Therapeutic Interventions in Oral Submucous Fibrosis: An Experimental and Clinical Study

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Abstract

Introduction Oral submucous fibrosis (OSF) is a chronic debilitating disease and premalignant condition of the oral cavity and is a serious public health issue in India and many parts of the world. The treatment is still elusive and empirical because of poorly understood etiopathogenesis, which is believed to be multifactorial including areca nut chewing, ingestion of chillies, genetic and immunologic processes, nutritional deficiencies, and many others. The present investigations was focused to understand the possible therapeutic interventions of anti-OSF agents in arecoline induced experimental in vitro model of OSF and clinical application of these anti-OSF agents in the restoration of various grade of the disease.

Materials and Methods The 127 subjects were selected from patients who visited the OPD of Department of Oral and Maxillofacial Surgery, Faculty of Dental Sciences, K.G. Medical University, Lucknow. Further the subjects were divided in two groups on the basis of clinical

examination. Group-1 subjects showed presence of fibrosis bands in the labial and/or buccal mucosa, loss of elasticity, difficulty to open the mouth and had a habit chewing areca-nut in some form. Group-2 subjects had no habit of chewing areca-nut, were apparently healthy with no mucosal disorder. The samples were collected and were immediately transported to Indian Institute of Toxicology Research, Lucknow, for isolation and cultivation of primary cultures of mucosal fibroblast cells. Then isolation and cultivation of oral mucosal fibroblast, identification of non-cytotoxic doses of arecoline, real time PCR, immunocytochemistry, cytokine determination in culture cells, western blot analyses, functional activity of collagenase, lysyl oxidase enzyme activity, collagen beads assay, cyclooxygenase (COX-2) expression analysis was done.

Results and Conclusions This study, shows that the reduction of phagocytic cells was strongly related to the arecoline levels in fibroblast culture when we exposed arecoline to normal oral mucosal cells (NOMC) and cells from OSF patient. An enhancement of phagocytic cells was observed following the pre exposure of cells to 1 µM dexamethasone, a glucocorticoids. In this study, histologic evidence is presented which supports the finding that COX-2 expression is upregulated in OSF specimens compared to normal oral submucosal cells. Strong immunostaining for COX-2 was detected in arecoline exposed NOMC and cells from OSF patient. Areca nut extract up-regulates prostaglandin production, cyclooxygenase-2 mRNA and protein expression of human oral keratinocytes. The number of phagocytic cells and phagocytic activity in cultured human oral fibroblasts from OSF site was lower than the fibroblasts from the normal regions of the same person.

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Introduction

Oral submucous fibrosis is a chronic debilitating disease and premalignant condition of the oral cavity and is a serious public health issue in India and many parts of the world [2, 22]. The treatment is still elusive and empirical because of poorly understood etiopathogenesis, which is believed to be multifactorial including areca nut chewing, ingestion of chillies, genetic and immunologic processes, nutritional deficiencies, and many others. The frequency and duration of chewing areca nut (without tobacco) is directly associated as the main etiological factor for OSF. Arecoline, an active alkaloid found in betel nuts, stimulates the fibroblasts to increase the production of collagen by 150 %. It has been demonstrated that arecoline, a major areca nut alkaloid, was found to stimulate human buccal mucosal fibroblasts (BMFs) proliferation and collagen synthesis in vitro [34]. Thus, the increased collagen synthesis or reduced collagen degradation may be a possible mechanism in the development of the disease [1].

There are numerous biological pathways involved in the above processes and, it is likely that the normal regulatory mechanisms are either down regulated or up regulated at different stages of the disease because of the interference of alkaloids and flavonoids of areca nut. In vitro studies on human fibroblasts using areca extracts or chemically purified arecoline support the theory of fibroblastic proliferation and increased collagen formation that is also demonstrable histologically in human OSF tissues [12]. An in vitro study suggests that arecoline and arecaine can enhance cell proliferation and affect fibroblasts to synthesize IL-6. Furthermore, IL-6 may be a contributing molecular factor in the pathological features noted in oral submucosal fibrosis [8]. The synthesis of collagens is influenced by a variety of mediators, including growth factors, hormones, cytokines, and lymphokines. A prominent mediator is the TGF- β , which stimulates the production of collagens and other matrix components.

