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ORIGINAL ARTICLE

Formulation of in-Vivo Experimental Model of Oral Submucous Fibrosis in Wistar Rat

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ABSTRACT

Background: Oral submucous fibrosis is a chronic, fibrotic and crippling disease that affects the oral mucosa. It leads to progressive fibrosis that mainly involves the buccal mucosa but other parts of the oral cavity such as the tongue and palate can also be affected. There is a general consensus that areca nut and its constituents are mainly responsible for the pathogenesis of the disease. On histological examination of a biopsy taken from the human oral mucosa, OSMF presents with atrophic epithelium and juxta-epithelial deposition of a vast amount of collagen.

Material and Methods:12 healthy male Wistar rats (age 5-6 weeks) were divided into two groups: group A (control) and group B (experimental) after ethical approval. Bleomycin is an anti-cancer drug that is used to create various animal models of fibrosis. In this study, bleomycin was injected into the buccal mucosa of Wistar rat at a concentration of 1mg/mL dissolved in 0.01 M phosphate-buffered saline (PBS) for 4-8 weeks daily. The control group was treated with normal saline. Body weight and mouth openings were recorded. A macroscopic examination was done along with a microscopic analysis of histopathological features and an immunohistochemical analysis of α-SMA expression. Masson's trichrome stain was used to visualize the collagenous deposition in sub-epithelial tissue.

Results: Clinically buccal mucosa showed lesions that mimicked OSMF. Changes in epithelium and lamina propria were observed in the experimental group as compared to the control group. At week 4, stratified squamous epithelium of buccal mucosa showed shortening of rete ridges and lamina propria showed deposition of thick separate collagen. At week 8, epithelium became flat, and atrophic with loss of rete ridges and lamina propria showed juxta-epithelial deposition of collagen which was completely hyalinized and α-SMA expression increased in myofibroblasts.

Conclusion: The results from our study suggested that Wistar rats are a reproducible and sustainable model for OSMF.

Keywords: Oral submucous fibrosis, animal model, bleomycin, fibrosis, alpha-smooth muscle actin.

INTRODUCTION

Clinically Oral submucous fibrosis (OSMF) is a chronic disorder of the oral cavity, it presents with limited mouth opening, formation of circumoral fibrotic bands, pale oral mucosa, xerostomia, ageusia, ankyloglossia and ulceration of oral mucosa.[(1)] OSMF is characterized histo-pathologically into four grades. In grade 1 (very early stage), the overlying epithelium is of normal thickness whereas lamina propria presents with fine fibrillar collagen, strong fibroblastic response and dilated blood vessels. In grade 2 (early stage), epithelium presents with keratinization and shortened rete ridges whereas lamina propria shows thick and separate collagen with dilated vessels and plump fibroblasts. In grade 3 (moderate stage), epithelial atrophy is present with loss of rete ridges and lamina propria exhibits moderate collagen hyalinization, few constricted blood vessels and fibroblasts. In grade 4 (advanced stage), epithelial atrophy occurs with completely hyalinized collagen, obliterated blood vessels and no fibroblastic response in lamina propria.[(2)] Various triggering components such as genetic disorders, persistent infections, release of inflammatory cytokines and frequent exposure to irritants have been reported in the development of progressive fibrotic disease.[(3)] High consumption of paan and chaalia has been reported among school children and females with a poor socioeconomic background in India, Pakistan and Asian immigrants based in United states.[(4-6)]

Myofibroblasts are activated fibroblasts which play a crucial role in wound healing, tissue repair and regeneration, maintain tissue homeostasis by regulating the production and degradation of extracellular matrix in tissues after injury.[(7-9)]

Bleomycin was first discovered by Umezewa et al. in 1966, originally isolated from the bacterium Streptomyces verticillus.[(10)] Bleomycin is an antibiotic medicament, widely used for toxic cellular effects.[(11)]

Previously Zhang et al. created a successful OSMF model in female Sprague-Dawley rats using bleomycin, an anticancer drug, which clinically and pathologically closely resembled grade 4 (advance stage) human OSMF. Clinically the buccal mucosa appeared pale with the formation of fibrous bands.[(12)] We also tried to replicate the same model that features the characteristics of OSMF and investigate whether the same concentration of bleomycin can induce OSMF in a different strain of rat which is sustainable, reproducible and less time consuming so that we can use it to our advantage to test various treatment modalities that might prove beneficial to humans suffering from OSMF in future.

