Fibroblastic phenotype in oral submucous fibrosis- a cell culture analysis

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Abstract: Arecoline present in the arecanut permeates through the oral mucosa and induces increase in the collagen production and decrease in the collagen degradation. This indicates the probability of a different phenotype of fibroblast being operative in the causation of oral submucous fibrosis (OSF). The present study aimed to establish cell lines of fibroblast from normal buccal mucosa and OSF tissues of human, identify the various morphological forms of fibroblasts; understand and assess the response of the fibroblast cell lines to different concentrations of arecoline.

fibroblast cell lines were obtained from control samples as well as from OSF. The cell lines were treated with 50/100/150/300/500 ug/ml of arecoline from control and cases. The response of the cell lines were evaluated over a period of 8 days on the basis of cell counts. The fibroblast cell lines of the control and the cases exhibited different morphological forms i.e. F1 spindle, F2 epitheloid and the F3 stellate form. The F3 to F1 ratio was higher in OSF. There was dose dependent response observed in arecoline treated cell lines. The untreated cell lines showed an increase in the cell counts over the period of 8 days and the control fibroblasts showed a higher proliferation. It was found that arecoline at 50ug/ml was slight stimulatory and at 150ug/ml it was found to be cytotoxic to the cell lines. Arecoline can be stimulatory or enhance proliferation of the fibroblast at lower concentrations but acted as cytotoxic at higher concentrations. This is because of the response of the arecoline receptors at various concentrations of the arecoline.

Keywords: OSF, fibroblast, cell lines, arecoline.

INTRODUCTION

Oral submucous fibrosis is characterized by excessive deposition of collagen in the subepithelial tissue by the fibroblasts. Previous studies on the pathogenesis of OSF have suggested that the occurrence is due to stimulation of fibroblasts proliferation and collagen synthesis by the arecanut alkaloids, clonal selection of the fibroblasts with high amount of collagen production during the long term exposure to arecanut ingredients, stabilization of collagen structure by catechin and tannin from the arecanut. It was also found that there was a decreased secretion of collagenase, deficiency of collagen phagocytosis by OSF fibroblasts and increase in the crosslinking as caused by the release of lysyl oxidase by the fibroblasts and increased expression of the fibrogenic cytokines like TGF-Beta [1]. The role of fibroblasts in oral submucous fibrosis cannot be understated. There are many reports which assayed the potential of fibroblasts in the pathogenesis of oral submucous fibrosis [2]. The various modes of assay include light microscopy, immunohistochemical analysis, scanning electron microscopy etc., but none of these techniques have been successful in assessing the actual role of fibroblasts in the disorder. In vivo techniques like cell culture are more precise mechanisms for assessing the cellular behavior of the lesion. Cell cultures of fibroblasts from OSF have been increasingly carried out in various laboratories around the world and the reaction and behavior of these cells to a vast array of stimulants and chemicals have been assayed.

Cell cultures of OSF fibroblasts indicate multiple morphological patterns of the cells. The various morphological phenotypes reported of fibroblasts in cell cultures are of three types i.e. F1, F2, and F3. The F1 fibroblast is spindle shaped, highly proliferative and secretes low levels of type 1 and type 3 collagen. F2 is more epitheloid, less proliferative and synthesizes relatively more collagen, while F3, a large stellate cell and the least proliferative, produces large quantities of collagen type 1 and 3. It is known that...
these population of cells arise in a hierarchical pattern i.e. F1 gives rise to F2, F2 gives rise to F3 and this process seems to be irreversible. The shape of the fibroblast is determined by the pseudopods and the movement of the cells that involve the pseudopods. In cases of fibrosis it was found that F3 fibroblasts predominate and similar observation have been reported in oral submucous fibrosis [3].

Arecoline is the major alkaloid in betel quid and plays an important role in the pathogenesis. The salivary arecoline levels vary depending on the consuming habit, since arecoline levels varies based on geographical location. The response of the fibroblasts to arecoline is concentration dependent [4-6]. Cell culture techniques can be one of the most reliable methods to assess cellular characteristics in vivo. Fibroblasts seem to be one of the most versatile cells of the connective tissue amenable to be grown in the laboratory. These cells display their potential to differentiate into other cell types and involve phenotypic evolution. An accurate and in-depth evaluation of these growth characteristics can be evaluated by cell-culture techniques.