Increased and continuous deposition of extracellular matrix may take place as a result of disruption of the equilibrium between matrix metallo-proteinases (MMPs) and tissue inhibitors of matrix metalloproteinases (TIMP). This may enhance in the alteration in oral submucosal fibrosis. Increased tissue inhibitor of matrix metalloproteinase-1 and decreased matrix metalloproteinase-2 activity [7] may contribute to the extracellular components accumulation in OSF. The main histopathological characteristic of OSF is the deposition of collagen in the oral submucosa [15]. Current evidence implicates collagen-related genes in the susceptibility and pathogenesis of OSF [9]. The activity of lysyl oxidase (LYOXase), an extracellular enzyme which stabilizes the collagen and prevents degradation of collagenase in oral mucosa, was found to increase in fibroblasts cultured from OSF patients [21].

Table 1 Gender distribution of normal and OSF patients in group-1 and group-2

Group	Gender	
	Male, n (%)	Female, n (%)
Group-1 (n = 87) (OSF)	n = 73 (83.90 %)	n = 14 (16.09 %)
Group-2 (n = 40) (NOMC)	n = 28 (70 %)	n = 12 (30 %)

The study subjects included 87 histopathologically confirmed cases of OSF and 21 age and sex matched healthy controls. A detailed case history which included habit of chewing areca nut, pan masala, gutkha and smoking habit index was taken from each subject in the study. Subjects with any other long-term systemic illness and long term medication were excluded from the study

The individual mechanism operating at various stages of the disease—initial, intermediate and advanced—need further study in order to propose appropriate therapeutic interventions, since the use of anti-OSF agents is currently not popular. In last couple of decades, in vitro model systems got popularized for prescreening of drugs and chemicals as they surrogate the in vivo and clinical situations with more precise biochemical and molecular end points. Thus, the present investigations will be focused to understand the possible therapeutic interventions of anti-OSF agents in arecoline induced experimental in vitro model of OSF and clinical application of these anti-OSF agents in the restoration of various grade of the disease.

Methodology

Sample Collection

Following the given consent, 127 subjects were selected from the patients who visited the OPD of Department of Oral and Maxillofacial Surgery, Faculty of Dental Sciences, K.G. Medical University, Lucknow. Further the subjects were divided in two groups on the basis of clinical examination. Group-1 subjects showed presence of fibrosis bands in the labial and/or buccal mucosa, loss of elasticity, difficulty to open the mouth and had a habit chewing areca-nut in some form. Group-2 subjects had no habit of chewing areca-nut, were apparently healthy with no mucosal disorder (distribution of samples shown in Table 1). Detail case history including age, sex, occupation, past and present dental history were recorded. The samples were collected and were immediately transported to Indian Institute of Toxicology Research, Lucknow, for isolation and cultivation of primary cultures of mucosal fibroblast cells.

Isolation and Cultivation of Oral Mucosal Fibroblast

Human buccal mucous fibroblast cells were cultured following the protocols with desired modifications [5]. Prior to

Fig. 1 **a** Primary cultures of human oral mucous fibroblast cells have been established and characterized in our laboratory conditions. **b** Arecoline induced alteration in mitochondrial activity in normal oral mucous fibroblast cells (NOMC) by MTT assay. The values are presented as percent cell viability. The data are the mean $\pm$ SE of three independent experiments and values were taken from at least six wells from each experiment (* $p < 0.01$). **c** Arecoline induced alteration in the lysosomal activity in NOMC by NRU assay. The

values are presented as percent cell viability. The data are the mean $\pm$ SE of three independent experiments and values were taken from at least six wells from each experiment (* $p < 0.01$). **d** Arecoline induced loss in membrane integrity in NOMC by LDH release assay. The values are presented as percent LDH released. The data are the mean $\pm$ SE of three independent experiments and values were taken from at least six wells from each experiment (* $p < 0.01$)

cultivation, each batch of cells was checked for viability using trypan blue dye exclusion method and batches showing cell viability more than 95 % were employed in the study. Batches used in the study were also screened for their genetic stability prior to use (Fig. 1a). Cells were cultivated as monolayer at 37 °C in 5 % CO₂ and 95 % air atmosphere with high humidity. At confluence, cells were detached by the treatment of 0.25 trypsin and 0.05 % EDTA for 2–3 min and aliquots of separated cells were subcultured or cryopreserved in freezing medium (Sigma) in liquid nitrogen.

