aggressive periodontitis, Chemotactic defect, Phagocytic defect, Respiratory burst failure, Intracellular killing.

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INTRODUCTION

The periodontal diseases are now recognized as 'ecogenetic' diseases. Hence, it is appropriate to consider not only the effects of bacteria on the tissue but also how host immune and inflammatory response, local and systemic environment factors might impact on the gingival and periodontal connective tissue structure and function.[1]

Neutrophils (PMNs) constitute the host's first line of defence against infectious agents and have a key role in maintaining the balance between an effective host response to microorganisms in microbial plaque and the periodontal destruction. In this context, several investigators have shown that patients with aggressive periodontitis (AgP) display functional defects of PMNs. These defects impair either the chemotactic attraction of PMNs to the site of infection or their ability to phagocytose and kill microorganisms. The possibility of a PMN chemotactic defect particularly in prepubertal periodontitis was first indicated in the studies from Cianciola et al and Clark et al as early as 1977.[2,3] Later other studies have tended to support this idea.[4,5] Recently, Asif and Kothiwale[6] did show altered phagocytic activity of crevicular and peripheral neutrophils in subjects with AgP. Also, now newer aspect being studied is hyperresponsiveness of neutrophils in periodontal tissue damage.[7] There is limited literature in our country on these aspects. We undertook this case control study to determine to what extent neutrophil dysfunction constitutes to the pathogenesis of generalized aggressive periodontitis (GAP) in our country and to focus on each aspects of neutrophil function–*viz* chemotaxis, phagocytosis, respiratory burst activity and intracellular killing.

MATERIALS AND METHODS

The present study was carried out in the Department of Periodontics, PMNM Dental College and Hospital, Bagalkot. The study group was divided into two groups–case group and control group each comprising of 20 systemically healthy patients which were age and sex mathced (Table 1). Case group consisted of subjects with GAP with clinical generalized intraproximal attachment loss affecting atleast three permanent teeth other than the first molars and the incisors with the radiological investigation in an affected individual below 30 years and were selected from those visiting the department for various treatment needs. Control group comprised of subjects with nonperiodontitis (NP).

Inclusion Criteria

According to the criteria established by 1999 International Workshop for classification of periodontal disease and conditions,[8] in order to receive a diagnosis of AgP, patients were required to:

- Be systemically healthy.
- Display a severity of disease disproportionate to the amount of local etiological factors.
- Show evidence of rapid attachment loss and bone loss either with respect to their age or based on comparisons of available sets of clinical and radiographic records.

Exclusion Criteria

- Patients with known systemic disease like diabetes mellitus (DM), renal failures or hypertension and others.
- Patients who had undergone oral prophylaxis or any other periodontal therapy within 3 months prior to the study.
- Patients who had taken antibiotics for any reasons within 3 months prior to the study.
- Smokers.

Ethical clearance was obtained from the ethical committee of the institution. An informed consent was obtained from all patients before the start of study. A brief case history was taken which included the demographic data, chief complaint, medical and dental history, family history and personal history (via proforma). A complete periodontal evaluation was done which included intraoral examination, oral hygiene index-simplified (Greene and Vermilion), probing pocket depth (William's graduated periodontal probe), clinical loss of attachment, mobility (Miller), pathologic tooth migration, missing teeth and full mouth periapical radiographs.[9-11]

In each case 5 ml of peripheral venous blood was drawn with aseptic precautions, mixed with EDTA and transported to laboratory. The blood was diluted with an equal volume of Hanks-Mops solution and dextran (1 volume per 10 volume blood). After 45 minutes, the leukocyte rich plasma is pipetted out and layered over an equal volume of Ficoll-Triosil mixture in a conical centrifuge tube–this separates the neutrophils from lymphocytes and monocytes. A white cell count should be carried out on the final cell preparation and the test and control counts should be approximately equal.

The cells of each subject were subjected to each of neutrophil function tests namely, chemotactic assay with and without casein, nitroblue tetrazolium test (NBT) for phagocytosis and oxidative burst function, phagocytic assay using killed *Candida albicans* and intracellular killing assay using live *Candida albicans*. The descriptive methodology of each assay is beyond scope of this article and further details can be sought from the quoted reference.[12] Only the principle underlying each assay is mentioned.

Chemotactic Test (Fig. 1)

The migration of PMNs was assessed by using a modified Boyden chemotactic chamber in which movement of PMNs through filters is measured by microscopy in response to chemotactic reagents.