MATERIALS AND METHODS

The Ethical Review Committee of University of Health Sciences, Lahore provided guidelines to be followed to conduct study on health male Wistar rats age 5-6 months and weight 150-200 g and approval provided by UHS. Standard rat chow and water ad libitum was given one week prior to the experimental trial. The rats were placed in front of light (6 am -6pm) and in darkness (6 pm- 6 am). This experimental trial divided in to two group as Group A (Control) and Group B (Experimental), comprises of six rats in each assigned group.

Normal saline was used in control group and bleomycin was used in experimental group.

Both groups were sacrificed at 4 and 8 weeks respectively. A rat model was created using bleomycin (Cipla pharmaceuticals) at a concentration of 1mg/mL dissolved in 0.01 M phosphate buffered saline (PBS). Quantity of 100 µL of bleomycin solution was administered through using a 26-guage brown needle for 4-8 weeks daily into the buccal mucosa of rats.[(12)] Since week 0 from the beginning of the study body weights and recorded till week 8 of both experimental and control groups. Vernier caliper tool was used to assess the distance of upper and lower incisors teeth. On intra-oral examination of buccal mucosa, changes in color, texture, and flexibility were observed weekly.

The animals of both control and experimental groups were immolated at week 4 and week 8 by overdose of chloroform, the day after the final injection of bleomycin. [(13)] Antigen retrieval was done using 10mM sodium citrate buffer (pH 9) and 0.05% Tween-20 solution in a water bath at 95°C for 45 min. Positive expression of α-SMA was scored as no expression=(0), 1%-25% of positively staining cells=(1), <25%-50% of positively staining cells=(2) and

<50%-100% of positively staining cells=(3). Both scores were multiplied and staining index was obtained as following: undetectable=(0), low=(1-2), moderate=(3-4) and high=(6-9).[12] Human appendix tissue was taken as a positive control.

α-SMA expression used in control and experimental groups and SPSS software was used in this analysis. p-value of ≤ 0.05 was considered as statistically significant.

RESULTS

General observations: An overall increase in body weight of control and experimental animals was observed. However, the mean difference of gain in body weight of rats in experimental group sacrificed at week 4 (51.33±1.57 gms) and week 8 (49.34±1.96 gms) was less as compared to those of control group sacrificed at week 4 (93±1.47 gms) and week 8 (157± 2.21 gms). We speculate that less weight gain in experimental group could be due to minimal caliber of mouth opening and limited intake of food (Fig. no. 1).

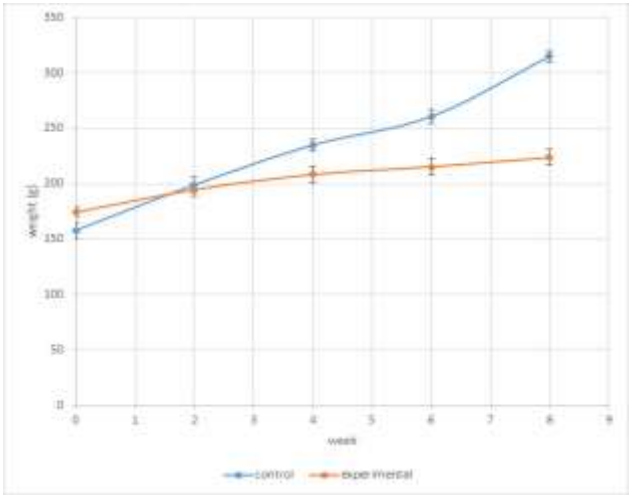

Figure 1: Graph showing comparison of weight gain in control and experimental groups.

Macroscopic observations: The buccal mucosa of control group clinically appeared pink in color and normal in texture at the beginning and at the closure of the experiment (week 8) in control group. The buccal mucosa of experimental group at the start of experiment appeared similar to that of control group. At week 4, the buccal mucosa appeared slightly pale whereas at the time of sacrifice at 8th week, the buccal mucosa appeared pale, leathery in texture and stiff (Fig. no. 2).

