Cytomorphometric Analysis of Buccal Smears in Treated Leprosy Patients and Healthy Individuals: A Comparative Study
Pooja VK, Anila K, Vanishree M, Santosh Hunasgi, Surekha R, Vardendra Manvikar

Abstract
Leprosy is a chronic multisystem disease caused by Mycobacterium Leprae. Oral manifestations occur in 20-60% of the cases. There is no available literature on oral cytological changes related to leprosy. The aim of the present study is to evaluate the cytological changes in the buccal smears of treated leprosy patients and healthy individuals. Materials and methods: The study was carried out in 30 treated leprosy patients and 30 healthy individuals. The buccal smears obtained were stained by rapid papanicolaou stain. The cellular and nuclear areas were measured using image analyzer and compared with 30 healthy individuals. Student’s t test was used for statistical analysis. Results: Cytomorphometric analysis showed a statistically significant increase in average nuclear area (p<0.0001) and significant decrease in average cellular area (p=0.0014) when compared to healthy individuals. Conclusion: Our study focused on oral cytological changes seen in treated leprosy patients. The changes observed can be attributed to Lepra bacilli or multi drug therapy used for longer duration. Furthermore light has to be thrown with larger sample size for the better understanding of the pathogenesis of these oral cytological changes seen in treated leprosy patients.

Keywords: Leprosy; Oral; Cytomorphometry; Xerostomia; Multidrug Therapy.

Introduction
Leprosy is a chronic infectious and granulomatous disease that mainly affects the skin, peripheral nerves and the mucous membranes caused by the organism Mycobacterium leprae. It was first established by Armeur Hansen in Norway in 1874 as a cause of leprosy. Leprosy is one of the oldest diseases recorded. Bony changes of leprosy were seen in skeletons dating to the second century B.C. Evidence suggested that its spread to east from India to China at around 500 B.C. and then to Greece in the west by the soldiers of ‘Alexander the Great’ after their Indian Campaign in the third century B.C.

The global burden of leprosy has declined dramatically, from 5.2 million cases in 1985 to 204,800 cases at the end of 2009, having a prevalent rate which is <1 per 10,000. In India, after the introduction of MDT, the leprosy case load came down from 57.6 cases per 10,000 population in 1985 to less than one case per 10,000 population in 2005. Although the exact route of transmission is not known, the high number of organisms in nasal secretions suggests that in some cases the initial site of infection may be from nasal or oro-pharyngeal mucosa. Oral mucosal lesions are seen in about 20-60% cases of lepromatous leprosy, while they are quite rare in the tuberculoid and the borderline forms. The lesions are proportional to the duration of the disease, indicating that these are late manifestations.

Oral exfoliative cytology is a quick, simple, less technically demanding, painless, and non-invasive laboratory procedure for microscopic investigation of different kinds of oral diseases. As a diagnostic tool, it has got an immense value in mass screening programs of high-risk adult population. It can be done chair side during routine dental examination. The use of oral exfoliative cytology in the past was limited due to the subjective nature of its interpretations and high false-negative results. These limitations were overcome by the introduction of quantitative methods such as image analysis systems, especially in the assessment of cytomorphic cellular alterations. Thus the aim of the present study was to evaluate the quantitative changes in nuclear area (NA), cytoplasmic area (CA) in cytological buccal smears of leprosy patients by comparing with normal healthy individuals.

Material and methods
The present study was carried out in 30 treated leprosy patients in the age range between 35-60yrs and 30 age and sex matched normal healthy individuals as controls group. The control group consisted of healthy individuals free of any systemic diseases, with clinically normal oral mucosa and free of any habits /nutritional deficiency. All the patients residing in leprosy colony in Raichur district, Karnataka, India were included irrespective of the type of leprosy. Treated leprosy patients with smoking and tobacco chewing habits, habitual alcohol intake, presence of oral sepsis, presence of other systemic diseases, and presence of clinically evident nutritional deficiencies were excluded from the study. Presence of Xerostomia was also noted by checking whether saliva pools in the floor of the mouth. Written informed consent was obtained and the proforma record was completed detailing name, age, gender, the clinical examination of the oral cavity and for publication of photographs.

