A novel approach to develop an animal model for oral submucous fibrosis

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Received: 4 February 2022 / Accepted: 26 May 2022 / Published online: 16 August 2022 © The Author(s), under exclusive licence to Springer Science+Business Media, LLC, part of Springer Nature 2022

Abstract
Epidemiological data have proved the association of consumption of areca nut with the causation of oral submucous fibrosis (OSF). OSF is a chronic inflammatory disease with the potential for malignant transformation from 7 to 13%. The establishment of animal models makes it easier for researchers to focus on the therapeutic options to combat this disease further. We developed and compared two areca nut extract (ANE) administration methods in Swiss albino mice to establish OSF. This study compared an invasive intrabuccal injection technique with a non-invasive intraoral droplet administration. The duration of induction was around 12 weeks. Histopathology (H&E, Masson’s trichrome staining) and gene expression analysis (COL-I, COL-II, and α-SMA) were performed using RT-PCR to confirm the OSF in animals. Our study showed that ANE administration through the intraoral droplet method exhibited significantly higher fibrosis than the intrabuccal injections, as evidenced by the H&E and Masson’s trichrome staining. Furthermore, intraoral administration of ANE significantly upregulated the mRNA expression of COL-I, COL-II, and α-SMA, as revealed by the RT-PCR analysis. The non-invasive droplet method could simulate the absorption of areca nut seen in humans through daily dosing. This study establishes the intraoral droplet method as an efficient and non-invasive method to administer the ANE to develop OSF. These findings will aid in the efficient development of OSF animal models for interventional studies, including screening novel drugs in the reversal of the OSF.

Keywords Oral submucous fibrosis · Animal models · Areca nut · Oral pathology · Oncology

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Introduction

OSF represents an oral potentially malignant disorder (OPMD) with a chronic progressive course resulting in severe debilitation. In 1952, Schwartz was the first to recognize the disease in five female patients, habitant from East Africa, and named it according to the visible clinical features “atrophica idiopathica mucosa oris” [1]. In India, Joshi SG studied 41 cases of submucosal fibrosis of pillars and palate and further labeled the entity as Oral Submucous Fibrosis in 1953, which is the most widely accepted terminology. Lal et al. was the first Indian dental professional to identify that the subepithelial connective tissue in OSF is replaced by acellular material and contains an extensive plasma-lymphocytic inflammatory infiltrate [2]. Most OSF cases are recorded in the Indian subcontinent [3], where a large population consumes chewable or smoking forms of areca nut products, which precipitates OSF with an incidence of 2 to 3% in both males and females [4]. Areca nut forms the essential ingredient of several chewing products used worldwide [5], and arecoline present in areca nut/betel nut is considered as one of the primary etiological factors for OSF. The detrimental effects of the areca nut are validated by its designation as a class I carcinogen by the International Agency for Research on Cancer (IARC) [6]. The malignant potential of OSF is estimated to be between 7 and 30% [7].

OSF is a complex disease and its etiopathogenesis, clinical presentation, histological features, and epidemiological prevalence of OSF have been investigated in detail. Varieties of therapeutic options are practiced in the pathophysiological reversal of OSF, such as nutritional intervention, anti-oxidant therapy, physiotherapy, intranasal injections of corticosteroids and IFN-gamma, laser therapy, and surgical intervention [2]. However, they offer limited benefits to the patients and are invasive. Furthermore, regenerative medicine (stem cells and their secretome) intervention has been proposed for the pathophysiological reversal of OSF [8]. This can be accredited to the lack of reliable animal models that would mimic the OSF’s in vivo features. Few studies have attempted to establish an animal model for OSF that utilizes administration of the areca nut extract (ANE) [9–15]. However, certain lacunae (mode of administration, exposure time and dosage, persistence of the OSF phenotype) still exist in terms of model establishment of OSF, limiting the testing of current and novel therapeutic options.

The primary objective of this study was to establish an economical and simple animal model in terms of cost and time to understand the pathophysiology of OSF and possible intervention for new drug delivery. The novel prepositions of the present study include the comparison between two different methods of administration of ANE (intrabuccal injections and intraoral doses) for induction of OSF, which has not been carried out in the previous studies.

Materials and methods

Ethical approval

The Institutional Animal Ethics Committee at Dr. D. Y. Patil Institute of Pharmaceutical Sciences & Research, Pune, India approved the experimental protocol (DYPIPS/IAEC/ Oct/20-21/P-12) for undertaking this study.

Preparation of areca nut extract

The extract was prepared at Dr. D. Y. Patil College of Ayurved and Research Center, Pune (India). The aqueous extract was prepared using endosperm of Areca catechu. A fine powder of areca catechu endosperms was obtained and dissolved in normal saline (0.9% of 50 mM Sodium chloride (Hi Media)) at the 50 mg/ml concentration. This was followed by centrifugation at 15,000 rpm for 30 min. The supernatant obtained after centrifugation was collected, filtered using a syringe filter, and stored at −80 °C.