Identification of Non-cytotoxic Doses of Arecoline

Prior to the use of arecoline to simulate the cellular and molecular events associated with the pathogenesis of OSF, the non-cytotoxic doses were ascertained by exposing the primary cultures to various concentrations of arecoline

(1–50 μM) for a period up to 72 h. Cytotoxicity assessment was done using standard endpoints including tetrazolium bromide salt MTT, neutral red uptake and lactate dehydrogenase release assays.

From all these experiment, we identified that a 10 μM dose of arecoline was non cytotoxic, so further we did the experiment by taking this dose.

Real Time PCR

Total mRNA was isolated from the cells (1×10^6) exposed to arecoline (10 μM) for 24 h. This mRNA was used to synthesize cDNA and further processed for real time-PCR for selected marker genes using primer specific to them. The selected marker genes were mainly associated with pro and anti-inflammatory cytokines viz., IL-1 β , IL-6, TNF- α , IFN- α , TGF- β .

Fig. 2 a Arecoline induced changes in m-RNA expression of cytokines and growth factor related gene in normal human mucosal cells (NOMC) and oral submucous fibrosis (OSF) specimens following non-cytotoxic exposure of arecoline (10 μM) by real time PCR. The values are presented as fold change and indicates the cytokine expression level were higher in arecoline exposed cells at 24 h in both normal oral mucosal cells (NOMC) and oral mucosal fibrosis (OSF)

specimens. **b** Arecoline induced changes in expression of IL-1β, IL-6, TNF-α and TGF-β protein in NOMC and OSF specimens following non-cytotoxic exposure of arecoline (10 μM) by immunocytochemistry. Figure indicates that cytokine expression levels were higher in arecoline exposed cells at 24 h in both NOMC and oral mucosal fibrosis (OSF) specimens

Immunocytochemistry

Cultured cells from group 1 and group 2 subjects were exposed with arecoline (10 μM) for 24 h, after completion of exposure period, cells were washed with PBS two times, fixed in 4 % paraformaldehyde for 30 min, permeabilized, with 0.5 % Triton X-100 for 15 min and again was washed with PBS, and blocked for 1 h with 2 % bovine serum albumin (BSA). Cells were then incubated with primary antibodies overnight at 4 °C. All the antibody against inflammatory cytokine and growth factor were used at 1:100 dilution as the primary antibody. Cells were then washed three times with PBS, followed by incubation with fluoresceinconjugated secondary antibodies (diluted 1:300) for 1 h at room temperature. After a second rinsing step with PBS, the cover slips were then transferred to a glass slide and labeled cells were examined with fluorescence microscopy (Fig. 2b).

Cytokine Determination in Culture Cells

Confirmation of the data of qRT-PCR was done by using commercially available “Ready-SET-Go! ELISA

Kit” (e-Biosciences, USA) to determine the levels of 5 cytokines in the cultured oral submucosal cells. These cytokines were IL-1β, IL-6, TNF-α, IFN- α and growth factor TGF-β.

Western Blot Analyses

Levels of protein expression were carried out using Western immunoblotting technique. The arecoline (10 μM) induced translational changes in the level of expression of selected pro-inflammatory cytokines and markers involved in collagen synthesis were assessed in normal human buccal mucosa and oral submucous fibrosis (OSF) at 24 h.

Functional Activity of Collagenase

The functional activity of collagenase, an enzyme of family metalloproteinases (MMPs) has also been carried out in culture supernatants of normal human buccal mucosa and OSF specimens following non-cytotoxic exposure of arecoline (10 μM) for 24 h. Collagenase activity for MMP-1, MMP-8 and MMP-13 in the culture supernatants was carried out.

Lysyl Oxidase (LOX) Enzyme Activity

Lysyl oxidase activity was assessed using Amplite™ Fluorimetric Lysyl Oxidase Assay Kit in both normal human buccal mucosa and OSF specimens, as per manufacturer instruction. Parallel set of under similar condition of arecoline treatment in primary cultures of normal human buccal mucosa and OSF specimens were also run with Anti-OSF agents β -aminopropionitrile (BAPN), an irreversible LOX inhibitor, using non-cytotoxic concentration.