Smears were prepared using a sterile metal spatula by gentling scraping buccal mucosa of each individual. Slides were then fixed in absolute alcohol and stained using “PAP” technique. Each smear was assessed with the image analysis system (Image Pro-Express Version 6.0) under research microscope (BX51M) and digital CCD camera at ×40 magnification. Pap-stained smears were observed in a stepwise manner, moving from left to right and then down across, in order to avoid measuring the same cells again. An average of 20 clearly defined cells selected in each case, were projected on to the monitor via the camera at ×40 magnification and images were captured.

Cytomorphometric analysis was done by selecting the function “Measurement mode” with the icon specifying “Polygon”, and thereafter the auto-trace function was also enabled. Using the mouse, the cursor was placed at a point on the perimeter of the image of the cell on the screen and traced for a short distance. On double-clicking the mouse, the tracing automatically follows the perimeter and completes the outline. This outline of the region of interest calculates the number of pixels in the cell image and displays the area in square microns. Similarly, the perimeter of the nucleus was traced and the area determined. The measurements of NA and CA that were automatically displayed were recorded and the C/N ratio was calculated for all 20 cells, and the average was derived for each individual. All the above parameters were calculated by the image analysis software, thereby considerably reducing the subjective error. All the results were indicated as mean±SD for quantitative variables and as percentage for categorical variables. The statistical analysis was done using student...
‘t-test’, p value < 0.05 was considered as statistically significant.

**Results**

All the 30 treated leprosy patients examined were a multibacillary form of leprosy. The age of treated leprosy patients and control group was in the range of 35-60 years. Majority of the patients were in the age group of 46-50yrs (Table 1). In both treated leprosy patients and healthy individuals 16 were males and 14 were females. All the patients were under Multidrug regime therapy (MDT) as per the WHO guidelines. Among 30 treated leprosy patients 28 patients had xerostomia and none of the healthy individuals had xerostomia. PAP stained leprosy smears comprised of superficial cells and intermediate type of cells arranged in singles and also in sheets. Few inflammatory cells and microorganisms were also seen (Fig 1a).

**Table: 1 Age group of treated leprosy patients and healthy individuals.**

<table>
<thead>
<tr>
<th>Age group</th>
<th>Leprosy patients</th>
<th>Healthy individuals</th>
</tr>
</thead>
<tbody>
<tr>
<td>35-40</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>41-45</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>46-50</td>
<td>14</td>
<td>14</td>
</tr>
<tr>
<td>51-55</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>56-60</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

The mean cellular area (CA) of cells in treated leprosy patients was 2600 ± 368.3 and in the cells of healthy individuals was 2800 ± 213.3. On statistical comparison of CA between treated leprosy patients and healthy individuals showed a significant difference with a P value of 0.0014 (Fig 1b and c). The mean nuclear area (NA) of cells in leprosy smear cells was 72 ± 14.06 and in the cells of healthy individuals was 56 ± 5.78. On statistical comparison of NA between treated leprosy patients and healthy individuals showed a significant difference with a P-value of <0.0001 (Fig 1d and e) [Table 2].

**Discussion**

Hansen’s disease or Leprosy classically presents as hypo pigmented cutaneous macules along with sensory and motor peripheral neuropathies, although the clinical manifestations vary along a disease spectrum. In addition to primary infection, patients may undergo a “reaction,” an acute inflammatory response to the mycobacterium, which leads to pain and erythema of skin. Oro-facial lesions in leprosy patients develop insidiously, generally asymptomatic and are secondary to nasal changes. Leprotic oral lesions which are more common in the lepromatous form of leprosy, indicate a late manifestation, and have a great epidemiological importance as a source of infection. The oral lesions usually appear as ulcerations on the hard or soft palate, gingiva in the anterior portion of the maxilla, uvula and the tongue.

