Evaluation of a Herbal Muco-adhesive Gel for Treatment of Oral Submucous Fibrosis in Wistar Rats

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ABSTRACT

The present study was designed to formulate and investigate the role of an oral muco-adhesive gel, having a novel combination of 1% curcumin and licorice, in an animal model of oral submucous fibrosis (OSMF), as a possible treatment modality. Animal model of OSMF was created by injecting an anti-cancer drug Bleomycin (1mg/mL) into buccal mucosa of animals daily for 8 weeks. The 24 male Wistar rats were equally divided into two groups; six rats (group 1A) were injected with normal saline (control group) while 18 rats (groups 1B, 2A and 2B, experimental groups) were injected with bleomycin. The six rats of control group (1A) and the six rats of experimental group (1B) were sacrificed after week 4 and week 8. Of the remaining 12 experimental rats (groups 2A and 2B), six were treated with muco-adhesive gel with active ingredients (group 2A) while the remaining six rats were treated with muco-adhesive gel with curcumin and licorice (group 2B) which were sacrificed after week 12 and week 16. Buccal tissues were evaluated for histological assessment. There was minimal increase in body weight of animals belonging to groups IB, 2A and 2B along with a decrease in mouth opening as compared to the control group (1A). However, in the OSMF groups, a gain in body weight, improvement in mouth opening and resolution of fibrosis was observed in experimental group (2B) treated with muco-adhesive gel with active ingredients (2B) than the one without it (2A). The findings of our study suggested that curcumin and licorice-based muco-adhesive gel led to improvement of fibrosis in OSMF animals.

INTRODUCTION

Oral submucous fibrosis (OSMF) is a chronic and insidious pre-cancerous condition seen in oral cavity that comprises of chronic inflammatory process due to persistent stimulation of oral mucosal tissues as a result of constant areca nut chewing (Sarode *et al*., 2018). OSMF severely affects the quality of life of an individual due to formation of fibrotic bands, limited mouth opening, intolerance to spicy food, pain and xerostomia (Sridhar *et al*., 2016). This condition is predominant in Indian subcontinent including Pakistan because of habitual use of gutkha, tobacco, affordable price and easy availability of areca nut in the form of commercial preparations. This premalignant condition is increasing not only in young Pakistani males but females and school going children are equally prone to this condition (Niaz *et al*., 2017; Shaikh *et al*., 2019). This may be due to poor socioeconomic status and lack of awareness of the potential hazardous effects of areca nut, gutkha, chaalia and tobacco chewing on human health (Gondivkar *et al*., 2019). The etiological factors involved in the pathogenesis of OSMF include an increased use of areca nut, excessive chilli consumption as a dietary component, immunological factors, genetic predisposition and nutritional deficiencies (Mohiuddin *et al*., 2016; Tilakaratne *et al*., 2016).

Histologically, OSMF in humans exhibits in buccal mucosa as juxta-epithelial hyalinization with collagen bundles infiltrating the submucosal tissues, epithelial atrophy, inflammatory cell infiltration of lamina propria, reduced vascularity and muscle degeneration (Peng *et al*., 2019). The various biological pathways implicated in the pathogenesis of OSMF include imbalance of collagen metabolism causing excessive collagen deposition and reduced collagen degradation, epithelial cells to mesenchymal cells transformation, down regulation of matrix metalloproteinases (MMPs) and up regulation of tissue inhibitors of matrix metalloproteinases (TIMPs), activation of fibroblast to myofibroblast ultimately resulting in development of OSMF (Rai *et al*., 2019). No definitive cure is available that can completely reverse

the condition however several treatment strategies include termination of areca nut chewing habit, physical regimen which include forceful mouth opening using splints, medicinal care i.e., intralesional corticicosteroid and proteolytic enzyme injections, vitamin supplements, antioxidants and surgical interventions (Arora, 2019). Still OSMF poses a grave challenge to the clinicans and also to the patients in regards to its lack of a definitive treatment modality. Medicinal herbs such as curcumin and licorice have been used since ancient times as a remedy for oral diseases such as gingivitis, periodontitis and oral cancer (Nagpal and Sood, 2013; Sidhu *et al.*, 2018). Curcumin is the active constituent of turmeric, which is a rhizome of *Curcuma longa* that belongs to the ginger family i.e., Zingiberaceae and is a flavourful yellow-orange spice. In addition to curcumin, other core components of turmeric include demethoxycurcumin, bisdemethoxycurcumin, volatile oils, resins and proteins (Meng *et al.*, 2018). Curcumin has shown its effectiveness against oral diseases due to its anti-inflammatory, anti-carcinogenic, antiseptic, analgesic and anti-oxidant properties (Chaturvedi, 2009). It has also been successfully tried in the form of turmeric extracts and oil in various precancerous conditions such as oral submucous fibrosis, leukoplakia and lichen planus due to its anti-tumor and onco-preventive potential and has also shown to improve clinical symptoms in OSMF patients when given in the form of oral tablets, capsules, lozenges and oil (Nagpal and Sood, 2013; Al-Maweri, 2019).

