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Original Article

Effect of areca nut on rabbit oral mucosa: evidence of oral precancerous condition by protein expression and genotoxic analysis

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ABSTRACT

Background: In humans, areca nut induces oral submucous fibrosis (OSMF), a potentially malignant disorder, characterised by the deposition of collagen in the lamina propria. This study aimed to determine whether OSMF-like characteristics develop in a rabbit model following treatment with areca nut and lime.

Results: The oral epithelial tissue upon treatment with areca nut extract at 6-day intervals for up to 6 months showed progressive changes in thickness from 3 months onwards, leading to blanching, ulceration, irregular growth and, finally, restricted mouth opening. The protein expression pattern of OSMF-like tissues of rabbit buccal mucosa was determined by 2-DE gel and MALDI-TOF and compared with normal buccal mucosa of the control group of rabbits. Three major proteins, namely tropomyosin beta chain (in the skeletal muscle), actin and collagen alpha-1(I) chain, have been identified in areca nut-treated rabbit tissues as compared to control. The genotoxic effect of areca nut was evaluated in the rabbit model by comet assay in the blood. A significant ($p < 0.0001$) DNA damage in areca nut-treated rabbits was observed as compared to the control group.

Conclusion: Histological characteristics, comet assay and protein profile show the development of OSMF-like features in the mucosal tissue of rabbit followed by areca nut treatment. The increased expression of collagen alpha-1(I) chain in areca nut-treated rabbit correlated with the progressive development of the OSMF symptoms in the rabbit buccal mucosa, which might serve as a potential biological marker in the pathological development of OSMF.

1. Introduction

Oral submucous fibrosis (OSMF), regarded as a pre-cancerous condition of the oral cavity, is a well-known chronic, progressive, virtually irreversible, and juxtaepithelial inflammatory disease of the oral mucosa that is more common in the Indian subcontinent and Southeast Asia, with sporadic occurrences in other parts of the world as well. The development and progression of OSMF are closely associated with adverse habits such as chewing areca nut and its commercially popular forms such as pan masala and gutkha. The strongest risk factor for OSMF is the chewing of betel quid containing areca nut. The amount of areca nut in betel quid and the frequency and duration of chewing betel quid are clearly related to the development of OSMF [1].

The common signs and symptoms of OSMF are burning sensation, dry mouth, blanching of the oral mucosa and ulceration. Blanching of the oral mucosa is caused by the impairment of local vascularity and increased fibrosis, and a lesion appears. In the advanced stage of the disease, the important characteristic is a fibrous band that restricts mouth opening and causes difficulty in mastication, speech, swallowing and maintaining oral hygiene. Development of fibrous bands in the lip and cheeks makes them thick, rubbery and rigid and makes the mucosa difficult to retract. In the more advanced stages of the disease, OSMF is characterised by the formation of thick bands of collagen and hyalinisation extending into the submucosal tissues and decreased vascularity.

Abbreviations: 2-DE, Two-dimension electrophoresis; MALDI-TOF, Matrix-assisted laser desorption ionization-time of flight; MS/MS, Tandem mass spectrometry; IPGs, Immobilised pH gradient strips.

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Pathogenesis is believed to involve juxtaepithelial inflammatory reaction and fibrosis in the oral mucosa due to increased cross-linking of collagen through the up-regulation of lysyl oxidase activity [2]. OSMF is strongly associated with a risk of oral cancer, although the biology underlying this association is unresolved [3].

There are a few previous reports on the development of an animal model for OSMF. The oral epithelium of female BALB/c mice treated with areca nut for 300–600 days showed increased cellularity of fibroblasts and fibrosis of the connective tissue [4]. So far, the animal models included mice and rats, where the monitoring of clinical changes was difficult because of the small size of the oral cavity. This prompted us to assess rabbit as a model to have a better hold on the monitoring of the disease. The availability of a suitable animal model will enable a better understanding of the pathogenesis of OSMF. Considering this, the objective of the study was to (1) determine whether the features of OSMF appear in the rabbit model after treatment with areca nut and, if so, further confirm the disease genotoxically (2) identify differentially expressed proteins in the buccal mucosa of rabbits with OSMF features compared to the control group by two-dimension (2-DE) gel electrophoresis followed by in-gel digestion and MALDI-TOF MS. This is the first study to use a proteomic approach in oral OSMF tissues in an animal model.

2. Materials and methods

2.1. Animals and reagents

For the development of OSMF in animal model, adult New Zealand white rabbits weighing between 1 and 1.5 kg each were segregated into three groups, namely control (negative), positive control (phenol), and areca nut-treated animals, each group containing three animals. For preparing the areca nut extract, 25 g dried areca nut was dissolved in deionised water, mixed using a cyclomixer and air dried. From this, 2 g power was mixed with slaked slime and 5 ml water and filtered, and the filtrate was used for the treatment of rabbits. Rabbits from each group received 1 ml submucous injection of the specified agents at 6 days interval: first group received 1X PBS (control), second group received phenol solution (4%) and third group received the filtered areca extract with lime. The doses were continued for the next 6 months. The oral mucosa was checked regularly. The study was duly approved by CPCSEA (New Delhi) and Institutional Animal Ethics Committee (Registration number 147/1999/CPCSEA). Housing conditions of the animals were maintained under standard conditions of 12 h/day/night cycle with water and food. The temperature of the animal houses was 22–23° C and humidity was 50–70%. All animals were deeply anaesthesitised by peptobartitine sodium solution before biopsy.

