INTRODUCTION

The blood supply of the oral mucosa is extremely rich and the vascularity of the various parts of oral mucosa differs. There are very few studies, which are conducted on the vascularity of normal buccal mucosa (NBM) and leukoplakia of buccal mucosa. It is a very well-appreciated fact that in developing countries like India approximately 94% of all oral malignancies are squamous cell carcinoma (SCC) and buccal mucosa is also one of the favored sites.

Hence, the present study “Computerized histomorphometric analysis of vascularity in normal buccal mucosa, leukoplakia, and squamous cell carcinoma of buccal mucosa” perforates angiogenesis.

AIMS AND OBJECTIVES

The study attempted to

- Evaluate vascularity in NBM, leukoplakia, and SCC of the buccal mucosa
- Evaluate the correlation between morphometric aspects of vascularity in NBM, leukoplakia, and SCC of buccal mucosa
• Investigate its usefulness in the histopathological classification and prognosis.

MATERIALS AND METHODS

The study involved the use of formalin-fixed, paraffin-embedded tissue of previously diagnosed cases of leukoplakia and SCC of buccal mucosa from the Department of Oral Pathology.

The study sample consisted of
• 15 cases of NBM
• 30 cases of leukoplakia of buccal mucosa
• 30 cases of SCC of buccal mucosa.

The relevant information of age, sex, site, histopathological grading, lymph node status, metastasis, and recurrence was obtained. Mean vessel density (MVD) was assessed of hematoxylin and eosin (H and E) and Masson’s trichrome (MT) stained, 15 NBM, 30 leukoplakia, and 30 SCC of buccal mucosa sections. Most intense vascular area was found by light microscopy in ×40 magnification and counting performed in ×400 field. The assessment was made by single trained observer. Images of histological samples were captured using Lawrence and Mayo (×75) microscope and Nikon digitized camera. The images were saved to the computer and examined using high resolution RGB display monitor and the morphometric analysis was performed manually by using “Image-J” image analysis software.

The assessment included
• The vascular tissue was identified by the presence of red blood cells and endothelial cells.
• For MT method, blood vessels were encircled with red line; lymphatics were devoid of encircling line. The presence of red blood cells was also considered.
• Most intense vascular area was found by light microscope in ×40.
• Three such fields were selected under ×40 for each slide, with slide moving in clockwise direction.
• The area representing vascular tissue in the three digital images were imported to image analysis software and the area counted. This indicated vascular tissue area.
• The area representing total tissue in the three digital images were imported to image analysis software and the area counted. This indicated total tissue area.
• MVD: The ratio of vascular tissue area to total tissue area in the digital images imported to image analyzer software [1] [Figure 1].

STATISTICAL ANALYSIS

Descriptive statistics that included mean, standard deviation (SD), minimum and maximum values were calculated for each group and for NBM, leukoplakia and SCC of buccal mucosa for each clinical stage and histopathological grades. The Student’s t-test was used to determine significant

![Figure 1](image-url)
Vascularity in normal buccal mucosa, leukoplakia, and squamous cell carcinoma

Pujari, et al. 336

One-way analysis of variance (ANOVA) was used for the variance between groups and Student’s t-test for variance within group using Statistical Package for Social Sciences (SPSS) 12.0 statistical package.

RESULTS

A total number of 75 cases were taken for the study and the mean MVD of all the cases in H and E was 0.1112 and for MT 0.2150 with a P value of 0.0017, which was statistically significant [Table 1]. The percentage of distribution of study subjects by three groups and gender in normal revealed 33.33 and 66.67% for female and male, respectively. In leukoplakia 3.33% for females and 96.67% for males and in SCC 46.67% are males [Graph 1]. The mean age of normal, leukoplakia, and SCC is 35, 43, and 51 years, respectively [Graph 2]. The mean MVD in 15 controls for H and E is 0.0611 and for MT 0.1314 with P value of 0.2823. Though the MVD was higher in MT but it was not significant statistically. The MVD in leukoplakia of buccal mucosa (30 cases) and SCC of buccal mucosa (30 cases) for H and E and MT groups were compared. The MVD for H and E was 0.0352 and for MT 0.1263 in leukoplakia of buccal mucosa with a P value of 0.0000 which is statistically significant. The MVD for H and E and MT groups in SCC of buccal mucosa was 0.2122 and 0.3455, respectively with a P value of 0.0413 which was statistically significant [Table 2].

