A Novel Approach to Develop an Animal Model for Oral Submucous Fibrosis

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Abstract

Epidemiological data has proved the association of consumption of areca nut with the causation of oral submucous fibrosis (OSMF). OSMF 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 applications to combat this disease further. We developed and compared two areca nut extract (ANE) administration methods in male Swiss albino mice to establish OSMF. We used an invasive intra-buccal injection technique and compared it with a non-invasive oral droplet administration method. The duration of induction was around 12 weeks. Histopathological analysis was performed at regular intervals to assess the establishment of the disease. Our study showed that ANE administration through the oral droplet method was superior in all aspects compared to the intra-buccal injection technique. Through daily dosing, OSMF was established within 12 weeks, as evidenced by histopathological analysis. The non-invasive droplet method could simulate the absorption of areca nut seen in humans. The present study focuses on developing a novel droplet method and its comparison with the intrabuccal injection method of ANE administration to develop OSMF features in Swiss albino mice. Our results suggest that the oral droplet method is an efficient and non-invasive method to administer the ANE to develop OSMF. These findings will aid the inefficient development of OSMF animal models for interventional studies, including screening novel drugs in the reversal of the OSMF.

1. Introduction:

The long-living disease ‘oral submucous fibrosis (OSMF)’ has been chewed upon the most for its malignant transformation to the most dreaded form of cancer affecting humanity. 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 as "atrophicaidiopathica 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 D was the first dental professional to replace the subepithelial connective tissue layer with acellular material and extensive plasma-lymphocytic inflammatory cell infiltrate [2]. OSMF is a chronic, insidious and debilitating disease affecting more than a single part of the oro-pharyngeal region [3]. The disease progresses gradually to a condition designated as a precancerous condition of the head and neck region. OSMF carries a high risk of malignant transformation to oral squamous cell carcinoma ranging from 7–30%. This disease's prevalence is mainly attributable to the Asian population, with more incidences occurring in the Indian sub-population [4]. Although multifactorial etiology is reported for OSMF, the master of causation is almost always tilted to consuming areca-nut (arecoline) or betel-nut in varying forms and proportions. Areca-nut forms the essential ingredient of several chewing products used worldwide [5]. In India, which happens to be a densely populated nation, a large amount of the population consumes chewable or smoking forms of areca-nut products, which precipitates in OSMF with an incidence of 2 to 3% in both males and females [6], making this disease a subject of crucial concern. Recent evidence shows that the

areca nut is a class I carcinogen responsible for the malignant transformation of OSMF and other premalignant lesions [1].

OSMF is a complex disease and its etiopathogenesis, clinical presentation, histological features, and epidemiological prevalence of OSMF have been investigated in detail. Varieties of therapeutic options are practiced in the pathophysiological reversal of OSMF, such as nutritional intervention, anti-oxidant therapy, physiotherapy, intralesional injections of corticosteroids and IFN-gamma, laser therapy, and surgical intervention removal of the fibers[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 OSMF[7], although its therapeutic potential is not translated from the laboratory to clinics as yet. This can be accredited to the lack of reliable animal models which would mimic the in vivo features of the OSMF. To date, few studies have attempted to establish an animal model for OSMF which utilizes administration of the areca nut extract (ANE), a potent precursor of the OSMF and subsequently oral cancer in humans[5, 8–14]. However, certain lacunae (mode of administration, exposure time and dosage, persistence of the OSMF phenotype) still exist in terms of model establishment of OSMF, limiting the testing of current and novel therapeutic options.

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

2. Materials And Methods:

2.1 Ethical Approval:

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

2.2 Preparation of areca-nut extract:

The extract was prepared at Dr. D. Y. Patil College of Ayurved and Research Centre. The aqueous extract was prepared using endosperms of Areca *catechu*. A fine powder of areca catechu endosperms was obtained and dissolved in normal saline (0.9% of 50mM Sodium chloride (Hi Media)) at the 50 mg/ml concentration. This was followed by centrifugation at 15000 rpm for 30 mins. The supernatant obtained after centrifugation was collected, filtered using a syringe filter, and administered.