Real Time PCR

Total mRNA was isolated from the cells (1×10^6) exposed to arecoline (10 μ M) for 24 h. This mRNA was used to synthesize cDNA and further processed for real time-PCR for selected marker genes viz. (Col1A2, Col3A1, Col6A1, Col6A3, Col7A1) involved in collagen synthesis using primer specific to them in primary cultures of normal human buccal mucosa and OSF specimens.

Collagen Beads Assay

To investigate the inhibition of collagen phagocytosis in the cells, normal fibroblast and the cells from OSF specimen were incubated with arecoline 10 μ M for 24 h. Coated fluorescent latex beads were incubated with human oral mucosa fibroblasts and the fluorescence associated with internalized beads was measured by fluorescence microscopy.

Cyclooxygenase (COX-2) Expression Analysis

The effect of arecoline on COX-2 expression in normal human buccal mucosa and OSF specimens were studied by immunocytochemistry and western blot analysis.

Results

Isolation and Cultivation of Oral Mucosal Fibroblast

Primary cultures of human oral mucous fibroblast cells have been established and characterized in our laboratory conditions. Cells were found to be proliferative retaining the entire characteristic features (Fig. 1a).

Identification of Non-cytotoxic Doses of Arecoline

The results of cytotoxicity assays (MTT, NRU and LDH) are summarized in Fig. 2a, b. In MTT assay, experimental

exposure of arecoline was found to be safe up to 10 μ M concentrations for all the exposure periods i.e., 24, 48 and 72 h. However, arecoline exposure for 72 h at 25 μ M was found to reduce cell viability significantly. The concentration 50 μ M was recorded statistically significant ($p > 0.01$) cytotoxic at all the time points in a dose dependent manner (Fig. 2a). The trend for NRU assay was also similar to MTT assay; however the magnitude of reduction was lesser than MTT assay (Fig. 2b). The data of LDH release assay also had good correlation with the other assays conducted for cytotoxicity assessment i.e., MTT and NRU. However, the significant prominence of arecoline induced changes appeared even from 25 μ M concentration and got intense further with the increase in arecoline concentration. On the basis of cytotoxic studies; 10 μ M concentration of arecoline was selected for expression and activity analysis studies.

Real Time Analysis of Pro and Anti-inflammatory Cytokines

Real Time-PCR based studies have shown that normal oral mucosal cells (NOMC) from healthy patients are vulnerable to arecoline exposure (10 μ M $\times$ 24 h), since statistically significant induction in the expression of IL-1 α (RQ = 19.2), IL-6 (RQ = 6.8), TNF- α (RQ = 5.4), IFN- α (RQ = 3.9) and TGF- β (RQ = 5.2) could be recorded, while the effect in the expression levels were more intense in the cells isolated from OSF specimens after arecoline exposure in same condition Fig. 3.

Immunocytochemistry of Pro and Anti-inflammatory Cytokines

Arecoline induced changes in expression of IL-1 β , IL-6, TNF- α and TGF- β protein in NOMC and OSF specimens following non-cytotoxic exposure of arecoline (10 μ M) by immunocytochemistry reveals high expression levels of these proteins in the arecoline exposed cells. Expression levels of IL-1 β , IL-6, TNF- α and TGF- β protein were more in OSF controlled cells in comparison to normal oral mucosal controlled cells (NOMC) (Fig. 4).

Cytokine Determination in Culture Cells

Figure 5a, b indicates that cytokine expression levels were higher in arecoline exposed cells at 24 h in both NOMC and oral mucosal fibrosis (OSF) specimens exposed to 10 μ M arecoline. The levels were maximum for TGF- β and minimum for IFN- α in both the groups. While expression levels in control cells of OSF specimen itself were significantly higher than NOMC.