Licorice is the root of *Glycyrrhiza glabra* which belongs to perennial plant of Fabaceae family (Parvaiz *et al.*, 2014). Licorice root contains a variety of components which include sugars, flavonoids, sterols, amino acids, resins, starch, essential oil and saponins and a number of active constituents such as glabridin, glycyrrhizin and licoricidin (Icer and Sanlier, 2017). Licorice has shown beneficial effects in various oro-dental diseases such as dental caries, periodontitis, aphthous ulcers, candidiasis and oral cancer as the pharmacological properties are attributed to its anti-oxidant, anti-microbial, anti-inflammatory and anti-tumor activities (Messier *et al.*, 2012; Wang *et al.*, 2015; Sidhu *et al.*, 2018). Glabridin, an isoflavone extract from licorice root, significantly have shown to exhibit anti-migratory, anti-contractile and anti-invasive effects in cultured human fibrotic buccal mucosal fibroblasts indicating licorice can effectively treat the precancerous OSMF (Lee *et al.*, 2018).

Despite various conservative and surgical treatment options available for management of OSMF, no definite cure has been demonstrated for the patients. Hence it is prudent to search for new experimental drugs or herbal formulations that can provide relief and even aid in resolution of this crippling disease in humans. As curcumin has already shown promising results in reducing the symptoms of OSMF to a great extent and licorice has proven to be effective against a plethora of other oral diseases, therefore the present study was designed to evaluate the effects of combination of curcumin and licorice-based muco-adhesive oral gel in an experimental model of OSMF in Wistar rats. No study has combined these two active components in a muco-adhesive gel form and investigating their effects on OSMF can lead to synthesis of an alternative herbal treatment modality in human population suffering from this debilitating condition.

MATERIALS AND METHODS

Ethical approval

Ethical approval of the proposed study was taken from the Ethical Review Committee of University of Health Sciences, Lahore under the no UHS/REG-18/ERC/1344 and all the experiments were conducted according to the guidelines set by the committee.

Preparation of muco-adhesive gel containing curcumin and licorice

To obtain curcumin extract, the roots of *Curcuma longa* were washed, dried and powdered in an electric grinder. Dry turmeric powder (150 g) was placed in a 4000 mL conical flask containing 1500 mL of 90% ethanol with intermittent stirring for 3 days. The supernatant was then filtered using Whatman No. 1 filter paper. Filtrate was concentrated on a rotary evaporator (Hei-VAP Core-036040055) and the resulting residue was incubated and stored at 4°C for further use (Yang *et al.*, 2007). For licorice extract, *Glycyrrhiza glabra* root was washed well with water, dried at room temperature in the dark, and then ground in an electric grinder to give a coarse powder. 100 g of the licorice powder was mixed with 1500 mL of 70% aqueous ethanol in a 4000 mL conical flask for 3 days with occasional shaking. The supernatant was then filtered, the filtrate was concentrated on a rotary evaporator (Hei-VAP Core-036040055) and the resulting residue was dried using a lyophilizer and stored at 4°C (Ajagannanavar *et al.*, 2014).

The semi-solid formulation was prepared by dissolving 4 g of hydroxyethyl cellulose powder (Daejung 4113-4405) in 77.5 mL of distilled water with constant stirring for 30 min and final mixture was kept at room temperature for 24 h. The ethanolic extracts of both the herbs were dissolved in 15 mL absolute ethanol and kept at room temperature for 24 h. Glycerine (Daejung 4066-4400) (2 g) and 0.5 g of sodium metabisulphite (Daejung

7593-4400) were dissolved in the above polymer-drug solution. This led to the formation of a muco-adhesive gel that was stored at 4°C until further use. Control gel was prepared by same procedure as described above without addition of curcumin and licorice (Kumar *et al*., 2012).

Rats, creation of rat model of OSMF and experimental grouping

Twenty-four healthy male Wistar rats (weighing 150-200 g, age 5-6 weeks), were acclimatized for 1 week before the start of the experiments and were maintained at constant 24 h light and dark cycles to ensure a stable biological clock and circadian rhythm (Bano *et al*., 2019). All animals received standard rat chow and water *ad libitum*. The animals were kept under controlled temperature of 24-26°C and humidity of 45-55% was maintained in the clean and well-ventilated environment of Experimental Research Laboratory of the University of Health Sciences, Lahore (Bano *et al*., 2019).