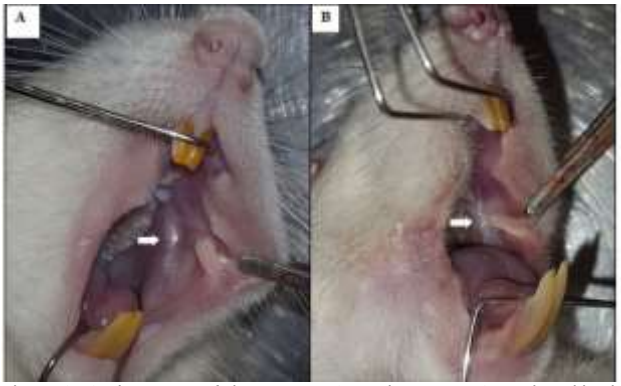

Figure 2: Oral mucosa of the rats; A, Control group at 8 weeks with pink buccal mucosa. B, Experimental group at 8 weeks with pale and stiff buccal mucosa

We speculate that administration of bleomycin led to fibrotic changes in buccal mucosa. The mean difference of mouth openings in rats of control group sacrificed at week 4 (2.70±0.16 mm) and week 8 (4.71± 0.14 mm) increased whereas those of experimental group sacrificed at week 4 (-4.77±0.11 mm) and week 8 (-7.1±0.15 mm) decreased (Fig. no.3).

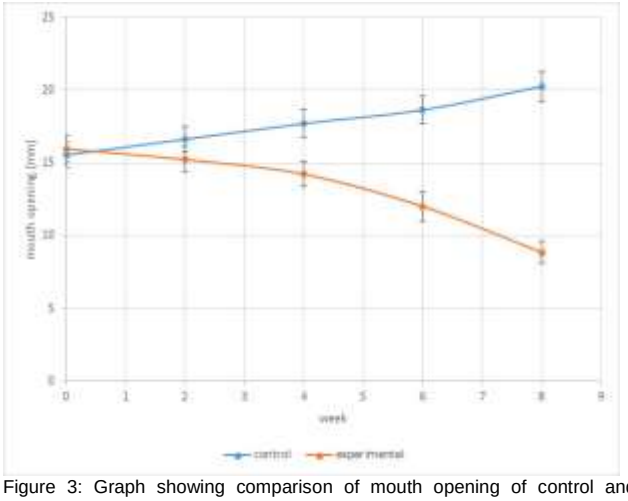

Figure 3: Graph showing comparison of mouth opening of control and experimental group.

Histopathological assessment of buccal mucosa: On histopathological examination, the buccal mucosa of control rats showed normal thickness of epithelium with long and broad rete ridges. The lamina propria showed minimal collagen and normal vasculature. The experimental rats at week 4 showed slightly decreased thickness of epithelium, shortened rete ridges and minimal collagen deposition in lamina propria. At week 8, the experimental rats showed decrease in epithelial thickness with loss of rete ridges. The lamina propria showed juxta-epithelial deposition of collagen which appeared in the form of bundles. Blood vessels appeared obliterated and skeletal muscle atrophy was also observed. These findings bear a close resemblance to grade 4 advanced OSMF in (Fig. no. 4).[14]

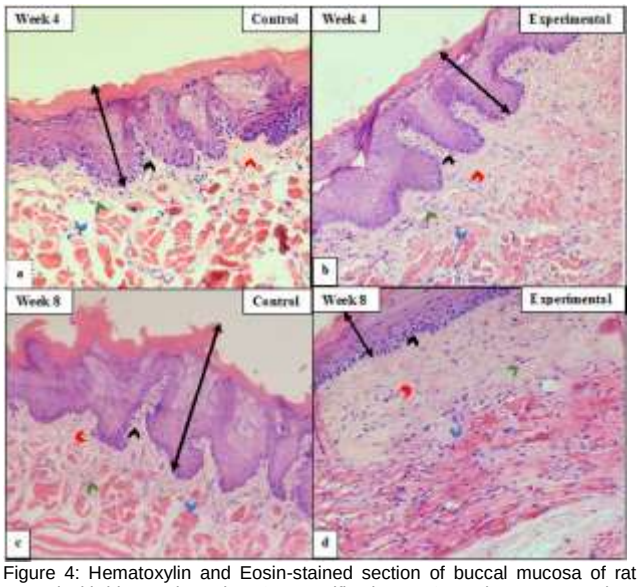

Figure 4: Hematoxylin and Eosin-stained section of buccal mucosa of rat treated with bleomycin under 10x magnification; a. Control group at week 4. b. Experimental group at week 4. c. Control group at week 8. d. Experimental group at week 8.

