Original Article

To investigate the in-vitro effect of areca nut aqueous extract on reconstituted human epithelium model

Syed Fareed Mohsin[1], Sajid Hanif[2], Kefi Iqbal[3]

ABSTRACT

Objective: To investigate the in vitro effect of areca nut aqueous extract on reconstituted human epithelium model by assessing the morphology of the tissue on formalin fixed paraffin embedded section.

Methodology: Aqueous extract of areca nut, and Phosphate Buffered Saline (as control) was applied to the surface of the buccal epithelium and gingival epithelium. The morphology of the stratified oral epithelial model was examined at 24 and 48 hours by using formalin fixed paraffin embedded tissue.

Results: It was found that after 24 hours areca affected the morphology of the tissue by causing intercellular spacing and vacuolation. After 48 hours these changes were more marked and there was disorganization of prickle cell layer.

Conclusion: This study has confirmed that aqueous extract of areca nut caused significant histological changes in the tissue examined.

KEY WORDS: Areca nut, Stratified epithelium, Keratinocytes, Morphology.

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INTRODUCTION

The use of betel quid has been common in South and Southeast Asia and in the Asia Pacific region for a long time. Its use is also common in migrated communities in Europe North America and Africa. It is confirmed that palm was originally native to Sri Lanka, West Malaysia and Melanesia.[1] Today it is farmed in the Middle-Eastern and Far-Eastern countries. The areca nut or *areca catechu linn* is the true botanical name of the endosperm of the *areca catechu palmae tree*. It is also known as betel nut. Betel quid or paan is chewed as a breath freshener. It is commonly offered to guest and visitors as a sign of hospitality and eaten at cultural event. Paan filling is generally a mixture of spices and fruits. Paan makers may use tobacco as an ingredient to their paan filling. Although many types of paan contain betel nut as filling and many other types do not. It has some medicinal value as well as ability to improve social and daily life. The chewing of betel quid/areca nut habit has been reported from many Countries such as Pakistan, Sri Lanka, Bangladesh, Thailand, India, Malaysia, Cambodia, Indonesia, China, Papua New Guinea and immigrant populations in Africa, UK, North America and Australia.[1]

The ingredients vary in ways of preparation of betel quid. In India betel quid preparation consist of half portion of a large betel leaf or medium small-sized betel leaves, smeared with slaked lime small amount of catechu to which is added few pieces of areca nut.[2]

1. Syed Fareed Mohsin,
Department of Oral Medicine/Periodontology,
2. Sajid Hanif,
Department of Oral Pathology,
3. Kefi Iqbal,
Department of Dental Material,
1-3: Baqai Dental College,
Karachi, Pakistan.

Correspondence:

Syed Fareed Mohsin,
Department of Oral Medicine/Periodontology,
Baqai Dental College, Karachi, Pakistan.
E-mail: fareedmohsin@hotmail.com

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In Mumbai, India a survey conducted during 1992 to 1994 reported that around 90% of the people use betel quid without tobacco, whilst 38% men and 30% women reported the use of betel quid with tobacco.[3]

Areca nut is derived from Areca catechu or commonly known betel nut tree is a species of palm tree which grows in much of the tropical Pacific, Asia, and parts of east Africa. It is a medium sized tree grows up to 20m tall. Areca catechu is grown for its economically important seed crop the Areca nut. The areca nut is an orange yellow color when ripe off and must be separated from its fibrous pericarp before use. Some believe that its use has some medicinal value, like the nut makes the gums strong, helps in digestion and prevent halitosis.[1]

In India areca nut is cut into small pieces using a special instrument called *sarota*. Areca nut is also sold in ready-to-eat pouches called *Pan Masala*. It is a mixture of many spices whose primary base is areca nut crushed into very small pieces. Some time Pan Masala also includes a small quantity of tobacco, in this case the product is called *gutka*.