MATERIALS AND METHODS

The study group comprised of 13 cases of oral submucous fibrosis from the department of oral and maxillofacial pathology. A detailed case history was recorded and clinical grading was done according to Lai DR et al classification (1995) based on mouth opening. The incisional biopsy samples of 0.5cmx0.5cmx0.5cm were obtained from the fibrotic areas of the buccal mucosa and were divided into two portions. One portion of the tissue was used for histopathological assessment and the other was transported from the clinic to the cell culture laboratory in the culture media which also acts as the transport media (Dulbecco Modified Eagles Media-Sigma Aldrich). During the initial assessment of the cell culture wells, it was noticed four wells got contaminated by adipocytes due to deeper biopsy and were discarded.

The control group comprised of subjects without arecanut chewing habits with clinically normal mucosa. A total of 12 biopsy samples were obtained, but due to contamination two samples had to be discarded. The control group biopsies were obtained from the non-inflamed posterior buccal mucosa during surgical extraction of the third molar.

The biopsy vials were sealed with paraffin coated tape and were transferred from the clinic to the laminar hood in the cell culture laboratory.

In the cell culture laboratory

The tissue is retrieved from the transport media and is minced into 1mm x1mm x1 mm pieces with the help of sterile BP blade No 15 on the petridish containing the culture media (DMEM). Then it is subsequently washed in Dulbecco Phosphate buffer saline (DPBS- Sigma Aldrich) supplemented with 100u/ml gentamycin and 2.5 u/ml fungicide.

Minced tissues were further incubated into 35 wells of 60 mm size and allowed to adhere and maintain in 5ml of DMEM supplemented with 10% bovine calf serum and gentamycin. These petridishes were incubated at 37 degree centigrade in a humidifying atmosphere of 5 % carbon dioxide for 24 hours in water jacketed carbon dioxide incubator (Thermo Electron Corporation). This was followed by centrifugation at 2000 rpm for 5 minutes. The sediment was then plated using pipettes onto 30 mm tissue culture wells containing the working media for 48 hours. This ensures the attachment of the cells to the culture plate.

The cells were later observed every alternate day under inverted microscopy during culture. In addition the fibroblastic nature of the cells in the early passage (3rd passage) is confirmed by the determination of the morphological phenotypes. The primary fibroblasts were treated with 0.05% trypsin (Sigma Aldrich) in 0.1% EDTA (Nice Chemicals) containing phosphate buffer of PH 7.4, the process of trypsinization leads to the separation of the coalesced cells and helps in the passaging of the cells.

Cell counting

For the determination of growth rate and the cumulative population doubling level of the cell passage. The cells were counted in manual cell counting chamber after staining with 0.4% trypan blue.

During these observations the media was changed every 3rd day till one week, till the cells were sub cultured for obtaining next passage of cell lines to reach the confluence. These cultures were maintained in 37 degree centigrade in a humidifying atmosphere of 95 % air and 5% carbon dioxide in water jacketed Carbon dioxide incubator.

Passages or sub-culturing

The third passage was taken into consideration for the morphological assessment of the fibroblasts and the effects of arecoline (Sigma Aldrich) and collagenase (Himedia) on a concentration on dose dependent manner.

Assessment of the fibroblasts subpopulation based on morphology

Cell lines from the third passage were seeded on three 30 mm tissue culture plates at a concentration of 10 [4] and maintained for four days, during which they expressed their specific cell type morphology. The assessment of the morphological phenotypes was done 24hours after the revival of the cell culture plates from the ultra-freeze (New Brunswick Scientific) for both the cases and controls. An assessment of the correlation of
the varied morphology of the fibroblasts in respect to the histopathological grading was done.

**Morphological phenotypes of fibroblast cells**

Using inverted microscope the cells were observed under 20X magnification and counted daily for eight days. Fibroblast subpopulations were classified using two models. The morphology identification was based on the rat skin model model where F1, F2, F3 (F1 are spindle shaped, F2 are epitheloid and F3 are stellate shaped cells) were identified as fibroblast morphological phenotypes.

**Assessment of the cell count after treatment with arecoline**

At various concentrations of 50ug, 100ug, 150ug, 300ug, 500ug of arecoline, the cell population was assessed for 8 days after obtaining the third passage of sub-cultured fibroblasts of cases and control tissues.