Injecting 1% bleomycin in buccal mucosa of Sprague-Dawley rats has also led to creation of a successful model of OSMF after 8 weeks (Zhang *et al*., 2016). Therefore, 100 µL of bleomycin solution was daily injected into the buccal mucosa of 12 rats till 8 weeks using a 26-guage brown needle for successful creation of animal model of OSMF.

Group 1A comprising of 6 rats were injected with normal saline (considered as the control group) and group of 18 rats (1B, 2A and 2B, considered as the experimental groups) were injected with 1% bleomycin solution till 8 weeks.

Three rats from the control group were sacrificed after 4 weeks while the remaining three of the control group were sacrificed after 8 weeks. Likewise, three rats from the experimental group were sacrificed after 4 weeks and another three were sacrificed after 8 weeks (group 1B) (Fig. 1).

Of the remaining 12 experimental rats (2A and 2B) which had OSMF were given daily application of muco-adhesive gel with (2B) or without (2A) active components (curcumin and licorice) from 8th week onwards till the time of their sacrifice, three on week 12 and three on week 16 (Fig. 1). The gel application was done on the buccal mucosa of these animals with the help of a cotton bud. The details of grouping and dose administration of these animals are shown in Table I and Figure 1.

Recording of body weight, sample collection and histological analysis

Body weights of all the animals were recorded daily and mouth openings were recorded weekly from the beginning of experiment (week 0) till the end of experiment (week 16) of all groups. Mouth openings were recorded by measuring the distance between upper and lower incisors using a digital vernier caliper (Kuraji *et al*., 2019).
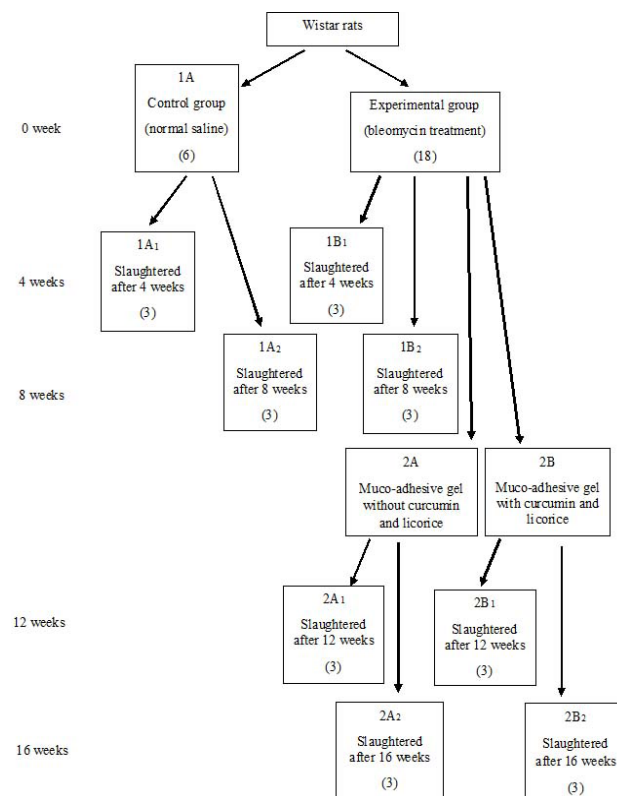

Fig. 1. Experimental flowchart.

The animals of all groups were sacrificed at week 4, week 8, week 12 and week 16 by overdose of chloroform (Sumeth Perera *et al*., 2007). The buccal mucosa was removed from each animal and fixed with 10% formalin solution for 24 h. After fixation, tissues were processed in automatic tissue processor (Microm STP-120) containing serial dilutions of 70%, 90% and 100% ethanol and xylene for tissue dehydration (Bancroft and Gamble, 2008). The tissues were then embedded in paraffin wax and cut into 3µm thick sections using electronic rotary microtome (Thermoscientific Microtome HM340E). Then the sections were stained with haematoxylin and eosin (H and E) for assessment of histopathological features.

Histological analysis was done using light microscope (Olympus BX51TF) with camera (Infinity-1). The histopathological assessment of OSMF was done by allocation of grade 1, 2, 3 and 4 as proposed by Pindborg and Sirsat (Pindborg and Sirsat, 1966) based on histopathological features in human OSMF since no grading system has been established for rat model of OSMF. This grading method included recording of scores

Table I. Grouping of animals and dosage administration.