Reactions can occur at any time during the course of leprosy, but they tend to be precipitated by treatment. Although leprosy may have a protracted onset and be difficult
to recognize, but cure is achievable with appropriate multidrug therapy. Because untreated leprosy can result in permanent, irreversible nerve damage and secondary transmission, early diagnosis and treatment are essential to minimize morbidity. Many factors affect the cytomorphic changes of the cells collected from the oral mucosa. Some of these factors are systemic diseases, e.g., anemia and diabetes mellitus; radiotherapy; alcohol consumption; and smoking. It is well studied in the literature that these factors play an important role in causing morphometric changes on oral mucosal cells. However in the literature there are no studies relating cytomorphic assessment in leprosy patients, apart from few studies done on biopsy tissue. Therefore in the present study, Cytomorphometric assessment of the exfoliated buccal mucosal cells was done in treated leprosy patients and compared with healthy individuals. In our study, we have measured the NA, CA of exfoliated buccal mucosa cells using an image analyzer software in the treated leprosy patients ($n=30$) and control subjects ($n=30$).

It was found that there was a significant increase in the mean CA among the treated leprosy patients when compared to the healthy controls. These changes can be attributed to action of Mycobacterium Leprae on oral mucosal cells. Studies have shown the presence of Lepra bacilli in oral smears as a viable source of infection in leprosy patients. Mycobacterium Leprae prefers temperature less than body temperature for its living in cooler areas of the body such as the chin, malar eminences, earlobes, knees and distal extremities. Considering this fact, a pathophysiological mechanism is postulated for oral involvement: A nasal lesion with obstruction leads to mouth breathing; this causes decrease in the intra oral temperature harboring bacilli for multiplication.

According to Morgado et al (2006) even clinically apparent normal oral cavity may show some microscopic mucosal changes in multi bacillary cases. The molecular and immunological studies have shown that oral mucosa may be a secondary source of infection of Lepra bacilli, nasal cavity being the primary. These factors may contribute to nuclear and cellular changes observed in our study.

In the present study, we have avoided all the other possible causes that can give rise to increase in NA (as mentioned in the exclusion criteria for patient sample selection). The possible hypothesis for explaining the increase in mean NA is as follows: though multi drug therapy is able to clear Mycobacterium Leprae effectively but a small number of persisting organisms may still remain. This could act as a causative factor for the cytomorphic alterations seen in treated leprosy patients. In addition, the qualitative and quantitative changes found in the oral smears of treated leprosy patients may be attributed to xerostomia resulting in a dry, atrophic mucosa with accompanying mucositis as well as lepra bacilli infection with an increase in polymorph nuclear leucocytes, in response to the microbial colonization. The other reason could be multi drug therapy used for longer duration may be the cause of these cytomorphometric changes. Drugs have the potential to affect the oral cavity in a number of ways. Accordingly many authors have reported that multidrug therapy used for treating leprosy can cause anemia, thereby causing cytological change in treated leprosy patients.

Costa et al. (2003) has emphasized the epidemiological importance of oral lesions in leprosy patients as an infection source, since bacilli and tissue changes have been detected in these lesions and also in apparently normal buccal mucosa by histopathological examination of biopsied tissues. However our study is the first study in English language literature where cytomorphomteric analysis is done in buccal mucosal cells of treated leprosy patients.

Conclusion
Exfoliative cytology is an additional tool to aid in better understanding of the disease and its pathogenesis. Hence our study focused on oral cytological changes seen in treated leprosy patients. Cytomorphometrically there was decrease in mean cellular area and increase in mean nuclear area in leprosy patients when compared to healthy individuals. These changes can be attributed to Lepra bacilli or multi drug therapy used for longer duration. Furthermore light has to be thrown with larger sample size for the better understanding of the pathogenesis of these oral changes seen in treated leprosy patients.
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