**Assessment of cell count after treatment with collagenase type**

Two groups of fibroblasts cell lines i.e. the controls and cases which were treated with 50 ug, 100 ug and 150 ug of arecoline as well as the untreated groups were subjected to 50ul of 0.1% type 2 collagenase and were evaluated over a period of 8 days to observe the morphological forms of fibroblasts and the variation in the cell count.

**RESULTS**

The present study involves culturing of the human oral fibroblasts obtained from the normal buccal mucosa and oral submucous fibrosis tissues and to know the various morphological phenotypes of the fibroblasts in those tissues and assess the response of those cultured fibroblasts to various concentrations of arecoline. The oral submucous fibrosis tissues were histopathologically assessed and the other portion was used for culturing the oral fibroblasts.

**Assessment of fibroblasts cell culture lines in the study**

The assessments of the response of the fibroblasts were based on the cell counts from each culture well which confirmed viable cells assayed trough trypan blue staining (figure 1a). The cells were visualized by inverted microscope. The cell counts were determined by cell counting chamber. The cells were subcultured from the first passage cell lines showing viable cells and it was continued till the third passage. The third passage yielded more pure cell lines of fibroblasts which were needed for assessment. The cell counts were noted after the third passage over a period of 8 consecutive days.

**Morphological assessment of fibroblasts**

There are three different morphological phenotypes of fibroblasts noticed in the normal buccal mucosa and the OSF tissues. It was noticed that in both the groups the epitheloid form (figure 1c) of the fibroblasts predominated followed by spindle shaped fibroblasts (figure 1b). Stellate varieties (figure 1d) of fibroblasts were very scanty in the cell culture wells. The F3 to F1 ratio in OSF cell lines was 0.27 which was higher than the normal buccal mucosa fibroblast cell lines based on the mean cell count of each fibroblast form (table 1).

**Mode of assessment of the fibroblasts response (cell count)**

The response of the fibroblasts with or without the addition of arecoline and collagenase were analyzed based on the cell counts over period of 8 days. The cells were counted based on 5 different high power fields (x100) and multiplied to 10 [4].

**The assessment based on varying concentrations of arecoline**

**Untreated fibroblasts**

It was found that over a period of time i.e. from the 1\textsuperscript{st} day to the 8\textsuperscript{th} day there was an increase in the cell count in both the controls (normal buccal mucosa) as well as in the cases. The fibroblasts showed a progressive proliferation over a period of time (Figure 2)

50 ug arecoline - When this concentration of arecoline was added there was a steep fall in the cell count levels compared to the untreated ones on the 1\textsuperscript{st} day. There was a decrease cell counts observed progressively over a period of 8 days both in the controls and cases (Figure 3-7)

100ug arecoline - There was progressive decrease in the cell counts observed over eight days in both the groups of treated fibroblasts. The cases showed a decreased fibroblasts count compared to the controls in all the days. The control cell counts showed a plateau on the 2\textsuperscript{nd} and the 3\textsuperscript{rd} day, and there was a decrease in the cell counts which was noticed (figure 6-7).

150 ug arecoline – There was a higher cell counts in the control cell cultures compared to the cases in all the days. It was noticed that there was a progressive decrease in the cell counts in the controls and cases (figure 6-7).

300ug arecoline - There was progressive decrease in the cell counts observed over eight days in both the groups of treated fibroblasts. There was a slight proliferation of fibroblasts seen at all days in cases. There was a sharp inhibition of cell proliferation which was noticed from the 6\textsuperscript{th} day to the 7\textsuperscript{th} day and thereafter progressive decrease (figure 6-7).

500ug arecoline – There was a uniform inhibition of cell growth over the progressive days in the controls and the cases. The cell count was always seen higher in the controls on all the days (figure 6-7).
Assessment of fibroblast response based on the days

1\textsuperscript{st} day- The cell count was seen almost similar in all the groups of added concentrations of arecoline. There was a decrease cell count upon adding 50 ug of arecoline to both the groups. It showed the highest cell count among all the days. There was a slight increase in the mean cell count when adding 300 ug of arecoline both in the controls as well as in the cases. After adding 500 ug of arecoline the cell count was found to be more in the controls. The changes seen in the cell counts with different concentrations were not significant (Figure 3, 6; 7; table 2).