Four alkaloids have been identified in areca as confirmed by biochemical studies. These are, arecoline, arecaidine, guvacine, guvacoline, out of these arecoline is the main component.[4] Arecoline forms at least four nitrosamines upon nitration; these products include N-nitrosoguvacine, N-nitroguvacoline, 3-(methyl-nitrosamino) propionitrile (MNPN) and 3-(methylnitrosamino) propionaldehyde (MNPA) and two unknown N-nitrosamines.[5,6] MNPN is a potent carcinogen which is produced in even higher yields from arecaidine.[5,7]

The role of areca nut constituents has been studied in detail over the last two decades. The increase in collagen synthesis and reduced collagen degradation as a possible mechanism in the development of disease.[8]

The effect of areca nut extract on fibroblast have proved that areca nut alkaloids cause direct stimulation of fibroblast and collagen synthesis which results in sequential connective tissue changes in OSF.[9,10] Arecoline is the major alkaloid in areca nut is hydrolyzed to arecaidine in vivo, which is the main stimulator of human buccal mucosal fibroblast.[4] The dramatic increase in deposition of type 1 collagen and reduction of type III collagen is associated in the progression of OSF.[11]

Membrane permeability studies have been proven that arecoline and arecaidaine diffuses across a stratified epithelium. This research was justified because genotoxic and mutagenic effects have not been proven to occur when mucous membrane was exposed to betel compound.[12]

The oral epithelium of clinically affected sites is markedly atrophic in more than 90% of cases.[13] The atrophic epithelium may have facilitated the migration of areca nut alkaloids towards connective tissue more readily.[14]

As little has been studied on whole epithelium, therefore, the aim of this study was to investigate the effect of areca nut on oral stratified epithelium.

METHODOLOGY

Preparation of Areca Nut solution: Standardized amount of Areca nut (4 g) was grounded for 15 minutes in 4 ml PBS using a conventional pestle and mortar. At the end of this period all the material was resuspended in a total of 20ml PBS and left for 4 hours at room temperature. The resultant extract was shaken and diluted 1:4 in PBS prior to application to the surface of the epithelium

Mucosal Model

Buccal Epithelial Model: The reconstituted human epithelium model used in the study was prepared and supplied by Skin Ethic Laboratories, Nice, France. It is a three-dimensional tissue culture model obtained by culturing transformed oral keratinocytes (TR146) derived from a buccal carcinoma.[15] The cells were seeded (8×10^5 cells/cm^2) on a 0.5cm^2 inert polycarbonate membrane and cultivated in a defined medium[16] for 12 days at the air- liquid interface. The resulting cultures formed a stratified epithelium with 10 - 12 cell layers devoid of stratum corneum. Skin Ethic Laboratories also supplied growth medium (MCDB 153 containing 5µg/ml insulin and 1.5mM $ca^{2+,}$ 25µg gentamycin and 0.4µg hydrocortisone) for use in the experiments.

Gingival Epithelial Model: The reconstituted human epithelium model used in the study was prepared and supplied by Skin Ethic Laboratories, Nice, France. It is a three-dimensional tissue culture model obtained by culturing transformed normal oral keratinocytes derived from gingiva age 23 years old**.**

Both of these models were shipped on agar and on arrival were transferred into a new 24 well culture plate containing 500µl growth medium per well and incubated overnight at 37°C in 5% CO^2 in a 100% humidified atmosphere. The cultures were transferred to 6 well plates containing fresh media of 1.5ml each well for all the experiment. During treatment of the epithelium with aqueous extract of areca and PBS as a control, supernatants were

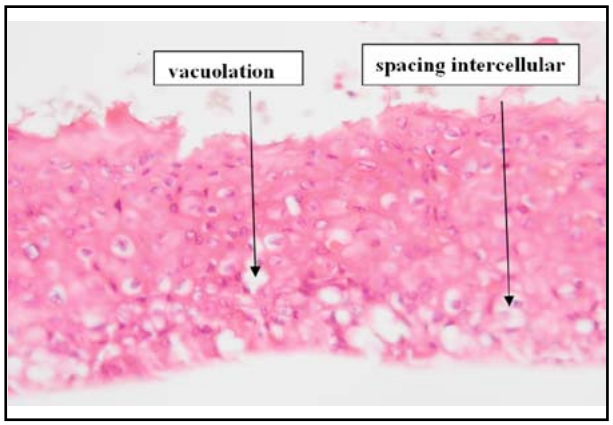

Fig.1: Areca on Buccal epithelium after 24 hours.

collected at 24 and 48 hours and replenish with a fresh media.