2.2. Histological staining of OSMF

The biopsy specimens of buccal mucosa from each group were obtained by punch biopsy and fixed in a buffered formalin solution at neutral pH. The tissue sections were paraffinised, sectioned, and stained with haematoxylin and eosin.

2.3. Protein extraction by tissue lyser and fractionation of total proteins

The biopsy samples from each rabbit were retained in 20 mM Tris–buffer (pH 7.5), washed with 5 mM $MgCl_2$, and homogenised using a tissue lyser (Qiagen, Hilden, Germany), and a protease inhibitor cocktail (Sigma, USA) was added. The homogenates were cleared to remove the cell debris by centrifugation at 14,000 rpm for 20 min at 4° C, and the supernatant was collected. The protein concentration of the resulting supernatant was determined using the Bradford method. The supernatant was treated with DNase/N nuclease at 37° C for 30 min and again centrifuged. The precipitate was kept in Trichloroacetic acid: Acetone (1:9) at −20° C overnight and then washed with 80% cold acetone. The precipitate was air dried and solubilised in rehydration buffer [5 M urea, 2 M thiourea, 2% CHAPS, 40 mM Tris, 2% SB3-10, 0.2% Bio-lyte (working pH range 3–10), 10 μl tributyl phosphine].

2.4. 2-DE PAGE

The first-dimension isoelectric focusing and second-dimension SDS-PAGE were performed according to the BIO-RAD manual provided with BIO-RAD 2-DE electrophoresis system. The protein (approximately, 200 μg in 125 μl) was adsorbed on to 7 cm IPG strips, pH 3–10, for 16 h and then isofocussed on an isoelectric focusing cell (PROTEAN IEF CELL, Bio-Rad) for 8000 Vh at 20 °C. Strips were rehydrated for 12–16 h by passive rehydration in the presence of samples. The total volume for the rehydration was always 125 μl for the total protein sample and was prepared in lysis buffer (BIO-RAD). Then reduction and alkylation of the focussed proteins was performed by equilibrating these strips in equilibration buffers I and II containing dithiothreitol (DTT) and iodoacetamide (IAA), respectively [375 mM Tris/HCl, pH 8.8, containing 6 M urea, 2% (w/v) SDS, 20% (v/v) glycerol containing either 2% (w/v) DTT or 2.5% (w/v) IAA], processed according to Bio-Rad manual. The equilibrated strips were placed on 12% SDS-PAGE separated in BIO-RAD gel apparatus stem (PROREAN II XI CELL).

2.5. In-gel digestion and identification of proteins by peptide mass fingerprinting

The gels were stained using colloidal Coomassie blue (R-250), and desired proteins were excised manually from the stained gel. The excised gel pieces were digested using a gel digestion kit (PIERCE) according to the standard manufacturer's protocol. The excised gel spots were destained by a destaining solution (100 mmol/L NH_4HCO_3 in 50% acetonitrile) and reduced by a reduction buffer (25 mmol/L NH_4HCO_3 and 10 mmol/L DTT) for 10 min at 60 °C.

Alkynation was done by an alkylation buffer (25 mmol/L NH_4HCO_3 and 55 mmol/L iodoacetamide) in the dark for 1 h at room temperature at until the gel was colourless. After drying, the gel was treated with trypsin solution (100 μg/mL) for overnight at 30 °C. After digestion, these tryptic digestive peptides were centrifuged, and the supernatant was dried on a Speedvac concentrator and desalted using ZIPTIP (C18 column-Millipore, USA). The elution of peptides was performed in 50% acetonitrile containing 0.1% trifluroacetic acid (TFA).

2.6. Identification of proteins by (peptide mass fingerprinting) MALDI-TOF-TOF mass spectrometry

The eluted peptides (0.5 μl) from the gel were mixed with α-cyano-4-hydroxycinnamic acid (Sigma) in 0.1% TFA/50% acetonitrile as the matrix (1:1), spotted on MALDI plate by using the matrix–sample–matrix sandwich method at room temperature and analysed on a 4800 MALDI-TOF-TOF mass spectrometer (Applied Biosystems, USA). All the data were analysed using the GPS explore software. To identify the proteins, a search was performed of the combined MS and MS/MS by NCBI and SWISS-PROT using the MASCOT software v2.1.

2.7. Comet assay

From each group of rabbits, blood was collected from the ear vein and suspended in PBS. The blood cells were embedded in thin

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