2.3 Animals:

Six-week-old, male Swiss albino mice with an average weight of 25 to 30 grams were procured from Crystal Biological Solutions. For the study, Pune, India (Reg. No. - 2030/PO/RcBiBt/S/18/CPCSEA). Since the habitual consumption of areca nut in humans predominates 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 fifteen 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 hours each. The feeding was carried out under standard protocols. Mice were fed with standard mouse fodder and water *ad libitum* at all times.

2.4 Experimental design:

A total of forty mice were randomized into four groups using the R statistical program, as shown in Figure 1. Each group consisted of 10 mice. The sample size calculation was performed according to the guidelines [15]. The first group was injected 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) intra-buccally using disposable syringes (Dispovan 31G Insulin Syringe 1 ml). The injections were given inside the right buccal mucosa every two 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 intra buccal injection group, and the fourth group is labeled as the intraoral dose group. Post administration, the mice in the fourth group were abstained from water for the next 3 hours. The ANE administration of intraoral dose was carried out every day in the fourth group of mice for 12 weeks. The time of administration was maintained at the same time throughout the entire experimentation for all four groups.

2.5 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 downwards towards 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.

2.6 Histopathological analysis:

For proper fixation, the tissue specimens collected were left in formalin for 24 hours. 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 hours. After tissue processing was complete, the tissues were embedded in paraffin wax. The wax was allowed to cool down using the cryo station. 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, Eosin, and Masson's Trichrome staining procedure was performed using a standard protocol[16,17]. 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. The estimated total fibrosis area was performed using Image J software and expressed as a percentage fibrotic area. 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.

3. Results:

3.1 Histopathological changes:

The changes associated with OSMF are best known to be expressed through histopathological analysis. The tissue sections obtained by the sacrifice of mice were inspected for microscopical changes associated with the concerned area of the oral cavity. The changes associated with epithelium and connective tissue were observed.

3.2 ANE administration by intraoral and intrabuccal method exhibits OSMF associated changes in buccal tissues:

The Hematoxylin and Eosin-stained tissue sections under a compound light microscope exhibited OSMF associated changes in the tissue, as shown in Figure 2. The Intra oral group of ANE administration exhibited a relatively higher level of fibrosis than the Intra buccal group. Our results closely resemble the histopathological OSMF features in humans and those documented in the published literature [3]. The changes observed in the histopathological slides are elaborated in Figure 2.

3.3 Intraoral administration of ANE results in more significant fibrosis than Intra buccal route:

Masson's Trichrome staining was performed to evaluate the ANE-associated fibrosis in the buccal tissues. As shown in figure 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 Image J software. The area in red denotes the fibrosis area in the last column (Figure 3). Our results revealed that ANE administration by the intraoral group exhibited a significantly higher percent fibrosis area as compared to the ANE administered through intra buccal 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 group (13.1356±0.00077 vs. 0.5496±0.00253). A highly significant increase in the fibrosis was evident in the ANE group, with a 48%-fold increase in the mean percent fibrosis area for

the intrabuccal injection group as 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 group (33.9246±0.00765 vs. 26.3775±0.00978).