Treatment: 50µl of diluted aqueous extract of areca, and PBS (as control) was applied to the surface of the epithelium and the tissue incubated for upto 48 hours at 37°C in 5 % CO_2 in a humidified atmosphere. Total 32 samples of reconstituted buccal and gingival epithelium were used in this study. At 24 and 48 hours of the experiment the epithelial surface was washed three times with PBS and the tissue used for morphological examination. The sample was applied as single suspension.

Histology: The morphology of the stratified oral epithelial model was examined by using formalin fixed paraffin embedded tissue. The tissue was processed to paraffin wax using an automatic tissue processor. 5µm sections were cut and stained with haematoxylin and eosin, examined under light microscopy whilst the image was recorded with digital photography.

RESULTS

The effect of the different treatment on tissue morphology was assessed using formalin fixed paraffin embedded sections and haematoxylin and eosin staining.

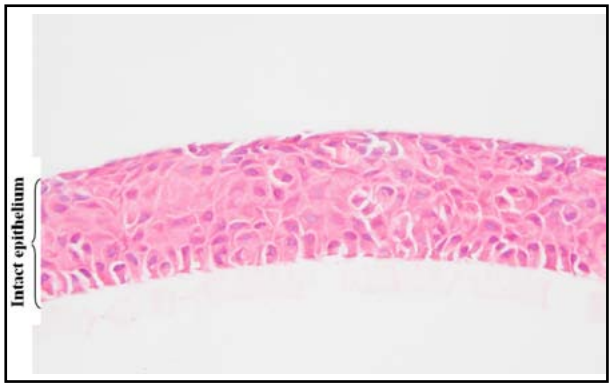

Fig.3: PBS after 48 hours on Buccal epithelium.

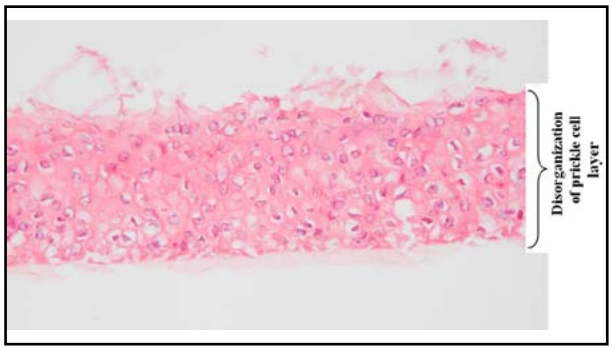

Fig.2: Areca on Buccal epithelium after 48 hours.

Buccal Epithelium: After 24 hours areca effect the morphology of the tissue by causing intercellular spacing and vacuolation, after 48 hours these changes was more marked and there was disorganization of prickle cell layer (Fig. 1 and 2).***Gingival Epithelium:*** Areca caused no significant effect on the morphology of the gingival epithelium, no disorganization of prickle cell layer (Fig.4).

PBS (Control): PBS used as a control shows normal appearance of the epithelium (Fig.5).

DISCUSSION

Oral submucous fibrosis is a localized response to topical contact with betel quid and its components. Early inflammation is an essential requirement for the initiation of the disease. Oral submucous fibrosis described as potentially a premalignant condition characterized by chronic inflammatory reaction followed by severe fibrotic change in the connective tissue and epithelial atrophy.[13] The aim of this study has been to investigate the initial effect of single application of areca nut on *in vitro* stratified epithelial model.