Groups	Intervention and dosage	Duration of administration	Route of administration	Duration of sacrifice
Group 1A	Normal saline	Daily for 4 ($1A_1$) and 8 ($1A_2$) weeks	Oral	At 4 ($1A_1$) and 8 ($1A_2$) weeks
Group 1B	Bleomycin solution at a concentration of 1mg/mL dissolved in 0.01M PBS.	Daily for 4 ($1B_1$) and 8 ($1B_2$) weeks	Intramucosal	At 4 ($1B_1$) and 8 ($1B_2$) weeks
Group 2A	Mucoadhesive gel without active component	Daily for 4 ($2A_1$) and 8 ($2A_2$) weeks	On buccal mucosa	At 12 ($2A_1$) and 16 ($2A_2$) weeks
Group 2B	Mucoadhesive gel containing 1% curcumin and licorice	Daily for 4 ($2B_1$) and 8 ($2B_2$) weeks	On buccal mucosa	At 12 ($2B_1$) and 16 ($2B_2$) weeks

after assessment of histological variables such as changes in thickness of stratified squamous epithelium, degree of epithelial keratinization, collagen deposition and fibroblastic response in lamina propria, presence or absence of chronic inflammation, vascular and muscle changes in the buccal tissues (Passi *et al.*, 2017).

Statistical analysis

All statistical analysis was done using IBM SPSS software (version 25.0, SPSS Inc.). Numerical variables such as body weight and mouth opening were presented as mean ± standard deviation (SD). Fisher's exact test was used for comparison of fibrosis in control and experimental groups. For all tests, a *p*-value of ≤ 0.05 was considered statistically significant.

RESULTS

Mouth opening of animals

The mean initial mouth opening of animals at the start of the study was 15.55±0.86 mm. At week 4, the control group 1A showed mouth opening averaged 19.40±0.36 mm while the mouth opening of experimental group 1B was 10.55±0.60 mm. However, at week 8, the control group 1A showed mouth opening averaged 20.26±1.01 mm while the mouth opening of experimental group 1B was 8.86±0.79 mm. The other two experimental groups 2A and 2B had an averaged mouth opening of 14.04±1.39 mm and 12.73±0.78 mm respectively at week 4. While at week 8, the mouth opening of experimental group 2A averaged 10.83±1.24 mm whereas the mouth opening of experimental group 2B was 9.03±1.80 mm. Thus overall, the animals of all three experimental groups at week 8 exhibited a decrease in mouth opening as compared to control group animals at this stage.

When muco-adhesive gel containing curcumin and licorice was given to the experimental group 2B, mouth opening showed an improvement at week 12 averaged 11.73±1.79 mm (which was initially 9.03±1.80 mm at week 8). This further increased to an average of 15.10±1.44 mm at week 16 in these animals. In comparison to experimental

group 2B, the mouth opening of experimental group 2A, which was given control gel had mouth opening averaged 11.86±1.35 mm at week 12 (which was initially 10.83±1.24 mm at week 8) and changed to an average of 12.91±1.27 mm at week 16. Thus a significant increase in mouth opening of group 2B after application of muco-adhesive gel containing curcumin and licorice was observed at week 16 as compared to group 2A.

Body weight of animals

During the entire course of experiment, an overall increase in body weight of all the groups was observed. The initial mean body weight of animals at the start of the study was 159.33±6.02 g. At the time of sacrifice of animals at week 4, the mean weight of control group 1A was 252.33±7.50 g while the mean weight of experimental group 1B was 211.00±6.08 g. At week 8, the mean weight of control group 1A was 315±5.00 g while the mean weight of experimental group 1B was 223.67±7.09 g. Thus the gain in weight in group 1B animals was less as compared to the control group 1A.

At the time of sacrifice of animals at week 12, the mean weight of experimental group 2A was 202.67±8.32 g while the mean weight of experimental group 2B averaged 222±6.24 g, however at week 16, the mean weight of experimental group 2A was 243.67±7.09 g, while the mean weight of experimental group 2B averaged 290±4.58 g. Altogether, these results suggested that group 2B gained more weight than group 2A at week 12 as well as at week 16.

Histological analysis and grading of OSMF

Control rats

At week 4 and week 8, on histopathological examination, the control group 1A rats had an epithelial thickness of buccal mucosa as 12-18 layers thick with slight to moderate depth of rete ridges into the underlying connective tissue. The lamina propria of group 1A rats exhibited moderate connective tissue density having few elongated fibroblasts in resting phase, some inflammatory cells (lymphocytes) and moderate number of blood vessels with scarce and loose arrangement of collagen fibers in