Fig. 3 **a, b** The cytokine expression levels were higher in arecoline exposed cells at 24 h in both NOMC and oral mucosal fibrosis (OSF) specimens. The level were maximum for TGF-β and minimum for

IFN-α in both the groups. **c, d** Band densitometry analysis of western blot showing arecoline (10 μM) induced cytokines after 24 h exposure in cells derived from normal oral mucosal and OSF patients

Western Blot Analyses of Pro and Anti-inflammatory Cytokines

Western blot analysis was synchronized with other measured values used for the determination of pro and anti-inflammatory cytokines. The expression levels were more than 2 fold higher in arecoline (10 μM for 24 h) exposed cells in both NOMC and oral mucosal fibrosis (OSF) cells. Expression levels were maximum for IL-1β in both the groups (Fig. 6a, b).

Functional Activity of Collagenase

The functional activity of collagenase indicates that the levels of MMP-1, 8 and 13 significantly decreased in arecoline induced normal oral mucosal and cells of OSF patients. While the levels were already very low in OSF specimen compared to NOMC. The anti-OSF agents or collagenase activator (colchicines) significantly induced the collagenase activity by up regulating the levels of MMP-1, 8 and MMP-13 (Fig. 7a, b).

Lysyl Oxidase (LOX) Enzyme Activity

Further we observed significant increase in LOX activity after 24 h exposure of arecoline (10 μM) in both the groups. β-aminopropionitrile (BAPN, 100 μM), an irreversible LOX inhibitor was found to significantly reduce the level of LOX near to control (NOMC), even below in cells from oral submucosal specimen (Fig. 8a, b). Further this result was confirmed at translational levels (Fig. 8a, b.)

Real Time PCR

RT-PCRq analysis showed significant up regulation of the marker genes associated with collagen synthesis. Cells exposed to arecoline 10 μM, 24 h significantly up-regulate the gene i.e., Col1A2 (RQ = 5.4), COL3A1 (RQ = 2.1), Col6A1 (RQ = 3.1), COL6A3 (RQ = 3.6) and COL7A1 (RQ = 2.5) in comparison to NOMC cells, while Col1A2 (RQ = 8.2), COL3A1 (RQ = 5.8), Col6A1 (RQ = 6.8),

Fig. 4 The functional activity of collagenase—an enzyme of family metalloproteinases (MMPs) indicates that the level of MMP-1, 8 and 13 significantly decreased in arecoline induced normal oral mucosal (a, c, e) and cells of OSF (b, d, f) patients. While the levels were

already very low in OSF specimen compared to NOMC, the anti-OSF agents or collagenase activator (colchicines) significantly induced the collagenase activity by up regulating the levels of MMP-1, 8 and MMP-13

COL6A3 (RQ = 7.1) and COL7A1 (RQ = 6.7) in comparison to OSF cells exposed to arecoline (Fig. 6a).

Western Blot Analysis of Collagen Synthesis Markers

Western blot analysis was very well synchronized with real time PCR analysis of collagen synthesis marker gene.

Figure 6b, c showed the translational expression levels of COL1A2 (2.8 fold), COL3A1 (2.37 fold), COL6A1 (3.1 fold), COL6A3 (3.9 fold) and COL7A1 (2.7 fold) in comparison to NOMC cells when exposed to 10 μ M arecoline for 24 h, while COL1A2 (3.5 fold), COL3A1 (4.8 fold), COL6A1 (5.8 fold), COL6A3 (2.9 fold) and COL7A1 (5.7 fold) in comparison to OSF cells exposed to arecoline.

Fig. 5 Arecoline (10 µM) induced lysyl oxidase (LOX) activity after 24 h exposure in NOMC (a) and in OSF patients (b). β-aminopropionitrile (BAPN, 100 µM), an irreversible LOX inhibitor significantly reduced the level of LOX near to control (NOMC), even below in OSF patients. Band densitometry analysis of western blot showing

arecoline (10 µM) induced LOX activity after 24 h exposure in NOMC (c) and in OSF patients (d). β-aminopropionitrile (BAPN, 100 µM), an irreversible LOX inhibitor significantly reduced the level of LOX near to control (NOMC), even below in OSF patients

Collagen Beads Assay

The Collagen bead assay analysis showed significant downregulation of the internalization of beads after arecoline (10 µM) treatment for 24 h, in NOMC (45 %). While cells from OSF tissue exhibited a 60 % reduction of the proportions of collagen phagocytic cells. Pre treatment with Dexamethasone (1 µM) significantly enhanced the proportion of phagocytic cells (Fig. 7a, b).