4. Discussion:

Our study was aimed to investigate the efficacy of ANE-induced OSMF animal model administered through previously reported routes of administration (intrabuccally and intraorally). Furthermore, an evaluation of the best possible method of administration that could closely simulate the occurrence of OSMF in humans was performed. Increasing cases of OSMF validates treatment strategy for assessing the safety and efficacy of the same. The very cause for OSMF, areca nut usage, has been shown in the present study to establish an animal model. Areca nut contains alkaloids like arecoline, preceding and quvacine, and other components like flavonoids and copper. These are responsible for disturbance in the extracellular matrix's normal homeostasis, mainly the collagen synthesis and degradation pathways, which terminally lead to OSMF [10]. Over the past decade, extensive research has been carried out on establishing an animal model for OSMF. Das et al.[11], Raghavendra et al.[13], Chiang et al.[18], and Maria et al.[19] used an intra-buccal 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 dosing) of ANE administration in animals, unlike other studies where only a single mode of administration was used. ANE through the intraoral dose method has been proved superior to the intra-buccal injection technique. We tried to establish an early model through the intra-buccal injection; it is an invasive method that can induce OSMF quickly but does not simulate the exact pathway of disease occurrence in humans [5,10,11,13,20]. 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.

Moreover, the repeated injections cause unnecessary inflammation, edema, and hyperemia, interfering with the usual pathway for OSMF establishment. Apart from the injection method of ANE administration, various methods have been used by authors like feeding with diet [8], topical application over the buccal mucosa [9], fed through drinking water [12]. All those methods were successful in the model establishment of OSMF, but none of them could simulate the absorption method in humans on the consumption of areca nut-containing products. 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 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 [9,14]. The histopathological analysis confirmed the diagnosis of OSMF, 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]. OSMF is principally diagnosed through histopathological analysis; thus, different staining procedures are enough to address the establishment of disease microscopically [5,20,21].

Currently, no definite treatment modality is available for the cure or at least reversal of the OSMF. 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 route showed noticeable histopathological features of OSMF and relatively higher fibrosis than the ANE administration through the intrabuccal route.

5. Conclusion:

The present study demonstrates an easy, economical, non-invasive method simulating the areca nut absorption seen in humans giving rise to an animal model for OSMF. Using mice as an animal model to study areca nut induced OSMF is a straightforward, reproducible, and sustainable method in a desired amount of time. However, OSMF related serum biomarkers need to be studied, which were lacking in our present study.

Declarations:

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Authors' contributions:

All authors contributed to the study's conception and design.

Conceptualization: [Supriya Kheur]; Methodology: [Madhura Shekatkar], [Avinash Sanap], [Vrushali Bhalchim]; Formal analysis and investigation: [Avinash Kharat], [Vaishali Undale]; Writing - original draft preparation: [Madhura Shekatkar]; Writing - review and editing: [Supriya Kheur], [Avinash Sanap]; Funding acquisition: [Supriya Kheur]; Resources: [Arati Dubewar]; Supervision: [Ramesh Bhonde].

Ethics approval:

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

Compliance with Ethical Standards:

The present work was carried out under the guidelines provided by the welfare of animals.

Declaration of Conflicting Interests:

The authors have no relevant financial or non-financial interests to disclose.

Data availability:

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Figures

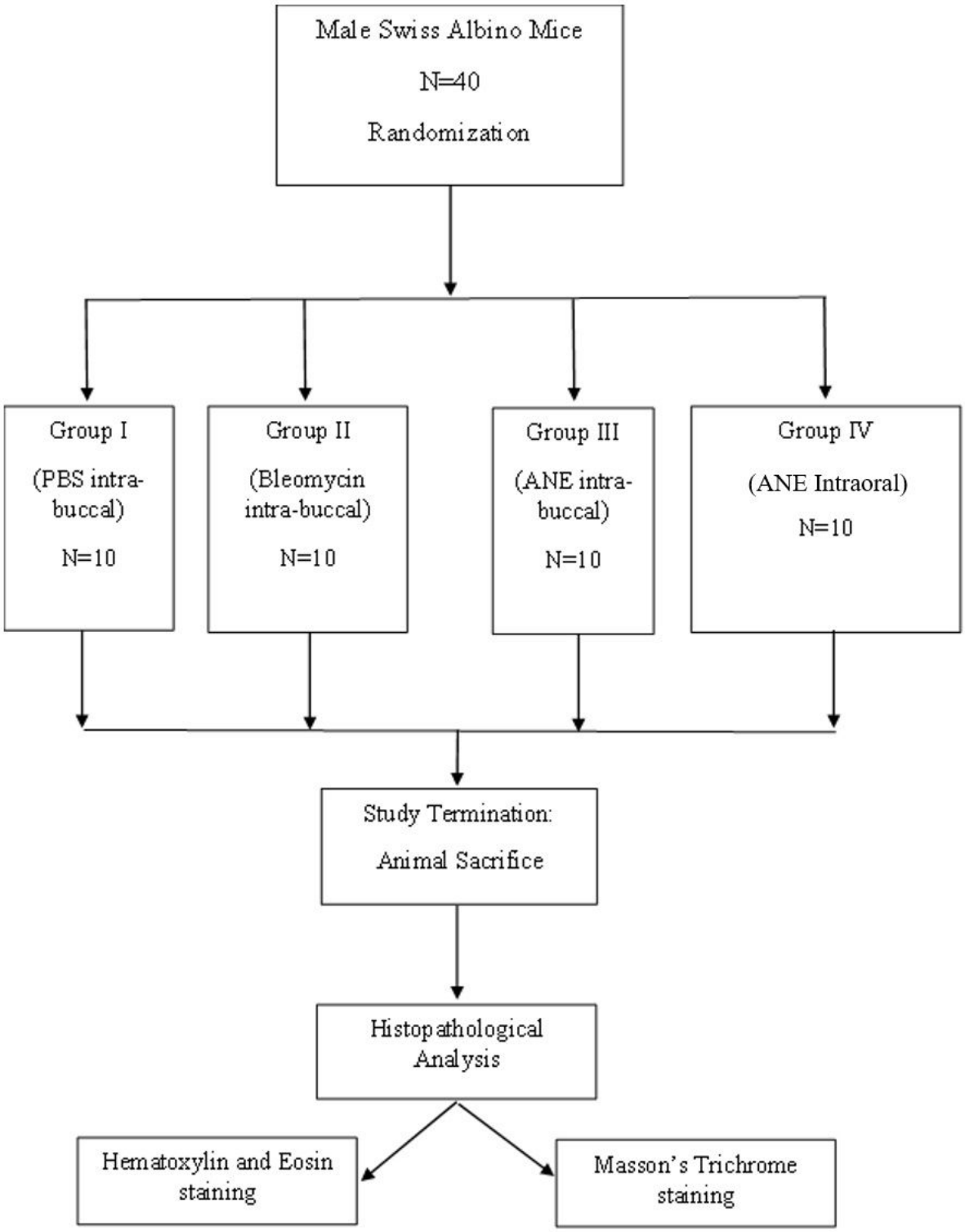

Figure 1

Flowchart representing experimental design.