2\textsuperscript{nd} day- There is a steep decrease in the cell counts seen in both the controls and the cases upon adding 50 ug and 100 ug of arecoline. The cell counts of the control were found to be higher than the cases at varying concentrations. There was an alternate fall and rise in the mean cell count in the control cell culture wells starting from the 50 ug to 500 ug. In the fibroblasts cell culture wells of the cases, the cell count was found to increase at 300 ug followed by a slight decrease at 500 ug. The response of the cell lines of the treated and the untreated fibroblasts differed significantly (p<0.05) (figure 6-7; table 2).

3\textsuperscript{rd} day- There was a steep decrease in the cell count on addition of 50 ug arecoline. In controls there was an alternate rise and fall of the cell count at varying concentrations from 50 ug arecoline to 500 ug arecoline. In cases the cell count were always less than controls in various concentration. In cases there was an inhibited cell proliferation in 100 ug and 150 ug of arecoline followed by a slight increase in the cell count in 300 ug arecoline and further decrease at 500 ug arecoline. The response of the cell lines of the treated and the untreated fibroblasts differed significantly (p<0.001) (figure 6-7; table 2).

4\textsuperscript{th} day- There was decreased mean cell count noticed in the cell lines treated with 50 ug arecoline. There was a slight increase in the cell count at 100 ug concentration of arecoline followed by a progressive decrease in cell count noticed in the controls till 500 ug of arecoline. In the cases there was a consistent decrease in the cell proliferation from 50 ug till 500 ug concentration of arecoline. The response of the cell lines of the treated and the untreated fibroblasts differed significantly (p<0.001) (Figure 4, 6; 7; table 2).

5\textsuperscript{th} day- The cell proliferation of the controls were found to be more in the controls than the cases at all concentrations of arecoline. Inhibition of cell proliferation was seen at 50 ug concentration of arecoline in both controls and cases. At 100 ug of arecoline there was increase in the mean cell count followed by an inhibited cell proliferation till 500 ug of arecoline in the controls. In cases at 300 ug of arecoline the mean cell count showed higher values, after that a fall in the cell count was noticed at 500ug of arecoline.

The response of the cell lines of the treated and the untreated fibroblasts differed significantly (p<0.001) (figure 6-7; table 2).

6\textsuperscript{th} day- A higher cell count was observed in the controls than cases in all concentrations of arecoline considered. In controls there was an increased mean cell count at 100 ug followed by a decrease in cell count till 300 ug of arecoline, after that the mean cell count showed constancy till 500 ug of arecoline. In the cases there was a progressive inhibition of the cell proliferation seen at all concentrations of arecoline. The response of the cell lines of the treated and the untreated fibroblasts differed significantly (p<0.001) (figure 6-7; table 2).

7\textsuperscript{th} day- The cell counts were higher in the controls than in the cases in all concentrations of arecoline. There was an increase in the cell proliferation seen at 100 ug of arceline followed by inhibition of cell proliferation in the control samples. In cases there was a significant decrease in the cell count progressively in the various concentrations of arecoline. The response of the cell lines of the treated and the untreated fibroblasts differed significantly (p<0.001) (figure 6-7; table 2).

8\textsuperscript{th} day- It showed the maximum fall of the mean cell count in all concentrations of arecoline in both the controls and cases. There was a slight increase in the cell count at 100 ug of arecoline in the control followed by inhibition of cell proliferation till 500 ug of arecoline. In cases there was a uniform inhibition of cell proliferation till 500 ug of arecoline. The response of the cell lines of the treated and the untreated fibroblasts differed significantly (p<0.001) (Figure 5-7; table 2).

Overall assessment of the observations

It was observed that the untreated fibroblasts cell lines of the control samples and the OSF samples showed an increased proliferation over a period of 8 days and the control tissue fibroblast showed more proliferation compared to the case tissues. The arecoline treated cell lines showed a progressive decrease in the mean cell count in both the groups. The mean cell count of the fibroblasts cell in the culture wells treated with various concentrations of arecoline was significantly. In the control samples it was noticed that at 100 ug concentration of arecoline, there was little increase in the cell count from 50 ug concentration of arecoline (figure 6). In cases the mean cell count was seen to be progressively declining over a period, but at 300 ug concentration of arecoline there was a slight augmentation of the cell proliferation till the 6\textsuperscript{th} day and gradually it flattened. The 100 ug concentration of arecoline was seen as a mark for the transition in the response of the fibroblast cell lines. The fibroblast cells when exposed to concentration above 100 ug of arecoline showed cytotoxicity to a greater extend and cell death at the peak concentrations (figure 7). On comparing the mean cell count among the various
groups, the changes were statistically significant from 2nd day onwards (table 2) The changes of the cell lines in terms of mean cell count was found to be statistically significant (Greenhouse-Geisser Test) on the days of evaluation where (p<0.001) (table 3).