Grading of OSMF of control and experimental groups: In experiment group, grade 2 and 4 changes are found compared to control group which shows grade 0 at week 4 and 8 **(Fig. no. 5)**.

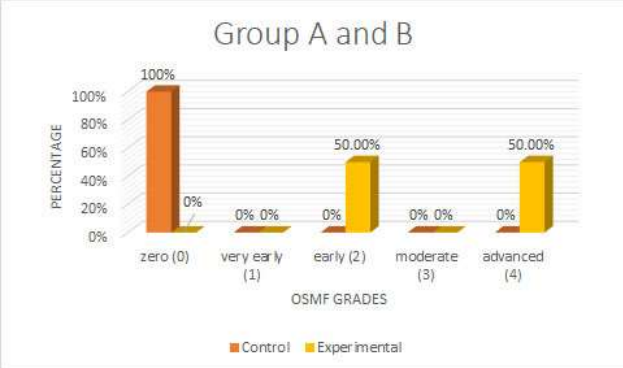

Figure 5: Comparison between Group A and B; Among group A 100% (n=6/6) showed grade zero (0). While among the group B, 50% (n=3/6) showed grade 2 and 50% (n=3/6) showed grade 4 indicating that OSMF grade is high in bleomycin-induced OSMF rat buccal mucosa as compared to control group. When OSMF grades of group A and B were compared with each other, Fisher's exact test showed a statistically significant association between them. P-value was found to be p=0.002

Changes in thickness of epithelium and collagen: In control group, the blood vessels and muscle fibers appeared normal. In experimental group, the epithelial rete ridges appeared flat with abundant deposition of juxta-epithelial collagen. The collagen was not confined to the superficial layer of lamina propria but invaded underlying connective tissue in the form of sheets with obliteration of blood vessels and skeletal muscle atrophy (Fig. No. 6).

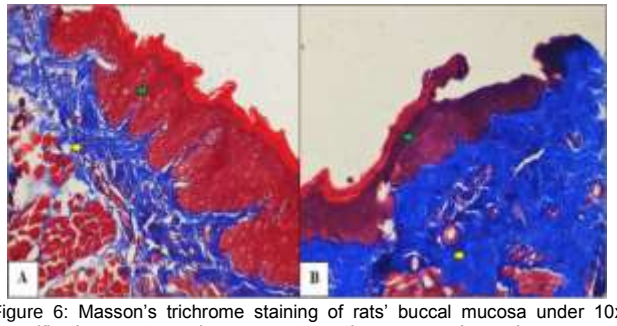

Figure 6: Masson's trichrome staining of rats' buccal mucosa under 10x magnification; A: Control group at 8 weeks. B: Experimental group at 8 weeks. Yellow arrow in control group showing sparse collagen and in experimental group showing abundant deposition of collagen leading to oral submucous fibrosis. Green arrow (epithelium), yellow arrow (collagen).

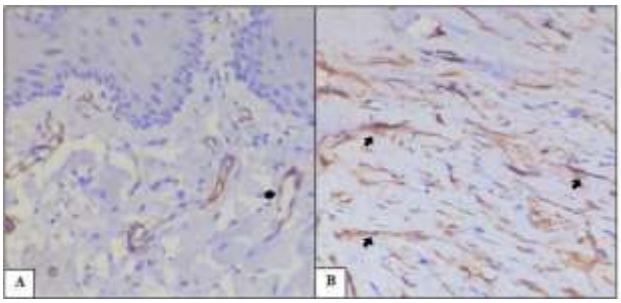

Figure 7: Immunohistochemical expression of α-SMA in buccal mucosa under 40x magnification; A: Control group at 8 weeks. Black arrow shows positive staining in blood vessels. B: Experimental group at 8 weeks. Black arrows showing positive staining in myofibroblasts

Immunohistochemical analysis of α-SMA expression: There was no immunohistochemical expression of α-SMA in control group but the α-SMA positive expression was present in myofibroblasts in connective tissue of buccal mucosa of experimental group at week 8 **(Fig. no. 7)**.