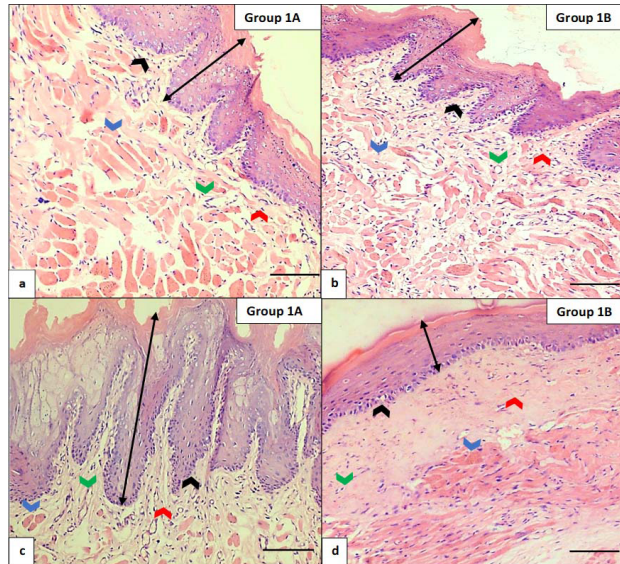lamina propria. The muscle fibers appeared close to the epithelium in the form of fascicles (Figs. 2a, 2c).

Fig. 2. Effect of bleomycin on histological structure of buccal mucosa of rat after treatment for 4 weeks and 8 weeks. (a) Control group 1A at week 4 (scale bar=100 µm). (b) Experimental group 1B at week 4 (scale bar=100 µm). (c) Control group 1A at week 8 (scale bar=100 µm). (d) Experimental group 1B at week 8 (scale bar=100 µm). In the 4th week, the control group 1A (a) showed normal buccal mucosa features i.e., thickened stratified squamous epithelium with orthokeratosis (double black arrows) having long and narrow rete pegs (black arrows), moderate number of vessels (green arrow), absence of inflammatory infiltrate, normal amount of collagen in connective tissue (red arrows) and muscle fibers in the form of fascicles (blue arrows). In the 4th week, the experimental group 1B (b) showed early stage OSMF features i.e., slight decreased thickness of epithelium with same degree of orthokeratosis (double black arrow), shortened rete pegs (black arrow), few constricted vessels and inflammatory infiltrate, pink filaments in connective tissue just below the epithelium (red arrow) and no muscular atrophy (blue arrows). In the 8th week, the control group 1A (c) showed normal buccal mucosa features i.e., thickened stratified squamous epithelium with orthokeratosis (double black arrows) having long and narrow rete pegs (black arrows), moderate number of vessels (green arrow), absence of inflammatory infiltrate, normal amount of collagen in connective tissue (red arrows) and muscle fibers in the form of fascicles (blue arrows). In the 8th week, the experimental group 1B (d) showed advanced stage OSMF features i.e., epithelial atrophy with increased orthokeratosis (double black arrows), absent rete pegs (black arrows), constricted and absent vasculature (green arrow), reduced cellularity, hyalinized change in collagen in connective tissue (red arrows) and muscular atrophy (blue arrows) (Stain: hematoxylin and eosin, Magnification: 10X).
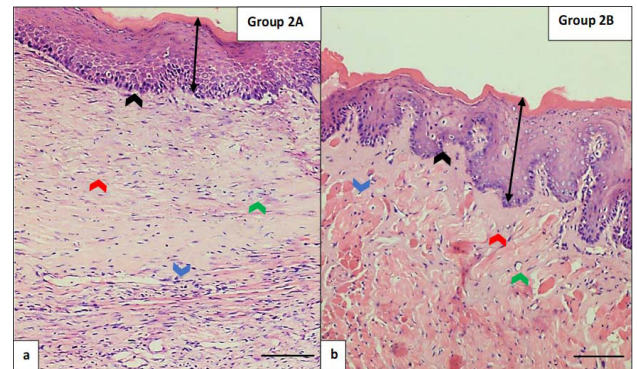

Fig. 3. Effect of muco-adhesive gel on oral submucous fibrosis in buccal mucosa of rat after 12 weeks of treatment. (a) Experimental group 2A at week 12 (scale bar=100 µm). (b) Experimental group 2B at week 12 (scale bar=100 µm). In the 12th week, the experimental group 2A (a) showed advanced stage OSMF features i.e., thin and atrophic epithelium with orthokeratosis (double black arrows), shallowed and absent rete pegs (black arrows), vascular constriction (green arrow), reduced cellularity, dense collagen in the form of sheets in connective tissue (red arrows) and muscular atrophy (blue arrows). In the 12th week, the experimental group 2B (b) showed moderate stage OSMF features i.e., varying thickness of epithelium with orthokeratosis (double black arrows), broad rete pegs (black arrows), dilated vasculature and vascular occlusion disappearance, few inflammatory infiltrate, dense collagen in connective tissue just below the epithelium (red arrows) and muscle fibers interspersed among collagen deposits (blue arrows) (Stain: hematoxylin and eosin, Magnification: 10X).