Table-1: Mean cell counts of the morphological forms of fibroblasts in cases and control.

<table>
<thead>
<tr>
<th>CELL FORM</th>
<th>CONTROL</th>
<th>CASE</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>2.7</td>
<td>1.1</td>
</tr>
<tr>
<td>F2</td>
<td>39</td>
<td>40.6</td>
</tr>
<tr>
<td>F3</td>
<td>0.6</td>
<td>0.3</td>
</tr>
</tbody>
</table>

*all cell counts = n10^4 ;(n = actual cell count)
F1 spindle shaped, F2 epitheloid , F3 are stellate shaped cells

Table-2: Comparison of cell responses among the different group of cell lines during the period of study

<table>
<thead>
<tr>
<th>DAY</th>
<th>MEAN</th>
<th>STANDARD DEVIATION</th>
<th>STAT VALUE</th>
<th>P-VALUE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>43.18</td>
<td>4.825</td>
<td>1.147</td>
<td>0.352</td>
</tr>
<tr>
<td>2</td>
<td>41.78</td>
<td>6.754</td>
<td>2.535</td>
<td>0.015</td>
</tr>
<tr>
<td>3</td>
<td>40.66</td>
<td>6.106</td>
<td>6.722</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>4</td>
<td>39.12</td>
<td>6.947</td>
<td>12.165</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>5</td>
<td>36.73</td>
<td>9.032</td>
<td>17.407</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>6</td>
<td>35.5</td>
<td>9.845</td>
<td>21.598</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>7</td>
<td>33.21</td>
<td>12.153</td>
<td>28.264</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>8</td>
<td>31.11</td>
<td>13.53</td>
<td>36.843</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

all cell counts = n10^4 ;(n = actual cell count) *P<0.05 (SIGNIFICANT)
**P<0.001 (HIGHLY SIGNIFICANT)

Table-3: Evaluating the level of changes in the mean cell counts of the individual group of cell lines over a period of 8 days as per concentration gradient.

<table>
<thead>
<tr>
<th>CONCENTRATION</th>
<th>MEAN VALUE</th>
<th>F-VALUE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CONTROL</td>
<td>CASES</td>
</tr>
<tr>
<td>Untreated</td>
<td>766.37</td>
<td>477.07</td>
</tr>
<tr>
<td>50ug/ml</td>
<td>1027.84</td>
<td>833.35</td>
</tr>
<tr>
<td>100ug/ml</td>
<td>1174.87</td>
<td>982.37</td>
</tr>
<tr>
<td>150ug/ml</td>
<td>1592.75</td>
<td>1279.24</td>
</tr>
<tr>
<td>300ug/ml</td>
<td>2536.54</td>
<td>2217.49</td>
</tr>
<tr>
<td>500ug/ml</td>
<td>2698.08</td>
<td>2118.37</td>
</tr>
</tbody>
</table>

all cell counts = n10^4 ; (n = actual cell count)
**P<0.001 (HIGHLY SIGNIFICANT)
Fig-1: A: Trypan blue assessment to ascertain the viability of the cell lines in the culture well. The non-viable cells uptake the stain. The lesser the stained cells, the more viable are the cell lines. B: Spindle form of fibroblasts (F1), the most proliferative population of fibroblasts and seen in the early stages of OSF. C: Epitheloid form of fibroblasts (F2), more committed towards production of type I and III collagen. D: Stellate form of fibroblast, least proliferative amongst all but produces more collagen and seen mostly in the advanced stages of OSF.