Animals

Six-week-old Swiss albino mice with an average weight of 25 to 30 g were procured from Crystal Biological Solutions, Pune, India (Reg. no.—2030/PO/ReBiBu/S/18/CPCSEA) for the study. Since the habitual consumption of areca nut in humans predominates in the male population, we selected male mice for close simulation of the occurrence of the disease. The mice were housed and left for acclimatization for 15 days before the onset of the study. The mice were maintained at standard laboratory conditions, within a standard temperature (25 ± 2 °C) and humidity (50–70%), with regular light and dark cycles of 12 h each. The feeding was carried out under standard protocols. Mice were fed with standard mouse fodder and water ad libitum.

Experimental design

A total of forty mice were randomized into four groups using the R statistical program, as shown in Fig. 1. Each group consisted of 10 mice. The sample size calculation was performed, considering the expected attrition of animals. G*power software was used for calculating the sample size [16]. The first group was injected with 50 µl of phosphate buffer saline (PBS) (Control) (Gibco) every day. The second group was administered 50 µl of bleomycin (Cipla, 15 IU)
(Positive Control) every day; the third group was injected with 50 µl ANE (50 mg/mL) intrabuccally using disposable syringes (Dispovan 31G Insulin Syringe 1 ml). The injections were given inside the right buccal mucosa every 2 days for 12 weeks. The mice in the fourth group were administered 50 µl of ANE with the help of a micropipette. The administration was done in the form of drops. For convenience, the third group is labeled as intrabuccal injection group, and the fourth group is labeled as the intraoral droplet group. Post-administration, the mice in the fourth group were abstained from water for the next 3 h. The ANE administration of intraoral drop was carried out every day in the fourth group of mice for 12 weeks. The administration time was maintained at a specific time every day throughout the entire experimentation.

**Study termination**

At the end of 12 weeks, animals in each group (n = 10) were sacrificed. The mice were sacrificed using an inhalational method with an overdose of chloroform. The cervical dislocation was performed to confirm the death of the animal. The right buccal mucosa specimen was obtained by cutting through the entire tissue length, extending anteroposteriorly from the corner of the mouth up to a line perpendicular downward toward the center of the eye. Furthermore, a cut was given supero-inferiorly from 2 mm below the eyes up to
the bony extension of the lower jaw for precise removal of ANE-exposed tissue. The tissue samples were transferred to a freshly prepared 10% neutral-buffered formalin solution.

**Histopathological analysis**

For proper fixation, the tissue specimens collected were left in formalin for 24 h. Post-fixation, the tissue specimens were grossed and labeled according to the mice group, packed with gauze into a cassette, and sent for an automatic tissue processing cycle that lasted for 16 h. After tissue processing was complete, the tissues were embedded in paraffin wax. The wax was allowed to cool down using cryostation. 3-µm sections were cut on a microtomy and retrieved on a clean glass slide. The slides were kept on a slide warmer so that the residual wax could melt away. Lastly, the hematoxylin and eosin and Masson’s trichrome staining procedures were performed using a standard protocol [17, 18]. After the staining procedure was complete, slides were left for drying and later mounted with DPX before examining under a compound microscope (OLYMPUS CX21i). Masson’s trichrome staining was performed to detect the collagen accumulation in the subepithelial region. ImageJ software was used to assess the percentage of the fibrotic area.

**Molecular expression (RT-PCR) analysis**

For gene expression analysis, tissue samples from the ANE-exposed area in the respective groups were collected and washed with PBS and total RNA was isolated by the TRIzol (Invitrogen) method as previously described [19]. Total RNA was converted into complementary DNA (cDNA) using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). RT-PCR was performed on QuantStudio 5 Real-Time PCR System using SYBR Green Chemistry (Applied Biosystems). Primer sequences and the PCR conditions of the genes (COL-I, COL-III, α-SMA) are given in Table 1. Fold expression analysis was performed using ∆∆Ct method.

**Statistical analysis**

Statistical analysis to determine the difference in fibrosis area was performed using Tukey’s Honest Significant Difference Test in IBM SPSS software version 20. Data were expressed as Mean ± SD, and significance levels were determined. *p < 0.01 was considered statistically significant.