Bleomycin-treated rats

The group 1B rats showed a gradual decrease in epithelial thickness, which became atrophic with loss of rete ridges from week 4 to week 8. At week 4, the lamina propria showed minimal collagen deposition, few constricted blood vessels and few fibroblasts, which appeared with no skeletal muscle atrophy. As the OSMF progressed to week 8, the buccal mucosa of group IB exhibited completely hyalinized collagen deposition with a progressive increase in connective tissue density, decrease in vasculature and number of fibroblasts within lamina propria. Muscle changes were obvious at week 8 illustrating muscle atrophy characterized by collagenous tissue invasion within the submucosal tissues (Fig. 2b, 2d). These findings bore a resemblance to histological picture of human OSMF condition (Passi *et al.*, 2017) indicating that OSMF-like histopathological changes had occurred at week 8 in these animals. The buccal mucosa of group 1B showed OSMF grade 2 features at week 4 indicating early grade OSMF features. While it had progressed to

grade 4 at week 8 indicating advanced OSMF features as compared to control group 1A which was at grade 0 with normal buccal mucosa at week 4 and week 8 (Fig. 2).

Effect of muco-adhesive gel with and without curcumin and licorice

After successful formation of OSMF model at week 8, animals that belonged to group 2A were treated with control gel while animals belonging to group 2B were treated with muco-adhesive gel containing curcumin and licorice.

It was found that the group 2A at week 12 and week 16 showed fibrotic features similar to group 1B at week 8, such as thin and atrophic epithelium with absent rete pegs. The lamina propria showed dense collagen, constricted blood vessels and muscular atrophy (Figs. 3a and 4b). At week 16, group 2A also showed varying thickness of epithelium with shortened rete pegs. The lamina propria showed dense collagen, few constricted and dilated blood vessels and muscular atrophy (Fig. 4a). Group 2A showed grade 4 features at week 12 indicating advanced OSMF and grade 3 and grade 4 features at week 16 indicating moderate as well as advanced OSMF was found. This indicated that no change in OSMF condition occurred in these animals despite the application of gel that was without active components.

Unlike group 2A, group 2B at week 12 showed partial regain in epithelial thickness with few rete ridges, dense collagen in lamina propria, few blood vessels, few muscle fibers dispersed among collagen fibers (Fig. 3b). Further application of gel on group 2B animals at week 16, led to a partial regain in epithelial thickness with partial reappearance of lost rete ridges. The lamina propria showed minimal collagen, increased vasculature and muscular fibers interspersed between collagen bundles (Fig. 4c, 4d). The buccal mucosa of group 2B showed OSMF grade 3 features at week 12 indicating moderate OSMF condition and grade 2 features were found at week 16 indicating early OSMF. This indicated that changes in OSMF condition occurred in these animals due to the application of gel having curcumin and licorice.

DISCUSSION

Oral submucous fibrosis is a pre-malignant, relentless and a chronic condition of oral cavity. It leads to juxta-epithelial deposition of collagen, chronic inflammation and epithelial atrophy leading to fibrosis and scarring of submucosal tissues with inability to open mouth. It is quite prevalent in Pakistan and many Asian countries mainly due to habitual use of areca nut containing substances (Peng *et al.*, 2019). In this experimental study, we induced fibrosis by injecting bleomycin in the buccal mucosa of Wistar rats till eight weeks. The resultant clinical and histological

parameters in the experimental animals then became much similar to that of human OSMF (Passi *et al.*, 2017). Muco-adhesive gel containing curcumin and licorice was applied on the buccal mucosa of Wistar rats of group 2B after the creation of a successful rat model of OSMF to discern whether it leads to improvement or resolution of induced fibrosis.
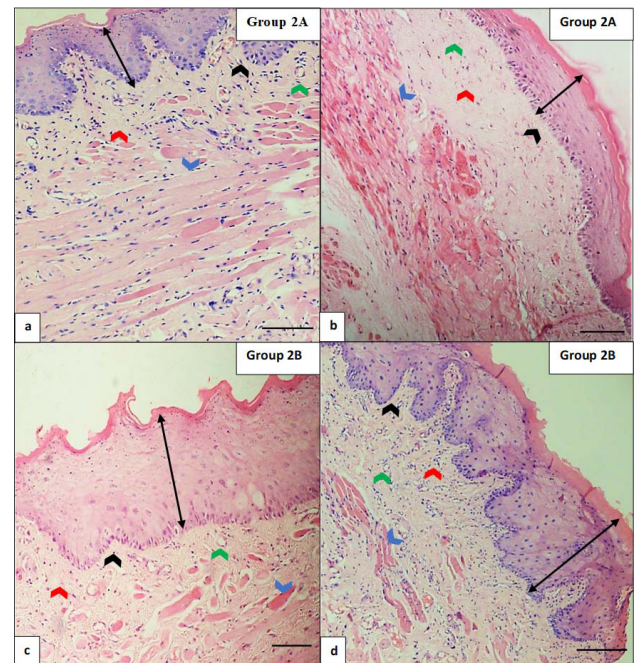