Phagocytosis Assay (Figs 2 and 3)

Killed *Candida albicans* are mixed with PMNs suspension, smear prepared and then under microscope, the number of ingested Candida associated with each cell is counted and the mean particle number calculated.

Nitroblue Tetrazolium Test (Fig. 4)

The cells are exposed to the yellow dye NBT. Unstimulated neutrophils do not ingest this dye but if the cells are stimulated by endotoxin to phagocytic activity, they take the dye into phagosomes and intracellular reduction of the

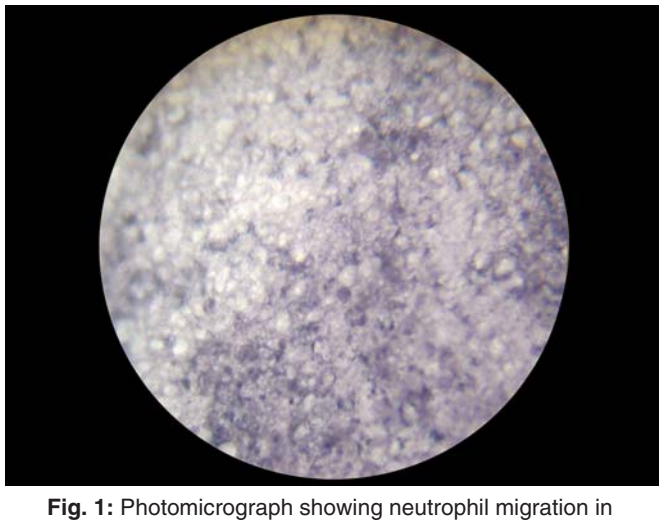

Fig. 1: Photomicrograph showing neutrophil migration in chemotaxis assay

dye converts it to an insoluble, blue crystalline from (formazan crystals). The NBT gives information about phagocytic function, since the dye is not taken into cells except by phagocytosis. It also gives information about metabolic function since intracellular reduction depends on hexose monophosphate shunt activation, an activation which is also necessary for normal microbicidal activity.

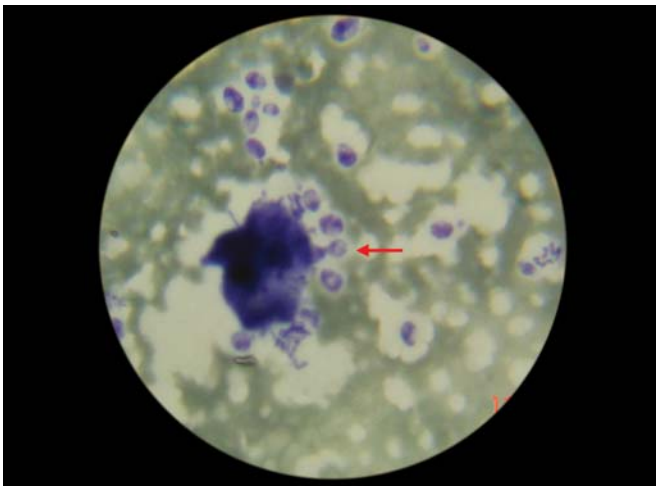

Fig. 2: Photomicrograph showing opsonization (binding of *Candida albicans* with receptors of neutrophils) in phagocytosis assay

Candidacidal Assay (Intracellular Killing Assay) (Figs 5 and 6)

Here live *Candida albicans* are mixed with PMNs suspension and the sodium deoxycholate added which lyses the leukocytes but does not damage the Candida. This is then stained with methylene blue and proportion of dead Candida counted, i.e. those cells which have taken methylene blue.

STATISTICAL METHODS

Chi-square test/Fisher's exact test has been used to find the significant association of study parameters between the patients and controls and Student t-test has been used to