Cyclooxygenase (COX-2) expression analysis

To examine the effect of arecoline on the COX-2 expression we did this experiment with two ways a. Immunocytochemistry and b western blot, and found synchronization in both the assays.

- (a) Immunocytochemistry: Arecoline exposed cells from normal tissues that had been incubated with

Fig. 6 RT-PCR analysis showed significant up regulation of the markers genes i.e., CoL1A2 (RQ = 5.4), CoL3A1 (RQ = 2.1), CoL6A1 (RQ = 3.1), CoL6A3 (RQ = 3.6) and CoL7A1 (RQ = 2.5) in comparison to NOMC cells while CoL1A2 (RQ = 8.2), CoL3A1 (RQ = 5.8), CoL6A1 (RQ = 6.8), CoL6A3 (RQ = 7.1) and CoL7A1 (RQ = 6.7) in comparison to OSF cells

exposed to arecoline (10 μM). *Western blot* analysis showed significant up regulation of collagen synthesis related protein in normal oral submucosal cells (NOMC) cells and the cells from OSF patients when treated with arecoline (10 μM) for 24 h. The cells from OSF patients showed higher expression than the NOMC cell without treatment

Fig. 7 Collagen beads assay—coated fluorescent latex beads were incubated with normal human oral mucosa fibroblasts and cells from OSF tissues. The fluorescence associated with internalized beads was measured by fluorescent microscopy. Analysis showed significant down regulation of the internalization of beads after arecoline

(10 μM) treatment for 24 h (45 %), in NOMC cells (a). while cells from OSF tissues exhibited a 60 % reduction of the proportions of collagen phagocytic cells. Pretreatment with dexamethasone 1 μM significantly enhanced the proportion of phagocytic cells (b)

10 μM arecoline showed significant up regulation in the expression of COX-2 protein; while cells from OSF specimens showed high expression of COX-2 in comparison to NOMC. The expression levels were maximum in arecoline exposed OSF cells (Fig. 8a).

(b) Western blot analysis: Band densitometry analysis of COX-2 protein was twofold higher in arecoline exposed normal submucosal cells, while the value was fourfold for arecoline exposed cells of OSF specimen (Fig. 8b).

Fig. 8 **a** Arecoline induced changes in expression of COX-2 protein in NOMC and the cells from OSF specimens following non-cytotoxic exposure of arecoline (10 μM) by immunocytochemistry. Arecoline exposed cells indicate that COX-2 expression levels were higher in

both NOMC and oral mucosal fibrosis (OSF) specimens. **b** Band densitometry analysis of western blot showing arecoline (10 μM) induced COX-2 activity after 24 h exposure in NOMC and in cells from OSF patients

Discussion

Oral submucous fibrosis is a pre-malignant condition, which has been described in detail in Asians and Asians settled in other countries. Various etiological factors have been suggested for OSF, which include local irritant such as capsaicin, pungent and spicy food and areca nut use [17]. In addition to the local factors, systemic factors have also been suggested to play a role in the development of OSF. These include anaemia, chronic iron and vitamin B deficiency [25]. Chewing areca nut in its various forms is widely prevalent in the Indian subcontinent, giving rise to increased prevalence of OSF, from an estimated 250,000 cases in 1980 [11] to an estimated 5 million people in 2002 [9]. The mean age of all cases affected with OSF has been reported as 28.8 ± 10.4 years, which is relatively a younger age when compared with south Indian (32.4 ± 10.4 years) and north Indian (30.42 ± 10.86) [26, 28]. In our case out of one hundred and twenty seven patients between the age ranges of 20–50 years, 83 % were males and 16 % females, which was similar to a male preponderance, reported by various authors [26, 28]. In the present study, there were 40 cases with no history of areca nut or tobacco chewing. Out of 40, 28 were males and 12 were females.

Primary cultures of human oral mucous fibroblast cells from normal and OSF patient have been established and characterized in our laboratory conditions (Fig. 1a). Figure 1a(I) Morphology of primary culture of normal oral submucosal cells (NOMC) and (II) morphology of primary culture of oral submucosal fibrosis cells from OSF patient. In OSF cells more cells became retracted or round-shaped with an increase of intercellular space. Similar effect was found in human endothelial cells when exposed to 0.4 mM arecoline for 24 h [32].