Fig. 4. Effect of muco-adhesive gel impregnated with curcumin and licorice on oral submucous fibrosis in buccal mucosa of rat after 16 weeks of treatment.
a and b, Experimental group 2A at week 16 (scale bar=100 µm). c and d, Experimental group 2B at week 16 (scale bar=100 µm). In the 16th week, the experimental group 2A (a) showed moderate stage OSMF features i.e., varying thickness of epithelium with orthokeratosis (double black arrows), shortened rete pegs (black arrows), dilated and constricted vasculature, inflammatory infiltrate, dense collagen in connective tissue just below the epithelium (red arrows) and muscular atrophy (blue arrows) and (b) showed advanced stage OSMF features i.e., thin and atrophic epithelium with orthokeratosis (double black arrows), shallowed and absent rete pegs (black arrows), vascular constriction (green arrow), reduced cellularity, dense collagen in the form of sheets in connective tissue (red arrows) and muscular atrophy (blue arrows). In the 16th week, the experimental group 2B (c and d) showed early stage OSMF features i.e., increased epithelial thickness with orthokeratosis (double black arrows), short, long and broad rete pegs (black arrows), increased and dilated vasculature (green arrow), increased fibroblast, disordered collagen in lamina propria (red arrows) and muscular fibers interspersed between collagen bundles (blue arrows) (Stain: hematoxylin and eosin, Magnification: 10X).

The mouth openings of experimental groups IB, 2A and 2B rats decreased till 8 weeks as far as bleomycin was administered due to presence of fibrosis formation in these animals. The mouth openings of group 2B rats improved because of reduction in fibrotic bands and decrease in rigidity of buccal mucosa due to application of muco-adhesive gel containing active components. Curcumin has been reported to be a fibrinolytic agent which possess fibrinolytic properties in lung and liver fibrosis (Punithavathi *et al.*, 2000; Venkatesan *et al.*, 2007; Rivera-Espinoza and Muriel, 2009). This fibrinolytic property of curcumin could be the reason for significant improvement in mouth opening in group 2B animals. The less weight gains in experimental group 1B at week 4 and week 8 was because of decreased mouth opening leading to decreased food intake in these animals. However, body weight of experimental group 2B animals showed significant increase as compared to group 2A because of improved mouth opening and increased food intake in these animals.

In our study, animals in experimental groups showed characteristic histopathological fibrotic features of OSMF beginning in the 4th week and maximum fibrosis was noted at 8 weeks. At week 4, the epithelial thickness of buccal mucosa of control Wistar rat appeared 12-18 layers thick with moderate degree of orthokeratinization and exhibited slight to moderate depth of epithelial extensions (rete ridges) into lamina propria. Similar changes were reported at week 8. Similarly, Thirion-Delalande *et al*. (2017) reported normal histological features of oral mucosa of rodents and found that cheek mucosa of rat displayed an orthokeratinized epithelium of 12-18 layered epithelial thickness with slight depth of rete ridges (Thirion-Delalande *et al*., 2017). The buccal mucosa of normal adult rat is found to have keratinized epithelium whereas in humans, the buccal mucosa is non-keratinized epithelium (Thirion-Delalande *et al*., 2017). The collagenous deposition in group 1B animals started at 4th week with mild inflammation in the superficial layers of lamina propria. The histopathological features of group 1B evident at 4th week included partial loss of rete pegs with varying degree of keratinization of stratified squamous epithelium. As evident from the results of our study, the characteristic fibrosis was evident at 8th week with juxta-epithelial hyalinization, complete loss of rete pegs, muscle atrophy underneath, reduced or constricted vessels and reduced cellular response. These findings suggested that bleomycin administration leads to changes in connective tissue of lamina propria that led to development of features similar to human OSMF (Passi *et al*., 2017).

The OSMF rats belonging to group 2A treated with muco-adhesive gel without active components displayed no noticeable change in the improvement of epithelial thickness, or decrease in degree of orthokeratosis and regaining of lost rete ridges. The connective tissue component of group 2A rat buccal mucosa exhibited completely hyalinized collagen, congested and constricted vessels with decrease in number of fibroblasts, few chronic inflammatory cells infiltrated the lamina propria and muscle atrophy characterized by collagenous tissue invasion within the submucosal tissues at week 12 as well as week 16. In contrast, the group 2B after application of muco-adhesive gel containing curcumin and licorice, exhibited an initiation of reversal of histopathological features of OSMF at week 12 and further improvement of these histopathological changes were observed at week 16. These findings included regain in partial thickness of epithelial layer and partial reappearance of epithelial extensions (rete ridges). However, no obvious change in decrease in degree of orthokeratinization of stratified squamous epithelium was noted. These findings were consistent with another study conducted by Navadagi *et al*., who reported a regain in epithelial thickness and reappearance of lost rete ridges from 3 months onwards after turmeric (curcumin) gel application in Gutkha-induced OSMF in albino mice (Navadagi, 2005). The connective tissue component of group 2B rat buccal mucosa at week 12 showed dense collagen in the form of separate bundles just below the epithelium, few constricted as well as dilated blood vessels, few fibroblasts and inflammatory cells and muscle fibers interspersed among collagen deposits.