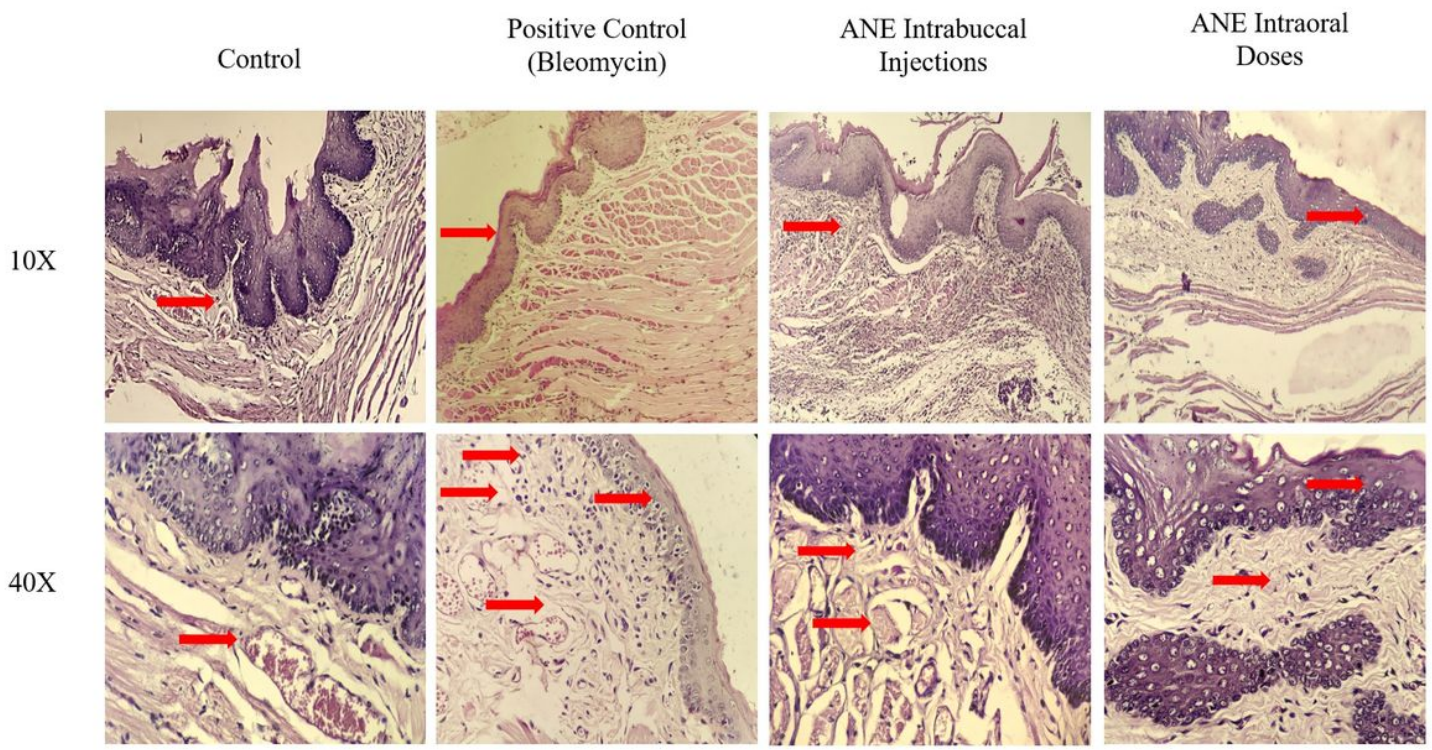

Figure 2

Photo micro-graphical presentation of histological changes associated with the mice group after Hematoxylin and Eosin staining (H & E): a) Control group: under 10X, the lesional tissue shows hyperplastic epithelium; the connective tissue is loosely arranged and composed of mild chronic inflammatory cells and large engorged blood vessels, 40X: similar findings are projected under high power. b) Positive control group: under 10X projects relative thinning of epithelium showing mild dysplastic features, 40X: mild chronic inflammation in the connective tissue along with engorged blood vessels of varying size and shape, and slightly greater collagen fibers than usual are evident. c) ANE intrabuccal injection group: under 10X, reveals that the overlying epithelium is hyperplastic at some places, 40X exhibiting mild to moderate dysplastic features like nuclear and cellular pleomorphism, loss of stratification, basal hyperplasia, and nuclear hyperchromatism. The underlying connective tissue is predominantly composed of severe chronic inflammatory cell infiltration, decreased blood vessels, muscle degeneration, and greater than standard bundles of collagen fibers. d) ANE oral droplet group: 10X shows the overlying epithelium is reduced in thickness and appears severely atrophic at certain places, 40X, exhibiting moderate dysplastic features as mentioned above. The underlying connective tissue is entirely composed of mature collagen fiber bundles.