**Results**

**ANE administration by intraoral droplet and intrabuccal injection method exhibits OSF-associated changes in buccal tissues**

The hematoxylin and eosin-stained tissue sections under a compound light microscope exhibited OSF-associated changes in the lamina propria and epithelium, as shown in Fig. 2. The intraoral droplet group of ANE administration exhibited a relatively higher level of fibrosis than the intrabuccal injection group. When ANE is administered through drops, the oral epithelium is exposed to the carcinogens present in the ANE. On the contrary, in injection group, ANE contacts the subepithelial connective tissue directly, bypassing the epithelium. Overall results of exposure of epithelium to the carcinogenic effect of ANE leads to epithelial atrophy, which is an established histopathological feature in OSF [20]. Our results closely resemble the histopathological OSF features in humans and those documented in the published literature [21].

**Intraoral administration of ANE results in more significant fibrosis than intrabuccal route**

Masson’s trichrome staining was performed to evaluate the ANE-associated fibrosis in the buccal tissues. As shown in Fig. 3, the collagen fibers were stained light blue compared to the other tissue components. As anticipated, the administration of bleomycin (positive control) promoted collagen fiber accumulation in the subepithelial region. Evaluation of the area covered with fibrosis was performed using ImageJ software. The area in red denotes the fibrosis area in the last column (Fig. 3). Our results revealed that ANE administration by the intraoral droplet group exhibited a significantly higher percent fibrosis area as compared to the ANE administered through intrabuccal injections (p < 0.01) as well as bleomycin administration (p < 0.01).

Our results showed a 23.9%-fold increase in the mean percent fibrosis area in the positive control group compared to the control/PBS group (13.1356 ± 0.00077 vs. 0.5496 ± 0.00253). A highly significant increase in the

| Gene | Forward primer | Reverse primer | Tm (°C) |
|------|----------------|----------------|---------|
| COL-I | CACTGCCCTCCTGAGCAATGG | CACGTCATCAGCACAGCG | 60 |
| COL-III | CAGGGCACTGGCAATGTAAGA | CTCATGGCCTTGCGTGGTATG | 60 |
| α-SMA | GTCCCAGACATCAGGAGTAA | TCGGATACTTCAGGTCAGGA | 60 |
fibrosis was evident in the ANE group, with a 48%-fold increase in the mean percent fibrosis area for the intrabuccal injection group compared to the control (26.3775 ± 0.00978 vs. 0.5496 ± 0.00253). Interestingly, a 61.72%-fold increase in the mean percent fibrosis area was observed in the ANE intraoral droplet group (33.9246 ± 0.00765 vs.26.3775 ± 0.00978) (Fig. 4).

ANE exposure through the oral droplet method exhibits a profound increase in COL-I, COL-III, and α-SMA mRNA expression

The ANE-mediated pathological development of OSF is a complex process and involves the altered expression of COL-I, COL-II, and α-SMA, which drive the fibrosis in the submucosal region [22, 23]. RT-PCR analysis of the mRNA expression in the intrabuccal-injected ANE administered through animals resulted in a significant increase in the expression of COL-I (43.9-fold), COL-III (30.4-fold), and α-SMA (16.4-fold). Interestingly, intraorally administered ANE exhibited a significant increase in the expression of COL-I (299.5-fold), COL-III (246.3-fold), and α-SMA (45.10-fold). mRNA expression of COL-I, COL-II, and α-SMA in intraorally administered ANE was significantly higher in intrabuccally administered ANE (ρ < 0.05) (Fig. 5).

Discussion

Our study aimed to investigate the efficacy of the ANE-induced OSF animal model administered through previously reported routes of administration (intrabuccal injection and intraoral droplet). Furthermore, an evaluation of the best possible method of administration that could closely simulate the occurrence of OSF in humans was performed. Increasing cases of OSF validate treatment strategy for assessing the safety and efficacy of the same. The very cause for OSF, areca nut usage, has established an animal model in the present study. Areca nut contains alkaloids, like arecoline, arecaidine, guvacoline, and guvacine, and other components, like flavonoids and copper. These are responsible for disturbance in the extracellular...
matrix normal homeostasis, mainly the collagen synthesis and degradation pathways, which terminally lead to OSF [11]. Over the past decade, extensive research has been conducted to establish an OSF animal model. Das et al. [12], Raghavendra et al. [13], Chiang et al. [10], and Maria et al. [4] used an intrabuccal injection of ANE as a method of administration, while Perera et al. [9] and Bo et al. [14] used topical application of ANE as a method of administration. With ANE as a causative factor, a dose-dependent relationship naturally exists in vivo that has been mimicked for the frequency and duration of exposure and development of a model.

This study simulated the human oral cavity environment with the mice after administering ANE in droplet form. The present study evaluated one conventional method (intrabuccal injections) and one non-conventional method (intraoral droplets) of ANE administration in animals, unlike other studies where only a single mode of administration was used. ANE through the intraoral droplet method has been proved superior to the intrabuccal injection technique. We tried to establish an early model through the intrabuccal injection; it is an invasive method that can induce OSF quickly but does not simulate the exact pathway of disease occurrence in humans [11, 12, 14]. Other drawbacks include less surface area for deposition of substance is available which hinders the absorption of ANE and causes discomfort to the animal, which further gains less co-operation from the animal.