In this study, we demonstrated that arecoline was cytotoxic to oral submucosal cells at concentration 25 μM or above in feasible time point. Cytotoxicity of arecoline was concomitant with marked morphological changes of NOMC cells, e.g., retraction and rounding, indicating the presence of endothelial cell damage such as loss of substrate adhesion or cell–cell adhesion as we have seen in the isolated cells from OSF patient (Fig. 1a(II)). That is why we further did all the experiment by taking concentration 10 μM of arecoline which was non cytotoxic to NOMC as examined by MTT, NRU and LDH assay.

Cytokines play an important role in regulating fibroblast function, such as proliferation, migration and matrix synthesis, and it is the balance of these mediators that is likely to play a key role in regulating the initiation and

progression of scarring in any fibrotic disease [20]. Both interleukin-1 (IL-1) and tumour necrosis factor alpha (TNF- α) stimulate fibroblast proliferation in vitro [27, 33], and intradermal injections of TNF- α stimulate the accumulation of fibroblasts and collagen [23]. Similarly, IL-6 has been implicated in the development of fibrosis [16]. On the other hand, IFN- γ is an antifibrotic cytokine [13]. Chou et al. [10] have shown that TNF- α inhibits adherence and phagocytosis of collagen; they suggest that inhibition of the collagen phagocytic pathway may contribute to fibrosis. In this present study we determine whether arecoline induced NOMC and cells from OSF patient have an altered cytokine profiling. The cytokines studied were IL-1 β , IL-6, TNF- α and IFN- α and TGF- β . Our result was correlative to the above finding. We also found that at noncytotoxic concentration of arecoline 10 μ M cells from NOMC and OSF patient significantly induced the expression of IL-1 β , IL-6, TNF- α and IFN- α and TGF- β at both m-RNA (Real time PCR) and protein levels (Elisa, immunocytochemistry and western blot), which may be responsible for development of oral submucosal fibrosis. We found very well synchronization in both (m-RNA & protein) related assays. Even the expression levels of these cytokines were themselves significantly high in comparison to normal oral submucosal cells which indicates that pathological fibrosis was already high in OSF patients.

Matrix metallo proteinases (MMPs) are a group of enzymes which together can degrade all the known protein components of the extracellular matrix (ECM). MMPs can be divided into the different subgroups one of them is known as collagenase. MMPs are highly regulated, with both secretion and activity levels under tight control. In general, in normal tissue, MMPs are expressed at very low levels. It has been described that areca quid as well as its components affect fibroblast proliferation, increase collagen synthesis, and promote an accumulation of ECM components in the oral mucosa. Many researchers have reported the etiology of submucosal fibrosis as an increase of collagen deposition resultant to a decrease of collagen degradation [4]. TIMPs play an important role in various physiological processes that involve tissue remodeling. They help maintain a delicate balance between physiological degradation and synthesis of the extracellular matrix. TIMPs are widely distributed in the tissues and fluids and are expressed by many cells including fibroblasts [3]. TIMPs play an important role in MMP inhibition and ECM turnover. In addition, TIMP-1 and TIMP-2 was reported to block collagen degradation in cartilage remodeling [14]. In our study we found the levels of collagenase an enzyme of matrix metalloproteinase, MMP-1, MMP-8 and MMP-13 were significantly decreased in arecoline induced NOMC and cells from OSF patients which clearly indicates the imbalance in increase of