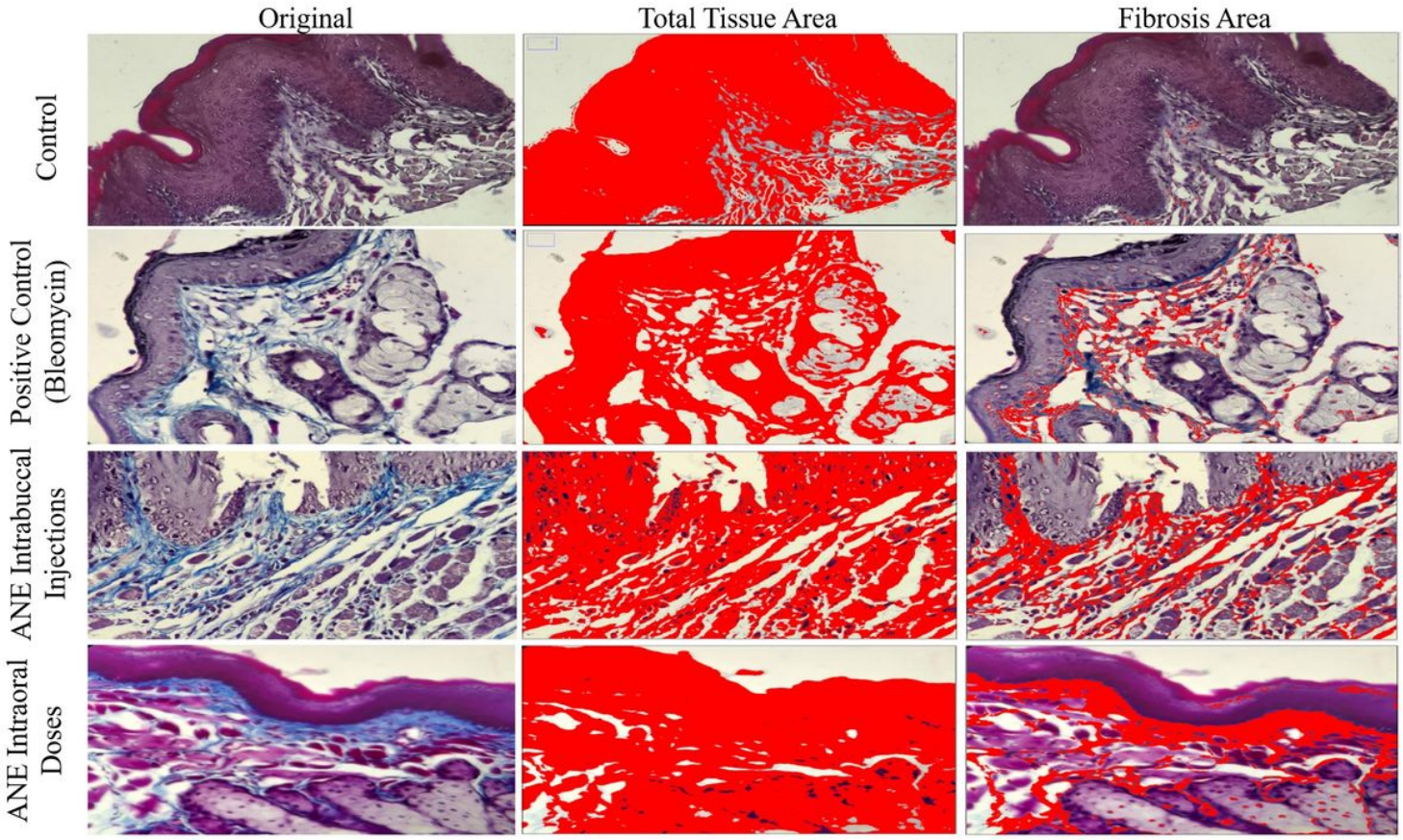

Figure 3

Composite photomicrographs depicting sub-epithelial fibrosis in the original images of Masson's Trichrome stained slides (Under 40X magnification), total tissue area after analyzing the image and finally total fibrotic area marked in red in all the four animal groups.