However, group 2B at week 16 displayed distinct change in sub-epithelial hyalinized collagen with prominent decrease in connective tissue density, reappearance of blood vessels and increased vasculature, more than few fibroblasts and few inflammatory cells along with decrease in distance between muscle fibers and overlying epithelium. These findings were in accordance with the study by Navadagi *et al*., who reported dense collagen deposition, presence of chronic inflammatory infiltrate and perivascular fibrosis at 1 month of turmeric (curcumin) gel application in Gutkha-induced OSMF in albino mice. After 3 months of gel application, dissolution of collagen in lamina propria with few inflammatory infiltrate invading the connective tissue appeared on histopathological examination (Navadagi, 2005).

Previous studies have reported the use of curcumin both systemically as well as topical application in the form of capsules, tablets and lozenges, oils and gels for symptomatic relief of OSMF and also has shown positive role in treatment of OSMF (AloK *et al*., 2015; Al-Maweri, 2019). Previous research also suggests effectiveness of licorice against OSMF such as although not directly related, but licorice root extract has also showed effective

results on cultured human fibrotic buccal fibroblasts indicating licorice can be used for effectively treating the precancerous OSMF (Lee *et al*., 2018). In another study, 29mg of licorice, in combination with other herbal antioxidants, was given to patients suffering from OSMF in a capsule form for 90 days. The findings showed that licorice, along with other medicinal herbs, was effective in treating the symptoms of OSMF such as improved the mouth opening and reduced the burning sensation of OSMF (Wollina *et al*., 2015).

This is the first study in which a combination of curcumin and licorice has been used for evaluating their combined role in treatment of OSMF. In our study the application of muco-adhesive gel containing active components i.e., curcumin and licorice led to encouraging results, which included improved mouth opening following fibrosis, regain of body weight and initiation of reversal of histopathological changes.

Histopathologic changes such as hyperplasia of epithelium, marked reduction in sub-epithelial hyalinization of connective tissue, reduction in inflammatory infiltrate and muscle fibers appearance close to the epithelium support the anti-inflammatory and fibrinolytic mechanistic properties of curcumin. The fibrinolytic mechanism of action of curcumin has been attributed to three basic properties i.e., it inhibits collagen synthesis, control and regulate cell divisions and inhibits lipid peroxidation (AloK *et al*., 2015). Curcumin has also been found to be anti-inflammatory because of inhibition of activation of nuclear factor kappa-B (NF-kB) pathway, a transcription factor implicated in the regulation of pro-inflammatory gene products and also reduces the expression of extracellular matrix genes through inhibition of connective tissue growth factor (CTGF), a main trigger for increased extracellular matrix (ECM) production (Deng *et al*., 2009; AloK *et al*., 2015). Similarly, licorice has been reported to possess anti-fibrotic and anti-inflammatory properties in bleomycin-induced pulmonary fibrosis via reduction in expression of pivotal inflammation mediatory pathways i.e., NF-κβ and transforming growth factor-β (TGF-β) pathways (Ghorashi *et al*., 2017). Glabridin, an isoflavonoid present in licorice root, has revealed anti-fibrotic effects on buccal mucosal fibroblasts by reducing the expression of TGF-β/Smad signaling pathway, ameliorated the trans differentiation of buccal mucosal fibroblast into myofibroblast and thus reduced the collagen synthesis (Lee *et al*., 2018). This suggested that combination of curcumin and licorice in gel form helped improved features of OSMF in experimental animals at week 12 and 16.

CONCLUSIONS

The application of muco-adhesive gel containing 1% curcumin and licorice led to resolution of fibrosis at week 16 in group 2B. These animals also exhibited a gain in body weight and mouth opening in Wistar rats because of anti-fibrotic, anti-oxidant and anti-inflammatory characteristics of curcumin and licorice.

FUTURE DIRECTIONS

Variation in dose amount of curcumin and licorice can be further explored. The different dosage of curcumin and licorice gel can be explored in other animal models of OSMF to investigate any variation in histopathological changes and efficacy of these herbs. The molecular mechanisms that underlay OSMF in animals models needs to be explored further to understand the disease process.

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Statement of conflict of interest
The authors have declared no conflict of interest.

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