DISCUSSION

We created a successful model of OSMF in Wistar rats by injecting bleomycin for 8 weeks in buccal mucosa of Wistar rats. The changes such as histological and clinical closely resembled grade 4 (advance stage) human OSMF.[15] In this study we observed that the buccal mucosa gradually became pale, stiff and experimental rats also showed limited mouth opening. Comparably Wen et al. (2017) demonstrated decreased mouth opening in arecoline-induced OSMF mice over a period of 5 months.[16] Similarly in another study conducted by Zhang et al. (2016), the mouth openings of bleomycin-induced OSMF rats decreased simultaneously. In this study, the weight gain of experimental rats was less as compared to controls and bleomycin-induced OSMF experimental rats' body weight dropped during first four weeks of bleomycin treatment with nearly no weight gain from 4-8 weeks. Similarly, in humans suffering from OSMF a positive correlation has been established between decrease in body weight and loss of appetite.[17] Previously it has been reported by Wen et al. (2017), arecoline-induced OSMF mice showed significant drop in weight gain during the early stages of experiment as compared to the controls.[16] However a study conducted by Zhang et al. (2016) in female Sprague Dawley rats demonstrated no statistically significant changes in weight in bleomycin-induced OSMF experimental rats as compared to control animals.[12]

We observed gradual collagen deposition at 8 week which was present in the form of sheets and led to obliteration of blood vessels. These changes when compared to human OSMF histology verify the formation of rat model of OSMF because similar changes are present in grade 4 human OSMF.[18] Comparably Zhang et al. (2016) demonstrated similar histological changes in female Sprague Dawley rats at week 8 with atrophic epithelium and abundant juxta-epithelial collagen deposition.[12] Immunohistochemical staining of connective tissue revealed positive expression of alpha smooth muscle actin (α-SMA) in experimental rats and negative expression of α-SMA in control rats. The positive expression of α-SMA proved the presence of myofibroblasts in the lamina propria. Similarly in human OSMF myofibroblasts have been identified as key cells in development of fibrosis and their presence positively correlates with the severity of disease.[19]

An important aspect pertaining to myofibroblasts is that they only appear in disease states such as pathological fibrosis and persist in the lamina propria.[20] It is well established that inflammatory cytokine TGF-β1 has a major role in pathogenesis of OSMF and the level of TGF-β1 is elevated in OSMF when compared to control. [21] Interferon-Y (IFN-Y) is downregulated in pathological fibrosis due to which levels of TGF-β1 increased.[22]

We speculate that administration of bleomycin might have led to activation of TGF-β1 culminating in fibrosis as TGF-β1 is implicated in the development of fibrosis from previous studies conducted on human OSMF and is the main reason for increased collagen production and decreased collagen degradation.[23, 24] We also speculate that bleomycin administration might have disturbed the balance of tissue inhibitors of metalloproteinases (TIMPs) and matrix metalloproteinase (MMPs) because from previous studies conducted on human OSMF suggest that development of fibrosis occurs due to imbalance between the levels of MMPs and TIMPs.[25, 26] Inactivity of certain MMPs leads to increased deposition of collagen and consequently increased activity of TIMPs leads to decreased degradation of collagen.[26]

CONCLUSIONS

In this study we have observed the effect of bleomycin on the buccal mucosa of Wistar rat which manifest in the form of clinically palpable fibrotic bands, juxta-epithelial fibrosis of lamina propria

and atrophy of stratified squamous epithelium. The pertinence of an animal model to a human pathosis relies on its ability to replicate the clinical and histopathological features that depict the disease in humans. Our model bears a close resemblance to the clinical and histopathological features of OSMF. Furthermore, this rat model is reproducible and can be replicated in a shorter time span when compared to other animal models. The findings from this study suggested that, Wistar rats are a reproducible and sustainable model for OSMF.

In the current scenario patients are reluctant to provide a biopsy due to the nature of this disease and clinicians are more focused on providing symptomatic relief to the patients only. This renders finding a cure for this disease difficult and elusive. In this regard animal models are a perfect tool and better suited to understand the disease process and explore various treatment modalities. Furthermore, our animal model may prove beneficial to devise therapeutic strategies which can be planned at different stages of this disease. This will ultimately aid in finding a relevant treatment option for the patients.

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