Original Article

Effects of Nicotine Treatment on Viability of Inflammed Reconstituted Model of Oral Mucosa

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ABSTRACT

Objective: The objective of this assignment is to evaluate the effects of nicotine for over a span of 5 minutes and more than 24 hours on an inflamed stratified epithelial layer.

Study Design: Observational research

Place and Duration of Study: This study was conducted at the Department of Oral Pathology at Bart's and the London Queen Mary School of Medicine and Dentistry at Queen Mary, University of London from July 2018 to July 2019 for a period of one year.

Materials and Methods: The effects of nicotine on an in vitro Viability of Inflamed Reconstituted Model of Oral Mucosa are the subject of this study. Skin Ethic Laboratories, Nice, France, produced and supplied the reconstituted human epithelial model used in this study. The MTT test was used to determine the effect of different nicotine treatments on tissue viability.

Results: The effect of nicotine on the viability of inflamed stratified epithelium layer viability after 5 minutes and 24 hours with working solutions (10M, 100M, 1mM, and 10mM) on inflamed oral mucosa was determined to be insignificant.

Conclusion: This study found that all nicotine concentrations applied after 5 minutes and 24 hours had no effect on the viability of inflamed tissue.

Key Words: Tobacco, Nicotine, Oral mucosa, viability

Citation of article: Sheikh MN, Hanif S, Atif M, Khan MS, Akhlaq H, Lone MA. Effects of Nicotine Treatment on Viability of Inflammed Reconstituted Model of Oral Mucosa. Med Forum 2021;32(11):46-49.

INTRODUCTION

Tobacco smoke is a main cause of early death globally [1, 2]. Tobacco smoking causes more deaths worldwide than other diseases like TB, HIV and malaria together [3, 4]. Tobacco smoking is responsible for around 6 million deaths per year in the world [5]. Tobacco smoking in countries such as west Europe, Australia, North America and the developing countries is increasing [6]. People use a wide variety of smoke tobacco products include cigars, cigarettes, bidis, kreteks, sticks, and snuff [7]. Use of all kinds of tobacco products lead to developing cancer in humans [8].

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Received: June, 2021
Accepted: August, 2021
Printed: November, 2021

Tobacco smoking is also responsible for serious conditions such as cardiovascular disease, COPD, stroke, pneumonia, aortic aneurysm and ischemic heart diseases [9]. Nicotine is a key component of tobacco [10]. The amount of nicotine consumed by individuals who use tobacco varies and it is well know that cigarette smoking has a direct link to nicotine addiction [11]. Tobacco smoking side effects are linked to time duration as well as a dose – response relationship to heavy smoking [6].

Tobacco smoking is a very popular habit and is associated with the development of cancers in humans [8]. The incidence of oral cancer is correlated to the use of tobacco products [12]**.** Oral cancer is considered as the 6th commonest cancer mostly affecting the male population of the world with a poor prognosis [13]. The higher incidence of cardiovascular morbidity and mortality is due to active and passive (environmental) cigarette smoking [14].

Active Tobacco
smoking significantly increases the risk of RCC compared with passive smoking [15].

Low birth weight, ectopic pregnancy, spontaneous abortion and limb reduction defects are risks associated with smoking in pregnancy [9]. According to WHO, the greatest risk ever found to world's health is tobacco use causing several congenital defects in children [16].

Precancerous lesions such as leukoplakia, erythroplakia, and smokeless tobacco keratosis, as well as cancerous lesions such as squamous cell carcinoma of the tongue, floor of mouth, lip, gingiva, and verrucous carcinomas of the buccal mucosa, gingiva, and alveolar ridge, are all linked to tobacco use [17]. Tooth stains, abrasions, smoker's melanosis, acute necrotizing ulcerative gingivitis, burns, keratotic patches, nicotinic stomatitis, peri-implantitis, and diseases such as increased plaque deposition, calculus depositions, gingival recession, and alveolar bone loss are all linked to tobacco use [18].

Nicotine concentration in oral snuff and pipe tobacco is equivalent to that in cigarette tobacco, however nicotine concentration in cigar and chewing tobacco is half that of cigarette tobacco [19].

The average tobacco rod contains 10 to 14 mg of nicotine. During smoking, 1 to 1.5 mg of nicotine is absorbed systemically on average.