Fig-2: The untreated fibroblast cell lines of the control and a case sample. A: The cell line of a control sample on day 1. B: The cell line of a control sample on day 4. C: The cell line of a control sample on day 8. D: The cell line of a case sample on day 1. E: The cell line of a case sample on day 4. F: The cell line of a case sample on day 8.
There is an increased proliferation of the cells over the subsequent days and the epitheloid form of fibroblasts is found in large counts compared to others.

**Fig-3:** shows the fibroblasts cell lines after being treated with 50ug/ml arecoline on day 1.

A: Control sample  
B: Case sample

**Fig-4:** shows the fibroblasts cell lines after being treated with 50ug/ml arecoline on day 4. There was a slight decrease in the cell count in both the groups.

A: Control sample  
B: Case sample

**Fig-5:** The cell counts reduced drastically in both control sample and case due to cytotoxicity cause due to arecoline at 50ug/ml on day 8.

A: Control sample  
B: Case sample
DISCUSSION

Arecanut is considered as the main etiological factor for the causation of OSF. The major constituents of arecanut include carbohydrates, fats, proteins, crude fibres, polyphenols (flavonols and tannins), alkaloids and minerals. Alkaloids form the most important biological components, of which arecoline, arecaidine, guvacoline and guvacine are the most important [7]. The pathogenesis concept was more strengthened by documenting the dose-response relationship of arecoline, the major alkaloid responsible in development of OSF. These alkaloids have been found to be genotoxic and carcinogenic. They undergo nitrosation and give rise to N- nitrosamines, which have a cytotoxic effect on the cells. Arecoline, the most abundant alkaloid has been shown to influence the process of fibrosis and has been implicated in the pathogenesis of OSF [8]. Arecoline penetrates through
the epithelial barrier of the oral mucosa and comes in contact with the connective tissue components and induces fibroblastic proliferation and increased collagen synthesis leading to the formation of excessive connective tissue characterizing OSF [9, 10].

The subepithelial change which takes place in this disease condition is mainly due to response of the fibroblasts to the penetrated components like arecoline. It is already well known that fibroblast has dual potential of formation as well as degradation of collagen matrix. The mechanisms of increased collagen were also analyzed by southern blot, northern blot and slot blot techniques. The OSF fibroblasts strains with increased collagen production also contained more than normal levels of procollagen gene expression which were derived from cell cultures [11]. TGF-beta, an important growth factor is secreted by fibroblasts and counteracts the mitotic effect of KGF (Keratinocyte growth factor) on keratinocytes. TGF-beta 1 is the most important isoform among the three isoforms that have shown to down regulate epithelial growth, induce differentiation and apoptosis in keratinocytes [12].

In our study we used the third passage of cells as the yield of more pure lines of cell populations were obtained compared to first passage where there can be an admixture of cells. The study was carried out for 8 days based on the previous literature review it was found that till 8 days significant viable cells can be appreciated in the cell lines, after that there may be a progressive decrease in the cell count which would have made the assessment of the observations difficult.

In previous studies done on fibroblasts cell culture, there were three morphological forms of fibroblasts which were appreciated. Based on the animal model experiments these forms were classified into F1 (spindle shaped), F2 (epitheloid shaped) and F3 (stellate shaped). F1 form produces low levels of collagen (type 1 and 3), F2 is proliferative and secretes more collagen. F3 produces high levels of collagen (type 1 and 3) and it was also found that F3 are terminal fibroblasts which arise from F2, while F2 arises from F1 [3,13,14]. In our study the maximum count of fibroblasts were of epitheloid form (F2) followed by spindle forms (F1) of fibroblasts in both the controls as well as OSF cases. This could be because of the fact that most of the cases belonged to early OSF and moderately advanced stages, the fibroblasts were still in the proliferative stage where most of the spindle cells gave rise to F2 population of cells. F3 are the terminal cells which are responsible for synthesizing high amounts of collagen were very scanty. The typical morphology of the fibroblasts i.e. spindle form were found scanty in our study. This could be because of the consideration of the 3rd passage of cell lines where the spindle form of fibroblasts probably has transformed to epitheloid variety over progressive sub-culturing.

The F3 to F1 ratio in OSF cell lines was 0.27 which was higher than the normal buccal mucosa fibroblast cell lines, which was found to be consistent to previous studies where F3 population were found to higher in OSF than in F1 which signified the prevalence of senescent fibroblasts in OSF. The cell populations were evaluated during initial days of cell culture of third passage but an evaluation of morphological phenotype was not done over a period of 8 days. A review of literature showed that in OSF there is a progressive shift of F1 to F3 population of fibroblasts and these fibroblasts become less proliferative and more committed towards collagen production hence producing OSF like condition [3, 15].