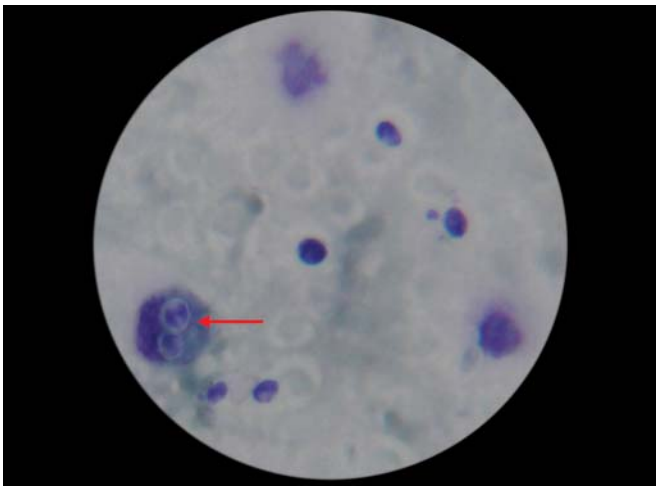

Fig. 3: Photomicrograph showing engulfment of *Candida albicans* by neutrophils in phagocytosis assay

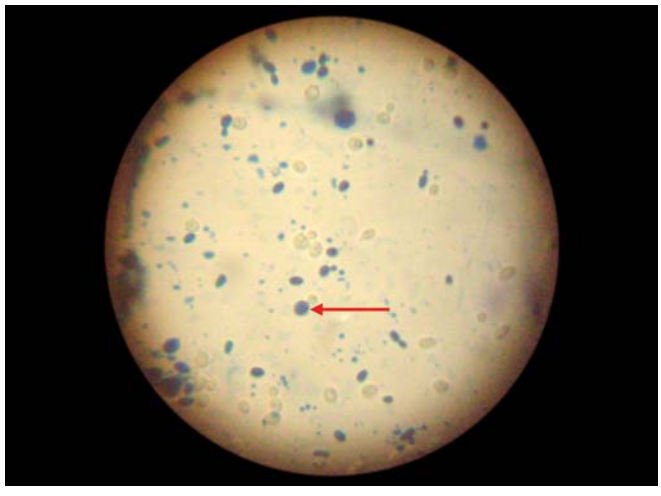

Fig. 5: Photomicrograph showing dead cells (taken blue dye) and clear viable cells (without blue dye) in candidacidal assay

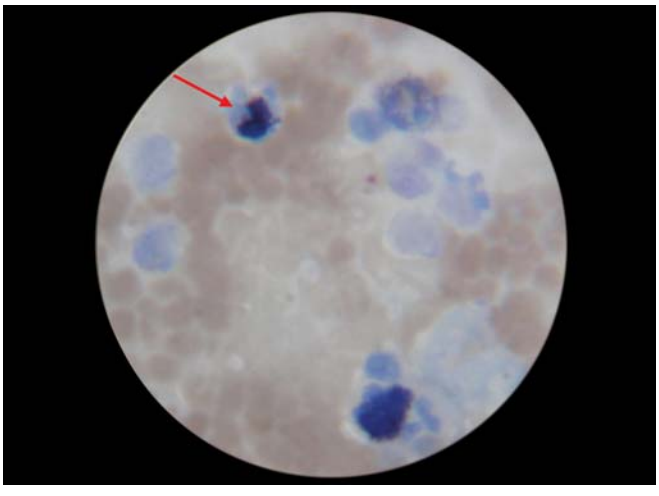

Fig. 4: Photomicrograph showing neutrophil intracellular reduction with formation of formazan crystals in NBT assay

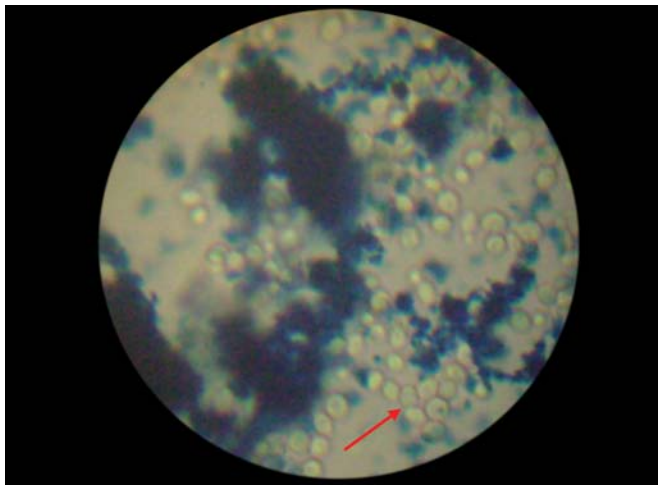

Fig. 6: Photomicrograph showing more number of viable cells suggestive of defective intracellular killing by neutrophils in candidacidal assay