Because of pH effects, buccal absorption of nicotine is reduced even when flue-cured tobacco smoke is held in the mouth [20]. Smoke from air cured tobacco used in pipes, cigars, and certain European cigarettes, which is the major tobacco, is more alkaline (pH6.5 or higher), contains significant unionised nicotine, and is easily absorbed in the mouth [21].

Nicotine enables smokers to operate more efficiently and with more concentration , by making them calm under stressful situation, as it is a type of psychomotor stimulant [11].

MATERIALS AND METHODS

The objective of this assignment is to evaluate the effects of nicotine for over a span of 5 minutes and more than 24 hours on an inflamed stratified epithelial layer. A modified MTT assay will be used to assess the tissue viability. In this study, The model utilised was a reconstructed human epithelium which was a model of three-dimensional tissue culturing and was TR146 altered oral keratinocytes (TR146) originating from a buccal cancer were cultured.This model SkinEthic Laboratories, Nice, France, produced and provided the product [22]. The model cultures were transported on agar and then rearranged into fresh 24 well culture plates (Costar, UK), each well containing 500l maintenance medium, and incubated for 2 hours in a humified environment at 37oC in 5% CO2. For all tests, each of the 24 cultures was moved to a fresh medium housed in a new 24 well plate. To make nicotine working solutions (10µM, 100µM, 1mM, and 10mM), a 2.5M stock solution was utilised. (Sigma, United Kingdom).

A phosphate buffered saline was used to dilute the working solutions. TNF-α solutions at (1000u/ml) was also prepared immediately before use. All solutions were discarded after each experiment Under experimental conditions, the percentage of cells that did not survive were quantified by designed viability assays. In current study, modified MTT assay was used. A modified MTT assay The activity of mitochondrial dehydrogenase in the cells is measured. was used to assess the viability of exposed cultures. A colour reaction is produced by the MTT assay which allows a measure of the cell activity. A pale-yellow substrate got transformed into an insoluble dark blue formazan product when living MTT (3-(4, 5-dimethyl-thiazol-2-yl)-2, 5-diphenyltetrazolium bromide) was used to incubate the cells. A densitometry was used to measure the amount of formazan uptake.

Once the treatment period ended, a 300µl MTT (0.5mg/ml in PBS) solution contained in a new 24 well plate was used for the transfer of the cultures. Aluminum foil was used to wrap the plates and then, in a humidified atmosphere at 37°C in 5% CO2 , incubated for 60 minutes. After incubation, 750l of isopropyl alcohol was sprayed to the epithelium's surface to transfer the cultures to a fresh 24-well plate containing 750l isopropyl alcohol each well.

To prevent evaporation, the plates were sealed with Parafilm®, and incubated at 37°C for 2 hours for extraction of formazan. All the surface solution which remained was preserved in the well, when insert was removed. To equilibrate the colour density the plate was agitated and all the cultures expended were discarded.

Three 200l aliquots were transferred from each well to a 96-well plate (Costar UK), and the optical density was determined using a Titertek Multiskan Plus plate reader (OD). The absobance at 570 nm was used to measure epithelial viability.

RESULTS

5 Minute Nicotine Treatment on Inflamed Mucosa:
There is no significant effect of nicotine on TNF-α stimulated stratified epithelial layer viability after 5 minutes at 10µM (108.38 ± 23.51 %), 100µM (94.13 ± 15.31 %), 1mM (121.86 ± 12.79 %) and 10mM (107.79 ± 6.26 %) as compared to the PBS control concentrations (100 ± 14.35 %), (Table 1 and Figure 1).

Table No.1: MTT findings on TNF-stimulated tissue after 5 minutes of nicotine administration

Treatment	Mean viability %	Standard deviation	n
10µM	108.38	23.51	3
100µM	94.13	15.31	3
1mM	121.86	12.79	3
10mM	107.79	6.26	3
PBS	100	14.35	3

Nicotine Treatment on Inflamed Mucosa for 24 Hours: There is no significant effect of nicotine on the inflamed stratified epithelial layer viability after 24 hours at 10µM (117.46 ± 9.29 %), 100µM (118.48 ± 10.21 %), 1mM (102.69 ±12.93 %) and 10mM (122.03

± 15.31 %) as compared to the PBS control concentrations (100 ± 24.60 %), (Table 2 and Fig 2).