The comparison of normal, leukoplakia, and SCC in H and E and MT by one-way ANOVA test resulted in a P value of 0.0006 and 0.0000, respectively, which was statistically significant. [Tables 3 and 4]. Pair wise comparison of three groups by Student’s t-test is studied for H and E and MT. For H and E the P value for normal and leukoplakia is 0.0413 respectively with a P value of 0.0008, respectively, which are

Table 1: Comparison of H and E and MT groups in total of all the three groups

| Group       | Mean±SD      | t value | P value |
|-------------|--------------|---------|---------|
| H and E (n=75) | 0.1112±0.1937 | −3.1937 | 0.0017* |
| MT (n=75)    | 0.2150±0.2042 |         |         |

H and E: Hematoxylin and Eosin; MT: Masson’s trichrome; SD: Standard deviation

Table 2: Comparison of H and E and MT groups in NBM, leukoplakia and SCC of buccal mucosa

| Group       | Mean±SD      | t value | P value |
|-------------|--------------|---------|---------|
| NBM         |              |         |         |
| H and E (n=15) | 0.0611±0.0783 | −1.0964 | 0.2823  |
| MT (n=15)   | 0.1314±0.2358 |         |         |
| Leukoplakia |              |         |         |
| H and E (n=30) | 0.0352±0.0428 | −9.5308 | 0.0000* |
| MT (n=30)   | 0.1263±0.0301 |         |         |
| SCC         |              |         |         |
| H and E (n=30) | 0.2122±0.2704 | −2.0866 | 0.0413* |
| MT (n=30)   | 0.3455±0.2221 |         |         |

H and E: Hematoxylin and Eosin; MT: Masson’s trichrome; SD: Standard deviation; NBM: Normal buccal mucosa; SCC: Squamous cell carcinoma

Table 3: Comparison of three groups (normal, leukoplakia, and SCC) in H and E by one-way ANOVA

| Source of variation | Degree of freedom | Sum of squares | Mean sum of squares | F value | P value |
|---------------------|------------------|----------------|---------------------|---------|---------|
| Between groups      | 2                | 0.52           | 0.2583              | 8.2300  | 0.0006* |
| Within groups       | 72               | 2.26           | 0.0314              |         |         |
| Total               | 74               | 2.78           |                     |         |         |

H and E: Hematoxylin and Eosin; MT: Masson’s trichrome; SCC: Squamous cell carcinoma; ANOVA: Analysis of variance

Table 4: Pair-wise comparison of three groups by student’s t test for H and E

| Group               | Mean±SD      | t value | P value |
|---------------------|--------------|---------|---------|
| Normal (n=15)       | 0.0611±0.0783| 1.4405  | 0.1570  |
| Leukoplakia (n=30)  | 0.0352±0.0428|         |         |
| Normal (n=15)       | 0.0611±0.0783| −2.1085 | 0.0409* |
| SCC (n=30)          | 0.2122±0.2704|         |         |
| Leukoplakia (n=30)  | 0.0352±0.0428| −3.5397 | 0.0008* |
| SCC (n=30)          | 0.2122±0.2704|         |         |

H and E: Hematoxylin and Eosin; SD: Standard deviation; SCC: Squamous cell carcinoma
statistically significant [Table 5]. For MT the \( P \) value for normal and leukoplakia was 0.9052 which was not significant; whereas, the \( P \) value for normal and SCC; and leukoplakia and SCC are 0.0046 and 0.0000, respectively, which were statistically significant [Table 6].

The percentages of distribution of leukoplakia study group according to histopathology grading are 56%, 33%, and 10% for hyperkeratosis, mild dysplasia, and moderate dysplasia, respectively. The percentage of distribution according to histopathology grading of SCC are 86% for well-differentiated and 14% for moderately differentiated [Table 7]. The MVD of H and E and MT in Tumor, Node, Metastasis (TNM) stages III and IV are 0.2026, 0.2748, 0.3563, and 0.5312, respectively. The comparison of H and E and MT groups in stage III yielded a \( P \) value of 0.0320, which was statistically significant. The \( P \) value in stage IV was 0.2910, which was not significant statistically [Table 8].

Comparison of H and E and MT groups in grade I, II and III of leukoplakia yielded a \( P \) value of 0.0000. 0.2210 and 0.0011. Grade I and III were statistically significant; whereas, grade II was not significant [Table 9]. The comparison of H and E and MT groups in grade I of SCC yielded a \( P \) value of 0.0211 which was significant; whereas, for grade II \( P \) value is 0.7452 which was statistically not significant [Table 10].

### DISCUSSION

Various methods have been used till date to assess both quantitative and qualitative characteristics of angiogenesis in a tumor. These include India Ink perfusion,[21] demonstration of alkaline phosphatase in endothelial cells, selective erythrocyte staining and more.[19] The majority of authors have suggested that the vessel counts are an excellent predictor of metastasis and clinical outcome.