RESULTS

Table 1: Age and sex distribution in cases and controls

Age (in years)	*Cases*	*Controls*	*p-value*
Age (in years)	25.30 ± 3.70	25.05 ± 3.62	0.830
Sex			
Male	11 (55.0%)	10 (50.0%)	0.752
Female	9 (45.0%)	10 (50.0%)	
Total	20	20	Matched

Table 2: Proportion of subjects having neutrophil dysfunction based on chemotaxis assay in cases and controls (number of cases and percentage)

Chemotaxis assay	*Hyperactive/ hypofunctional*	*Cases (n = 20)*	*Controls (n = 20)*	*p-value*	*Significance*
Minimum essential medium (MEM)	Normal	16 (80.0%)	20 (100.0%)	0.106	NS
	Hyperactive	4 (20.0%)	–		
Case in	Normal	11 (55.0%)	20 (100.0%)	0.001	SS
	Hypofunctional	09 (45.0%)	–		

Chi-square test used; SS: Strongly significant; NS: Nonsignificant

Table 3: Proportion of subjects having neutrophil dysfunction based on phagocytosis assay in cases and controls

Phagocytosis assay	*Cases (n = 20)*	*Controls (n = 20)*	*p-value*	*Significance*
Normal	10 (50.0%)	20 (100.0%)	<0.001	SS
Hypofunctional	10 (50.0%)	–		

Chi-square test used; SS: Strongly significant

Table 4: Proportion of neutrophil dysfunction based on NBT assay in cases and controls (number of cases and percentage)

NBT	*Hyperactive/ hypofunctional*	*Cases (n = 20)*	*Controls (n = 20)*	*p-value*	*Significance*
Unstimulated	Hyperactive	4 (20.0%)	0	0.106	NS
Stimulated	Hypofunctional	8 (40.0%)	0	0.003	SS

Fisher's exact test used; Hyperfunctional neutrophils defined as >30% on unstimulated NBT assay; Hypofunctional neutrophils defined as <35% on stimulated NBT assay; NS: Non significant; SS: Strongly significant

Table 5: Proportion of subjects having neutrophil dysfunction based on candidacidal assay in cases and controls

Candid assay	*Cases (n = 20)*	*Controls (n = 20)*	*p-value*	*Significance*
Normal	14 (70.0%)	20 (100.0%)		
Hypofunctional	6 (30.0%)	–	0.020	MS

Chi-square test used; MS: Moderately significant

Table 6: Comparison of mean values of individual neutrophil assays by NBT, phagocytosis, candidacidal and chemotaxis in cases and controls

Study parameters	*Cases*	*Controls*	*p-value*	*Significance*
Chemotactic assay using casein	103.00 ± 32.21 (60-141 µm)	129.10 ± 8.98 (118-146 µm)	0.002	SS
Phagocytosis assay—no. of organisms ingested	3.45 ± 0.61 (2-4)	4.65 ± 0.49 (4-5)	<0.001	SS
NBT stimulated	42.70 ± 12.81 (26-80%)	44.00 ± 3.99 (37-50%)	0.669	NS
Candidacidal assay (% killed)	26.80 ± 3.17 (22-35%)	37.35 ± 2.16 (34-41%)	<0.001	SS

Results are presented in mean ± SD (min-max); Student t-test (Two-tailed and independent); SS: Strongly significant; NS: Non significant

Table 7: Overview chart showing abnormal neutrophil assays in individual subjects