Permeability of the oral mucosa is an important phenomenon to be considered in the pathogenesis and progression of OSF. It is a factor which governs the absorption of the components of arecanut into the connective tissue component through the epithelial barrier and it depends upon the degree of ionization of the penetrating compounds at any particular pH (pKa) [16]. A compound when consumed through oral route has a higher concentration of the component in the saliva, but due to various factors like permeability, tissue metabolism etc., when it reaches the tissue level the concentration reduces drastically. This phenomenon is known as first pass metabolism or first pass elimination [17]. It is only after the first pass elimination the cells of the tissue starts interacting with the components and the actual pathogenesis of the disease starts.

Fibroblasts population derived from the normal buccal mucosa and OSF cases exhibited similar response on getting exposed to 10 ug/ml of arecoline. The population doubling time was also almost similar in the cell culture wells [14, 18, 19]. We also saw a similar response in the fibroblasts of the controls as well as the OSF cases when they were exposed to different concentrations of arecoline. In the untreated groups of fibroblasts the cell count of the normal buccal mucosa was found to be slightly higher than the OSF cases. The cell lines belong to two different conditions and the phenotype exhibited by the OSF fibroblasts is different from that of normal buccal mucosa fibroblasts. This is probably due to decreased sensitivity of the OSF fibroblasts that have already get exposed to different components of arecanut.

The salivary concentrations of arecoline were found to be ranging from 5.66 ug/ml to 97.39ug/ml while chewing arecanut [20]. But the concentration drastically gets reduced upon reaching the tissue and that concentration actually initiates the response in the fibroblasts. We have considered the same principle of bioavailability and used different concentrations of arecoline ranging from 50-500ug/ml. A study estimated the concentrations of arecoline in saliva to be 140ug/ml during chewing betel quid; the concentration in the oral cavity.
tissue level was not ascertained [21]. Therefore various lower concentrations of arecoline were subjected to the cultured fibroblasts to observe the response. The concentrations of arecoline i.e. from 50 ug/ml to 500 ug/ml were taken in consideration for our study on noting the salivary concentrations of arecoline stated in the previous studies [20, 21]. We tried to hypothesize the situation of the OSF condition where arecanut was chewed over years and over a period of time the tissue level concentrations of arecanut increased.

In similar studies which used cultured human buccal mucosa fibroblasts and further treated with 50ug/ml, 100ug/ml, 150ug/ml, 300ug/ml and 500ug/ml of arecoline, it was found that the response of the cultured fibroblasts was dose dependent. The concentrations 50-100 ug/ml showed inhibited cell growth followed by the cytotoxicity and cell death [22, 23].

In our study there was a progressive decrease in the cell count which was noticed in comparison with the untreated cell lines at all concentrations. This is due to the inhibition of the cell proliferation caused by 50ug/ml concentration of arecoline and this inhibition was also noticed in fibroblasts treated with 100ug/ml of arecoline. It is interesting to note this response of the fibroblasts to varying concentrations of arecoline, two phased response seem to be evident. In the initial phase there is inhibition of cell proliferation as noted by progressive but limited decrease in cell count. At higher concentrations of 150 ug/ml of arecoline, the decrease was rapid; this is the phase of cytotoxicity. The clinical behavior of the disorder is consistent with the cellular changes. OSF is a self-limiting disorder in that increase in fibrosis is not perpetual probably due to the cytotoxicity of the arecoline in the chewers. The habit-grade relationship from epidemiological studies further supports this observation.