![Fig. 3 Composite photomicrographs depicting subepithelial fibrosis in the original images of Masson’s trichrome-stained slides (Under ×40 magnification), total tissue area after analyzing the image, and finally total fibrotic area marked in red in all the four animal groups.](image1)

![Fig. 4 Graphical representation of the total area of fibrosis after evaluating histological images of Masson’s trichrome-stained slides. The highest value is observed in the ANE oral doses group, which was statistically significant with a p value of 0.000 and 95% CI of [33.3492, 33.4009].](image2)
Moreover, the repeated injections cause unnecessary inflammation, edema, and hyperemia, interfering with the usual pathway for OSF establishment. Apart from the injection method of ANE administration, various methods have been used by authors, like feeding with diet [9], topical application over the buccal mucosa [13], and fed through drinking water [12]. All those methods were successful in the model establishment of OSF, but none of them could simulate the absorption method in humans on the consumption of areca nut-containing products. Administering ANE through water or feed was carried out using diluted concentrations of ANE, whereas the present study administered ANE at a higher concentration, ensuring more excellent absorption. ANE administration through water or feed can ensure the intake of areca nut, but the absorption through oral mucosa is minimal and thus requires more time to establish the disease.

The intraoral droplet method is easy to perform without much discomfort to the animals, making it user-friendly and animal ethics committee friendly, allowing us to use more animals. Last but not least, the benefit was derived in a short period, through daily administration. A single dose daily was sufficient to produce results compared to the twice or thrice daily dosage performed earlier [9, 13]. The histopathological analysis confirmed the diagnosis of OSF, which is characterized by atrophic/hyperplastic epithelium, increased fibrillar collagen in the connective tissue, and decreased vascularity in the subepithelial connective tissue increased chronic inflammatory cell infiltration [3]. OSF is principally diagnosed through histopathological analysis; thus, the present study employed a standard H and E histopathological confirmation and a special stain (Masson trichrome) to confirm the disease state microscopically [5, 21, 22]. Furthermore, we validated our finding by the mRNA expression profile of COL-I, COL-II, and α-SMA in the intrabuccal and intraoral administered ANE. Our results unequivocally demonstrate that intraoral administration of ANE is a better method for the OSF development, as evidenced by the histopathological analysis (H&E, Masson trichrome staining) and gene expression analysis of COL-I, COL-II, and α-SMA expression.

Currently, no definite treatment modality is available for the cure or at least reversal of the OSF. Therefore, developing a definite and pragmatic animal model at the earliest using the least invasive technique that could be employed for drug screening is of prime importance. The present study revealed that ANE administration through the intraoral droplet route showed noticeable histopathological features of OSF and relatively higher fibrosis than the ANE administration through the intrabuccal injection route. Our model focuses on covering the entire buccal mucosa as seen in the areca nut users. If injected through intrabuccal injections, ANE reaches the subepithelial connective tissue bypassing the epithelium and may exert localized effects only. Overall results of exposure of epithelium to the carcinogenic effect of ANE leads to epithelial atrophy, which is an established histopathological feature in OSF. When ANE is administered through drops, the oral epithelium is exposed to the carcinogens evenly throughout the oral cavity. Moreover, the oral method of administering the ANE mimics the natural progression of the disease. As per the advanced guidelines of animal ethics in experimentation, the oral drop method of administration is preferred as it causes less pain to the animals.

**Conclusion**

The present study demonstrates an easy, economical, and non-invasive method of inducing OSF simulating the areca nut absorption seen in humans. Using mice as an animal model to study areca nut-induced OSF is a simple, reproducible, and sustainable method in shorter time duration. Animals have always served as paradigms when developing and assessing the effect of drugs or vaccines. Change is a constant feature, and animal models should be regularly updated and improved to make them more reliable and illuminating. Breakthroughs in regenerative medicine and pharmaceutical industries have been possible only due to appropriate animal models depicting pathological conditions. Various alternative therapeutic options are emerging and have shown
promising results in in vitro studies. However, their clinical translation is obstructed due to the non-availability of a reliable and user-friendly animal model that mimics the OSF’s in vivo scenario. This study provides an economical, convenient, and reproducible method to develop OSF. This is a promising step toward drug development for the clinical management of OSF.

Acknowledgements The author would like to thank Dr. D. Y. Patil Dental College and Hospital, Pune, India for funding the present study and Dr. D. Y. Patil Institute of Pharmaceutical Sciences & Research, Pune, India for their support throughout the study.

Declarations

Conflict of interest All authors declare no conflict of interest.

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