Serial no.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	Total (%)
Age	28	26	21	25	23	24	29	19	19	28	29	25	27	17	27	29	29	27	28	26	-
Sex	F	M	M	M	F	M	M	F	F	M	M	M	M	F	F	M	M	F	F	F	-
NBT assay (US) hyperactive	N	N	N	N	Y	N	N	N	N	Y	N	N	N	Y	N	Y	N	N	N	N	04 (20%)
NBT assay (S) hypoactive	N	N	N	Y	N	Y	N	N	N	N	Y	Y	N	N	Y	N	Y	Y	N	Y	08 (40%)
Phagocytosis assay hypoactive	Y	N	N	Y	N	N	Y	N	N	Y	Y	Y	N	N	Y	N	Y	Y	N	Y	10 (50%)
Candidacidal assay hypoactive	N	N	Y	Y	N	Y	N	N	N	N	N	N	N	N	N	Y	N	Y	Y	N	06 (30%)
Chemotaxis assay (M) hyperactive	N	N	Y	N	N	N	N	Y	N	N	N	N	N	N	N	N	Y	N	Y	N	04 (20%)
Chemotaxis assay (C) hypoactive	N	N	N	N	Y	Y	N	N	N	Y	Y	Y	N	Y	Y	Y	N	Y	N	N	09 (45%)
Any abnormal assay	Y	N	Y	Y	Y	Y	Y	Y	N	Y	Y	Y	N	Y	Y	Y	Y	Y	Y	Y	17 (85%)
Hypoactive assay	Y	N	Y	Y	Y	Y	Y	N	N	Y	Y	Y	N	Y	Y	Y	Y	Y	Y	Y	16 (80%)
Hyperactive assay	N	N	Y	N	Y	N	N	Y	N	Y	N	N	N	Y	N	Y	Y	N	Y	N	08 (40%)
Combined hypo- and hyperactive assays	-	-	√	-	√	-	-	-	-	√	-	-	-	√	-	√	√	-	√	-	07 (35%)

NBT: Nitroblue tetrazolium test; US: Unstimulated; S: Stimulated; M: Minimal essential medium; C: Casein; Y: Yes; N: No

find the significance difference of study parameters, between cases and controls. The significant figures were taken as suggestive significance $0.05 < p < 0.10$, moderately significant $0.01 < p \leq 0.05$ and strongly significant $p \leq 0.01$. The statistical software namely SPSS 15.0, Stata 8.0, Medical 9.0.1 and Systat 11.0 were used for the analysis of the data.

DISCUSSION

The present study was a case control study design consisting of 20 subjects of GAP as cases and 20 NP as controls to determine various types of neutrophil dysfunction in patients with AgP.

The present study excluded all other risk factors for periodontitis like DM, human immunodeficiency virus (HIV), prolonged antibiotic use and smoking. The design of the study in itself eliminated the possible confounding factors. This study also looked at both extremes of neutrophil function, i.e. both hyperfunctional and hypofunctional activity.

A very high prevalence of neutrophil dysfunction was observed in this study. Seventeen out of 20 subjects (85%) had atleast one abnormal neutrophil assay; either hypofunctional or hyperfunctional. Among 17 subjects with abnormal neutrophil assays, 16 (80%) had hypofunctional assays and 8 (40%) had hyperfunctional assays. Seven (35%) subjects had both hypofunctional and hyperfunctional neutrophils at different phases of neutrophil function (Table 7).

We compared each individual neutrophil assay in GAP with that of NP with respect to (i) proportion of dysfunction and (ii) the mean values of respective assay.

The chemotaxis was studied both in presence as well as in absence of casein. In the casein stimulated chemotactic assay, 9 of 20 (45%) subjects had a chemotactic defect compared to none in controls which was statistically significant, $p = 0.001$ (Table 2). The finding of chemotactic defect in our study is comparable to study done by Suzuki et al[13] who reported 58% chemotactic defect in patients with generalized juvenile periodontitis. In our study, even when the mean chemotactic migration distance of the neutrophils in response to casein was considered, the value was very significantly less in GAP when compared to NP (103 *vs* 129 µm, $p = 0.002$, Table 6). The possibility of a PMN chemotactic defect particularly in prepubertal periodontitis was first indicated in the studies from Cianciola et al[2] and Clark et al[3] as early as 1977. Singh et al showed that neutrophil migration through the gingival crevice in response to challenge by the chemotactic agent, casein, was abnormal, providing *in vivo* evidence of altered neutrophil function in LJP.[14] So, it is now generally accepted that a PMN chemotactic defect is characteristic of most cases of early onset periodontitis.

The phagocytosis assay examined the ability of neutrophil to ingest organisms (here Candida), i.e. phagocytosis. In this study, 10 (50%) had defective phagocytosis in GAP group compared to none in NP group which was highly significant ($p < 0.001$; Table 3). This was the commonest neutrophil defect seen in patients with GAP. Suzuki et al[12] reported a prevalence of 29% in patients with generalized juvenile periodontitis whereas Cianciola et al[2] reported universal depression of phagocytic activity in juvenile periodontitis.