In a study done to observe the effects of arecanut extracts on the cultured kidney fibroblasts, it was found at 100ug/ml concentration of arecoline there was an enhanced proliferation [24]. At the same concentration of arecoline there was dose dependent fall in the cell count of the cultured buccal epithelial cells [25]. Hence it can be concluded that the response of the fibroblasts vary depending upon the origin or site. On observing the response of the fibroblasts in the controls to various concentrations of arecoline, there was an interesting finding which was noted. At 100 ug/ml concentrations of arecoline there was an increased cell count noticed at all days followed by a fall in cell count? This phenomenon can take place when the population of fibroblasts tries to overcome the toxic effects of arecoline and shows proliferation to adapt to the condition or arecoline shows slight stimulation of fibroblasts till it is treated with 100 ug/ml concentration of arecoline. In the cases the fibroblast cells lines showed progressive decrease in the cell count from 50-150ug/ml concentration of arecoline but at 300 ug/ml of arecoline a slight proliferation of the cells was noticed. This may be attributed to the role of the arecoline receptors in the OSF fibroblasts which have already got adapted to the exposure of various concentrations of arecoline. The arecoline receptors initially responded by showing cell proliferation inhibition at lower concentrations of added arecoline but later to probably due to changes in receptor sensitivity, decrease in fibrosis and cell counts results.

Previous studies done to evaluate the effect of arecoline on the cultured HBF found that 0.1-10ug/ml of arecoline showed increased DNA content of the fibroblasts till they were treated with 25ug/ml arecoline. At 50ug/ml the cell cultures showed inhibition of cell proliferation and at 100 ug/ml, there was marked reduction in the DNA content of 54% in the cells and cytotoxicity [25]. In our study at 50-100 ug/ml concentration of arecoline we found the cell counts were nearly constant following at 150ug/ml there was a noticeable fall in the cell counts in both the groups. We have not done any DNA damage analysis of the cells but the marked reduction of the cells definitely indicate the inability of the cells to sustain further or proliferate in the cell culture wells.

There has been no time bound analysis done for the response of the fibroblasts treated with different concentrations of arecoline in the previous literature. We found that there was a progressive decrease of the cell count seen on each day till the 8th day of the study period. The 4th day at 100ug/ml concentration of arecoline was found to be the maximum cultivation time. The decrease in the cell count is an indication to less remodeling and more stabilization of fibrotic deposit.

There was an interesting fact that we can derive from our observation that the fibroblasts cell lines which were treated with higher concentrations of arecoline simulates the condition when the patient chews arecanut over years and the tissue level arecoline increases. Hence cultured fibroblasts were also exposed to higher concentrations of arecoline i.e. 150ug/ml, 300ug/ml and 500ug/ml of arecoline and it was found these concentrations caused cytotoxicity and progressive cell death. Cytotoxicity creates a self-limiting environment which leads to perpetuation and stabilization of fibrosis condition. Inhibition of cell proliferation and cellular toxicity may also lead to reduced remodeling of collagen fibres and help in the progression of fibrosis condition.

Obtaining primary cell lines of fibroblasts from the OSF tissues was very challenging as they were susceptible to contaminations of the media as well as from environment. Hence with the cell lines we could obtain in our study we conclude that, there exist different morphological forms of fibroblasts and it is...
tissue specific. There was a dose dependent response of arecoline seen on the cultured fibroblasts over a period of eight days. Arecoline at 150 ug/ml was found to be the cytotoxic dosage for the cultures changes as it showed significant decrease in the cell counts; the 3rd and 4th day showed maximum cultivation of cells.

CONCLUSION

The experiment was carried out only after obtaining the viable population of cells in the normal buccal mucosa fibroblasts cell lines as well as the OSF fibroblast cell lines. The response of the fibroblasts of those two groups of cell lines differed at various concentrations of arecoline ranged from 50 ug/ml to 500 ug/ml. The untreated cell lines of the control tissues showed greater proliferation compared to the OSF cases over a period of 8 days. The cells when treated with various concentrations of arecoline showed a decrease in the cell count over the period which was dose dependent, but the cell lines of the OSF cases showed a slight peak of cell proliferation at 300 ug/ml of arecoline. This could be because of the variability and the sensitivity of the fibroblasts or the development of arecoline receptors in the fibroblasts in the cell lines from OSF samples to the different concentrations of arecoline. The rate of decrease in the cell colony count seems to indicate the action of arecoline concentrations which were initially inhibitory then frankly cytotoxic. Based on the reaction of the controls and OSF cell lines to different concentrations of arecoline, it is safe to assume that differential cell lines of fibroblasts are produced in OSF which react in different manner to arecoline as compared to controls.

There appears to be incontrovertible proof that arecoline is a potent stimulator of fibroblasts and primary compound in the development of OSF. Attempts to observe these pathways would be one of the primary steps in the targeted therapy of this disorder.

REFERENCES


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