The model was devoid of connective tissue so the response was purely from the epithelium only. The cultures were treated for 48 hours with areca

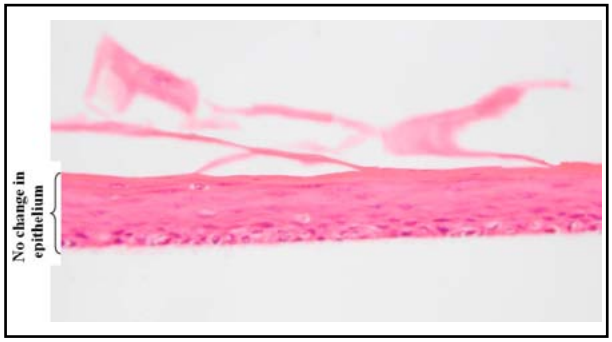

Fig.4: Areca after 48 hours on gingival epithelium.

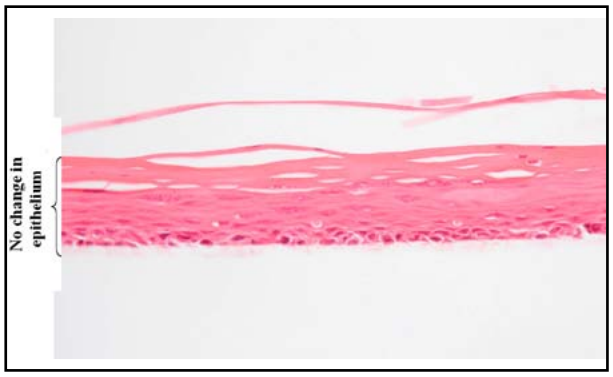

Fig.5: PBS after 48 hours on gingival epithelium. The epithelium was intact there was no change.

nut. The histological study was used to establish whether areca nut had an irritant action on the stratified epithelial model.

In this study after 24 hours the morphology of buccal epithelium shows intercellular spacing and vacuolation and these changes were more marked and there was disorganization of prickle cell layer after 48 hours of aqueous extract areca nut application. In contrast to gingival epithelium areca had no significant effect on the morphology after 48 hours of application.

Various studies have been carried out with regard to the cytotoxic and genotoxic effects of individual components and extracts of areca nut, which include aqueous ethanolic and acetic acid extracts of the nut.[17-19] Areca nut extract also induces unscheduled DNA synthesis by cultured gingival keratinocytes. However, only AN extract induced TDS and UDS in cultured GK within 6 hours of exposure. Induction of UDS by AN extract was concomitant with the presence of apparent intracellular vacuolization.[20,21]

Other studies have shown that the application of areca products to the oral mucosa of animals results in histological changes which may be indicative of DNA damage.[22,23] In addition to the local effects of areca on the oral mucosa there is also evidence that suggests that areca, when applied to the skin of animal models or included in the feed, may cause neoplastic changes in sites distant from the oral cavity such as the foregut, liver, kidneys and lung. 94±97. Areca (pan) chewing without tobacco causing oral cancer has been highlighted in a few recent studies. Furthermore, there is new evidence which suggest that areca in the absence of tobacco may be an independent risk factor for the development of oral squamous cell carcinoma (SCC). Areca nut extract can activate NF-κB in oral keratinocytes[24] hence it has been implicated in the development and progression of SCC.[25]

In this study the response of buccal epithelium was more aggressive as compared to gingival epithelium. This may be a reflection of the structure of buccal epithelium which is non-keratinized while gingival epithelium has stratum corneum. So the gingival epithelium has more permeability barrier against the effect of betel components on it.[26]

Further work would be required to confirm our observations. It is important to be aware that this experiment was limited to single application betel quid components over 48 hours. Furthermore, suspension of these compounds in PBS may induce a different effect to those found in saliva. Ultimately, our in vitro stratified model is grown using transformed oral keratinocytes (TR146) which may not respond in the same manner as cells in vivo.

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Authors Contributions:

The laboratory work was done by the corresponding author with help in manuscript writing by Dr. Sajid Hanif and Dr. Keafi Iqbal contributed in interpretation of results and discussion.