collagen deposition and decrease of collagen degradation. Our result was correlative with previously published data by Chang et al. [6, 7], who showed that arecoline reduced the MMP-2 secretion and increased the TIMP-1 levels resulting in increased deposition of collagen in the extracellular matrix. The mechanism(s) which cause down regulation of the activity of these enzymes in vitro seems rather speculative at present, which needs further research. Further we observed the reversal in the levels of MMPs by adding collagenase activator colchicine. This indicates certainly the involvement of MMPs in the development of oral submucosal fibrosis (Fig. 4). The activity of LYOXase, an extracellular enzyme stabilizing the collagen fibrillar array by covalent cross-links, was found to increase in fibroblasts cultured from OSF patients in comparison to NOMC. This is a copper dependent enzyme [19] and plays a key role in collagen synthesis. The possible role of copper as a mediator of fibrosis is supported by the demonstration of up regulation of this enzyme in OSF biopsies [30] and in OSF fibroblasts compared to normal fibroblasts grown in culture [21]. We also found a similar effect in our case and the effect was more prominent when these cells were exposed to arecoline 10 μ M, 24 h. β -aminopropionitril (BAPN, 100 μ M), an irreversible LOX inhibitor, was found to complete reversal of LOX activity as measured by ELISA and western blot analysis (Fig. 5), which hypothesized that LOX contribute to the pathogenesis of OSF.

Because several genes are involved in the metabolism and synthesis of collagens fibers we further assessed the expression and inducibility of collagen related genes in arecoline exposed NOMC cells and the cells from OSF patients. The genes Col1A2, COL3A1, Col6A1, COL6A3 and COL7A1 have been identified as definite TGF- β targets and induced in fibroblasts at early stages of the disease. There is evidence to suggest that collagen-related genes are altered due to ingredients in the quid [24]. Results in this study were correlative to above finding. We also found an increase in collagen related genes when NOMC cells and cells from OSF patients were exposed to arecoline at both transcriptional and translational levels (Fig. 6), which can be the indication of high expression of collagen synthesis in OSF pathogenesis. Chiu et al. [9] analyzed two groups of betel chewers, one with OSF and the other without, in order to compare the association of OSF and polymorphisms of six collagen related genes. They found that genotypes associated with highest OSF risk for collagen 1A1, collagen 1A2, collagenase-1, TGF- β 1, lysyl oxidase and cystanin C were CC, AA, TT, CC, AA, and AA, respectively in the low-exposure group whilst TT, BB, AA, CC, GG, and AA, respectively for the high-exposure group.

Degradation of collagen by fibroblast phagocytosis is an important pathway of physiological remodeling of the

extracellular matrix (ECM) in connective tissue. As OSF shows a gross imbalance in ECM remodeling, this putative mechanism was investigated in vitro [29]. A decrease of the phagocytic activity of fibroblasts in OSF patients when compared with the normal persons has been earlier reported [31]. The number of phagocytic cells and phagocytic activity in cultured human oral fibroblasts from OSF site was lower than the fibroblasts from the normal regions of the same person. This result demonstrated that the fibroblast assumes heterogeneity in function in different areas from the same patient. Similarity has been seen in our experiment as we saw decrease in phagocytic activity in our system. We have also shown in this study, that the reduction of phagocytic cells was strongly related to the arecoline levels in fibroblast culture when we exposed arecoline to NOMC and cells from OSF patients. An enhancement of phagocytic cells was observed following the pre exposure of cells to 1 μ M dexamethasone, a glucocorticoids (Fig. 7).

In this study, histologic evidence is presented which supports the finding that COX-2 expression is up-regulated in OSF specimens compared to normal oral submucosal cells. Strong immunostaining for COX-2 was detected in arecoline exposed NOMC and cells from OSF patients. This observation is in agreement with other study in which authors showed areca nut extract up-regulates prostaglandin production, cyclooxygenase-2 mRNA and protein expression of human oral keratinocytes [18]. We confirmed our result with western blot assay and found the synchronization with immunostaining assay of COX-2 expression.

Conclusions

This study, shows that the reduction of phagocytic cells was strongly related to the arecoline levels in fibroblast culture when we exposed arecoline to NOMC and cells from OSF patient. An enhancement of phagocytic cells was observed following the pre exposure of cells to 1 μ M dexamethasone, a glucocorticoids. In this study, histologic evidence is presented which supports the finding that COX-2 expression is up-regulated in OSF specimens compared to normal oral submucosal cells. Strong immunostaining for COX-2 was detected in arecoline exposed NOMC and cells from OSF patient. Areca nut extract up-regulates prostaglandin production, cyclooxygenase-2 mRNA and protein expression of human oral keratinocytes. The number of phagocytic cells and phagocytic activity in cultured human oral fibroblasts from OSF site was lower than the fibroblasts from the normal regions of the same person.

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