The roles of genetic and epigenetic factors have been studied and their expressions compared among tissues from these stages. The vascularity values increased significantly from normal oral mucosa to dysplasia and from dysplasia to carcinoma. The observations confirm that disease progression in the oral mucosa is associated with angiogenesis. Interestingly, vascularity is found to be significantly higher in histologically normal mucosa adjacent to tumors than in normal mucosa without concurrent lesion.[41] The data on tumor, node, metastasis (TNM) staging was missing in one patient. H and E: Hematoxylin and Eosin; MT: Masson’s trichrome; SD: Standard deviation; TNM: Tumor, node, metastasis

### Table 5: Comparison of three groups (normal, leukoplakia, and SCC) in MT by one-way ANOVA

| Source of variation | Degree of freedom | Sum of squares | Mean sum of squares | \( F \) value | \( P \) value |
|---------------------|-------------------|----------------|--------------------|--------------|-------------|
| Between groups      | 2                 | 0.85           | 0.4259             | 13.7177      | 0.0000*     |
| Within groups       | 72                | 2.24           | 0.0310             |              |             |
| Total               | 74                | 3.09           |                    |              |             |

MT: Masson’s trichrome; SCC: Squamous cell carcinoma

ANOVA: Analysis of variance

### Table 6: Pair-wise comparison of three groups by Student’s \( t \) test for MT

| Group               | Mean±SD         | \( t \) value | \( P \) value |
|---------------------|-----------------|--------------|--------------|
| Normal (\( n=15 \)) | 0.1314±0.2358   | 0.1199       | 0.9052       |
| Leukoplakia (\( n=30 \)) | 0.1263±0.0301 |              |              |
| Normal (\( n=15 \)) | 0.1314±0.2358   | −2.9862      | 0.0046*      |
| SCC (\( n=30 \))   | 0.3455±0.2221  |              |              |
| Leukoplakia (\( n=30 \)) | 0.1263±0.0301 |              |              |
| SCC (\( n=30 \))   | 0.3455±0.2221  |              |              |

MT: Masson’s trichrome; SD: Standard deviation; SCC: Squamous cell carcinoma

### Table 7: Histopathology grading for leukoplakia and SCC

|                 | Total | Percentage |
|-----------------|-------|------------|
| Leukoplakia (\( n=30 \)) |       |            |
| Hyperkeratosis  | 17    | 56.67      |
| Mild dysplasia  | 10    | 33.33      |
| Moderate dysplasia | 3   | 10.00      |
| SCC (\( n=30 \)) |       |            |
| Well-differentiated | 26  | 86.67      |
| Moderately differentiated | 4   | 13.33      |

SCC: Squamous cell carcinoma

### Table 8: Comparison of H and E and MT groups in different TNM stages

| Stage | Group       | Mean±SD    | \( t \) value | \( P \) value |
|-------|-------------|------------|--------------|--------------|
| III   | H and E (\( n=24 \)) | 0.2026±0.2676 | −2.2118 | 0.0320* |
|       | MT (\( n=24 \))      | 0.3563±0.2104 |            |             |
| IV    | H and E (\( n=5 \))   | 0.2748±0.3335 | −1.1305 | 0.2910 |
|       | MT (\( n=5 \))      | 0.5312±0.3822 |            |             |

The vascularity values increased significantly from normal oral mucosa to dysplasia and from dysplasia to carcinoma. The observations confirm that disease progression in the oral mucosa is associated with angiogenesis. Interestingly, vascularity is found to be significantly higher in histologically normal mucosa adjacent to tumors than in normal mucosa without concurrent lesion.

### Table 9: Comparison of H and E and MT groups in different grades of leukoplakia

| Grade | Group       | Mean±SD    | \( t \) value | \( P \) value |
|-------|-------------|------------|--------------|--------------|
| 1     | H and E (\( n=17 \)) | 0.0165±0.0086 | −50.6019 | 0.0000* |
|       | MT (\( n=17 \))      | 0.1505±0.0067 |            |             |
| 2     | H and E (\( n=10 \))  | 0.0635±0.0645 | −1.2677 | 0.2210 |
|       | MT (\( n=10 \))      | 0.0898±0.0116 |            |             |
| 3     | H and E (\( n=3 \))   | 0.0470±0.0085 | −8.3503 | 0.0011* |
|       | MT (\( n=3 \))      | 0.1103±0.0101 |            |             |

H and E: Hematoxylin and Eosin; MT: Masson’s trichrome; SD: Standard deviation

### Table 10: Comparison of H and E and MT groups in different grades of SCC

| Grade | Group       | Mean±SD    | \( t \) value | \( P \) value |
|-------|-------------|------------|--------------|--------------|
| 1     | H and E (\( n=26 \)) | 0.1923±0.2568 | −2.3808 | 0.0211* |
|       | MT (\( n=26 \))      | 0.3556±0.2376 |            |             |
| 2     | H and E (\( n=4 \))   | 0.3413±0.3630 | 0.3404 | 0.7452 |
|       | MT (\( n=4 \))      | 0.2795±0.0055 |            |             |