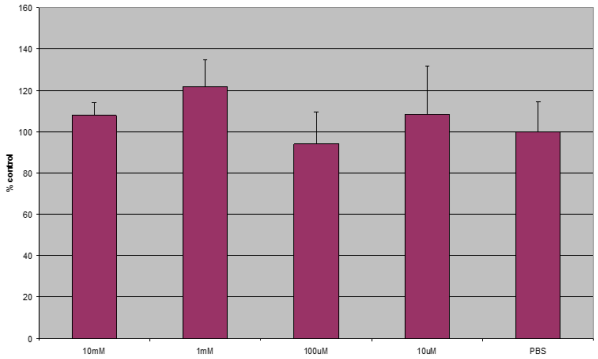

Figure No.1: Effects of a 5-minute nicotine administration on inflamed tissue viability

Table No.2: MTT findings on inflamed tissue following a 24-hour nicotine administration

Treatment	Mean viability %	Standard deviation	n
10µM	117.46	9.29	3
100µM	118.48	10.21	3
1mM	102.69	12.93	3
10mM	122.03	15.31	3
PBS	100	24.60	3

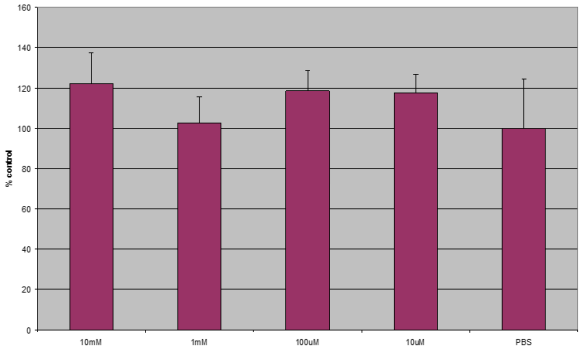

Figure No.2: Effects of a 24-hour nicotine administration on inflamed tissue viability

DISCUSSION

The purpose of this study is to see how nicotine affects a reconstituted oral mucosa that has been injured and activated by TNF. The epithelial model allows researchers to investigate the effects of nicotine on epithelial layers without the presence of mesenchyme. Stratified cultures were treated for 5 minutes and 24 hours, respectively.

The results indicated that there was no impact on viability after 5 minutes and 24 hours of the cells with the nicotine treatment of TNF-α stimulated reconstituted oral mucosa.

However, the viability studies suggest that there are only subtle changes in the membrane integrity from the nicotine, and most importantly there are no significant changes in the epithelium appearance. Surprisingly, nicotine has been shown to alter viability.

In a previous *in vivo* study by Anderson and Warfving, it was revealed that nicotine exerts its biological effect on the oral mucosa and resulted in changes in the appearance of the epithelium [(23)]. A similar study by Kwon *et al* found there was no effect of nicotine At concentrations of 10uM and 100uM, the viability of the cells was reduced dose-dependently with mucosal epithelial thickness.on reconstituted oral mucosa however it did reduce the viability of cells in the epidermal keratinocyte at a 100µM concentration [(24)].

A study by Alpar *et al* revealed that nicotine with higher doses (10.5-15.5mM) had a direct relationship in initiating changes in morphological appearance of the cells which were irreversible [(25)]. The findings of Squier and Johnson also stated that when on oral mucosa 0.2M nicotine was topically applied, after 2 hours it induced acantholysis and nuclear shrinkage within the epithelium [(26)].

The limitations of the findings in this study could be due to several factors such as the permeabilizing effect of nicotine on the mucosa. In addition, the tissue culture models used in this study were *in vitro* whereas many of the studies looked at tissue culture models *in vivo*. Therefore, it could suggest that the results obtained, with nicotine at the range of concentrations used, cannot be used to quantify the amount of mitochondrial disruption.

CONCLUSION

The viability of the inflamed oral mucosa was unaffected by nicotine concentrations ranging from 10µM to 10mM.

Author's Contribution:

Concept & Design of Study:	Muhammad Nauman Sheikh
Drafting:	Sajid Hanif, Muhammad Atif
Data Analysis:	Muhammad Sibghat Ullah Khan, Humera Akhlaq, Mohid Abrar Lone
Revisiting Critically:	Muhammad Nauman Sheikh, Sajid Hanif
Final Approval of version:	Muhammad Nauman Sheikh

Conflict of Interest: The study has no conflict of interest to declare by any author.

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