Further, in this study, the mean number of organisms ingested was significantly less in GAP when compared to NP (3.45 *vs* 4.65, $p < 0.001$; Table 6). The mean percentage of PMNs with phagocytosis of opsonized *C. albicans* was significantly reduced compared to controls in an Indian study by Asif and Kothiwale.[6] On further subanalysis, the stimulated NBT assay was impaired in 7 of 10 subjects with phagocytic defect and of these 7 subjects, 5 had normal candidacidal response. Such a finding is obvious since a stimulated NBT assay not only looks at respiratory burst function but also in itself is test for phagocytosis. An abnormal NBT reduction and normal bactericidal response indicates impaired phagocytosis.[5]

The NBT assay to assess respiratory burst failure was studied by examining it in both absences (unstimulated) as well as presence of endotoxin (stimulated). In the unstimulated NBT assay, 4 (20%) had hyperactive oxidative burst among GAP group suggesting that neutrophils in this subjects were in stimulated state probably because of pre-existing endotoxin in these subjects. Van Dyke in recent review article[15] suggested that neutrophil abnormalities in LAP are the result of chronic hyperactivated or 'primed' state of the LAP neutrophil. Leino et al also demonstrated that oxidative burst is increased in many localized AgP cases compared with healthy controls.[16] Interestingly, in our study all the 4 subjects with hyperactive oxidative burst had impaired chemotaxis which could explain why inflammation and periodontal damage may be seen at locations away from the site of infection. In fact, Debski et al showed that although the migration of neutrophil is delayed, experimental evidence suggests that activation mechanisms of the neutrophil are not impaired.[17] It may be argued, therefore, that release of granule contents in connective tissue rather than in direct proximity to the microbes might contribute to local connective tissue destruction.

In the stimulated NBT assay, 8 (40%) subjects had defective oxidative burst mechanism compared to none in controls which was statistically significant ($p = 0.003$; Table 4). Further, when the mean values were compared, it was less in cases compared to controls however this was not significant (Table 6). This was because of the hyperfunctional values seen in 4 subjects which would have confounded the overall mean values in the cases.

The candidacidal assay assessed the neutrophil function of intracellular killing (here taken as percentage of Candida killed). Six (30%) in GAP group had reduced intracellular killing compared to none in NP which was statistically significant ($p = 0.02$, Table 5). Also the mean percentage of Candida killed in GAP group was significantly less when compared to NP group (26.80 *vs* 37.35, $p < 0.001$, Table 6). Kalmar JR, Arnold and Van Dyke evaluated killing of *Actinobacillus actinomycetemcomitans* (Aa) by LJP neutrophils.[18] They observed that LJP neutrophil viability was retained in the presence of LJP serum and equivalent phagocytosis was observed; however killing Aa by LJP neutrophils was significantly reduced, suggesting a qualitative defect in LJP neutrophils in the killing of a specific pathogen. In the present study too, 4 out of 6 had normal phagocytic activity but had defective killing of Candida.

Under a new paradigm, as described by Van Dyke and Serhan,[14] the neutrophil is not hypofunctional or deficient, but hyperfunctional, and it is the excess activity and release of toxic products from the cells that are responsible, in part for the tissue destruction in chronic periodontal inflammation. More recent research supports this new paradigm.[7,19] In the present study, totally 8 (40%) subjects among the GAP group had hyperfunctional neutrophils in absence of stimulation; 4 with increased oxidative burst and 4 with increased chemotaxis. The difference of cumulative proportion of hyperactive neutrophils (4 + 4 = 8; 40%) was significant between GAP and NP. Thus, the presence of hyperactive neutrophils in the present study was in trend with this new paradigm. However, when the unstimulated NBT and chemotactic assay were compared individually, the difference was not significant. This means that larger cohort studies will be required to further understand the role of hyperactive neutrophils in the pathogenesis of AgP.

To sum it up, neutrophil defects are commonly seen in AgP which is seen even in absence of systemic risk factors. Such defects are predominantly hypofunctional and moreover many have multiple level defects.

CONCLUSION

Neutrophil dysfunction contributes significantly to the pathogenesis of AgP which is independent of systemic risk factors. It is the hypofunctional neutrophils which predominate, however, at the same time the role of hyperfunctional neutrophils cannot be undermined and needs to be studied further with larger cohort sample.

CLINICAL SIGNIFICANCE

Recognition of neutrophil dysfunction would help in planning and individualization of periodontal treatment in such patients. Such approach would translate into successful treatment outcome of patients with AgP.

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