H and E: Hematoxylin and Eosin; MT: Masson’s trichrome; SD: Standard deviation; SCC: Squamous cell carcinoma
There are various studies on angiogenesis with different parameters (mean vascular density (Mvd), mean vascular volume (Mvv), mast cell density (Mcd)) and immunohistochemical stains. However, none of the antibodies (CD34, CD31, and Factor VIII) could distinguish between blood vessels and newly forming blood vessels. αvβ3 integrin and tumor growth factor (TGF)-β receptor complex were till recently thought to differentiate neovasculature from preexisting blood vessel, which were not found useful in the subsequent studies.

Though the reasons for the observed variability are unclear, the possible explanations are anatomical variation of vascularity throughout different locations of oral cavity, methods of determining Mvd, methods of staining (antigens screened for CD 31, factor VIII, CD 34 and different antigens), methods of observation and reporting degree of angiogenesis. Hence, digital Mvd determination in MT stained sections is considered to be appropriate especially in developing countries like India, considering the cost and early detection of the tumor.

The present study included 75 cases of which 15 cases were of NBM, 30 cases of leukoplakia and 30 cases of SCC of buccal mucosa. The objective of the study was the morphometric correlation of vascularity, its usefulness for the histopathological classification and prognosis in NBM, leukoplakia, and SCC of the buccal mucosa.

Hence, the results obtained were that the mean MVD of all the groups by MT staining was 0.2150 which was more than that of H and E, where it was 0.1112. The compared values of both the staining were statistically significant (P = 0.0017). It is worth mentioning that to our knowledge none of the previous studies have studied the combined mean MVD of different groups.

The MVD analyzed in NBM was not significant between H and E and MT groups; whereas, it was significant for leukoplakia and SCC of buccal mucosa.

No statistical significant results were found when the MVD of NBM (0.1314) was compared to that of leukoplakia (0.1263). Lamaroon et al., showed in their study an increase in MVD from normal (39.17) to hyperkeratosis (50.00) to premalignant dysplasia (60.00). In this study, similar finding of increase in MVD from NBM (0.1314) to hyperkeratosis (0.1505) was also observed. These results strengthen the view that the normal adjacent tissue promotes angiogenesis under the influence of various cytokines, mast cells, macrophages and neutrophils which are elaborated by altered keratinocytes of hyperkeratotic or dysplastic epithelium.

Most of the previous studies have shown that mean MVD was remarkably more in different clinical stages and histological grades of OSCC than that of normal mucosa and leukoplakia. In this study too, the mean MVD in SCC (0.3455) for MT was higher than normal (0.1314) and leukoplakia (0.1263), which was statistically significant. No other study included baseline level of MVD for specific sites like buccal mucosa which is very much necessary, considering the variation in the vascularity at different anatomic sites. The mean MVD increased significantly from stage III (0.3563) to IV (0.5312) when studied in relation to different TNM stages. These observations are in accordance with previous studies of Ranieri et al., Chunan et al., Shang and Li. However, Pazouki et al., and Tae et al., refute the increase in MVD.

Several studies have suggested variations in angiogenesis in relation to different histopathological grading. Sedivy et al., and Li, et al., in their study, showed decrease in MVD from grade I to III; whereas, it increased from grade I to II and then decreased again in grade III. In the studies conducted by Schimming et al., and Shieh et al., such variations were noted even in our study. The MVD for H and E increased from grade I (0.1923) grade II (0.3413), comparatively decreased for MT from 0.3556 to 0.2795, which was statistically nonsignificant.

These variations could be attributed to the size of the sample and methods of assessment. This shift from prevascular to neovascular phase in and around dysplastic and malignant epithelium from normal is influenced by various genetic, epigenetic and immunologic factors. The tumor cells are also able to block production of inhibitors of angiogenesis and produce enzymes that release angiogenic factors.

There was no considerable difference between the percentage of MVD of NBM (13%) and leukoplakia (12%) as far as angiogenesis was concerned which is in contrary to the Tae et al., This difference in the observation could be due to the maximum number of hyperkeratotopic samples (17). There was an exponential increase in angiogenic activity from normal (13%) to OSCC (34%) through leukoplakia (12%) in this study. Hence, we agree with Samsoszuk et al., Lamaroon et al., Ranieri et al., and Sedivy et al., for suggesting MVD as a surrogate marker of angiogenic activity, which may occur at any time during neoplastic transformation and development of OSCC.

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