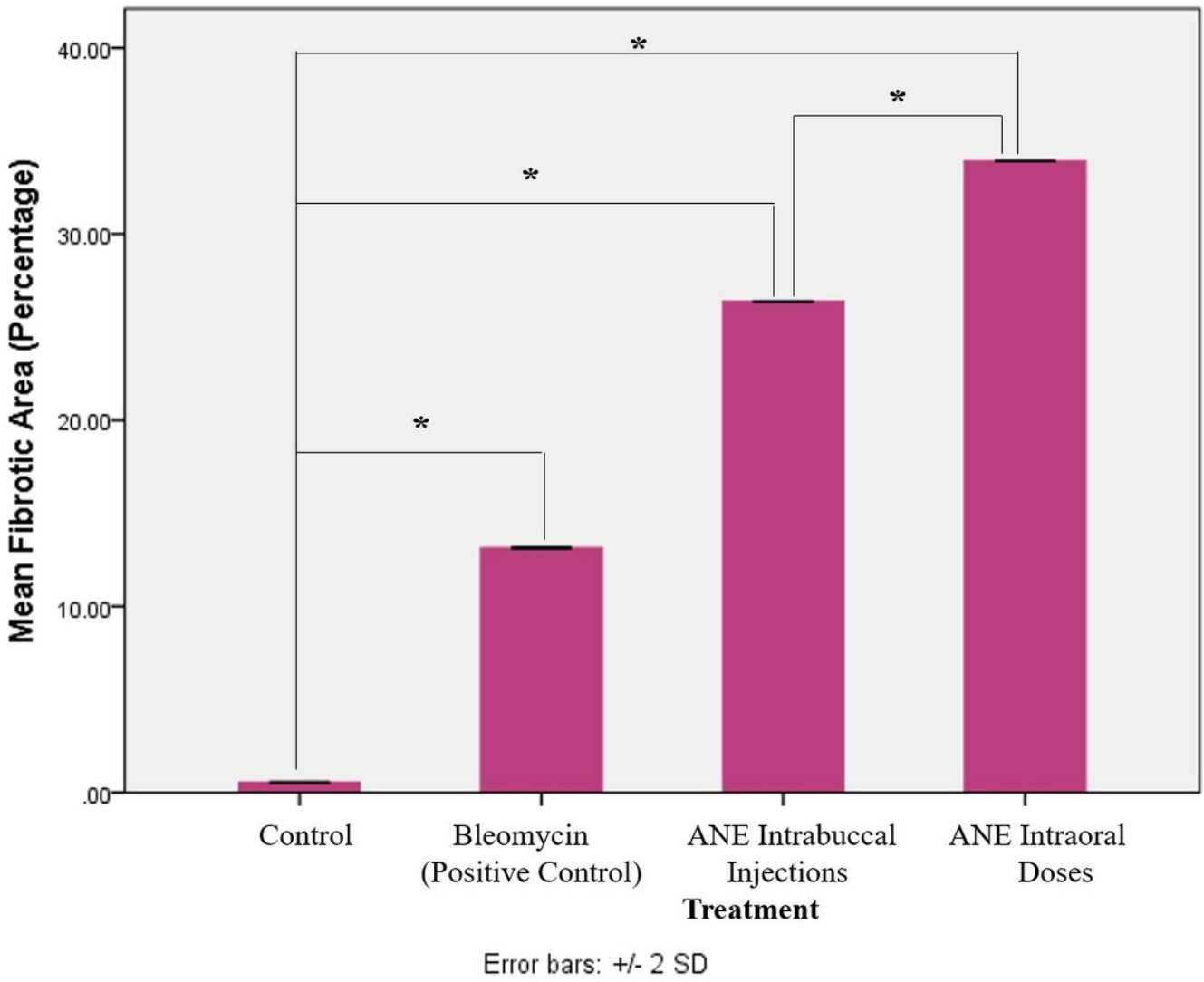

Figure 4

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 (*p-value <0.05)