ORIGINAL ARTICLE

EFFECTS OF NICOTINE ON AN *IN VITRO* RECONSTITUTED MODEL ORAL MUCOSA IN TERMS OF CYTOKINE PRODUCTION

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Background: The extensive use of tobacco and its associated problematic health issues have been a concern to mankind. The World Health Organization (WHO) estimates that approximately one-third of the global population aged 15 years or older are smokers and each smoker consumes an average of 15 cigarettes daily**.** The objective of this study was to establish the effect of nicotine on an in vitro reconstituted oral mucosa model, the effect of treatment with this compound was measured in terms of cytokine production. **Method:** Observational laboratory based study design was used to carry out the experiment. The reconstituted human epithelium model used in the study was prepared and supplied by Skin Ethic Laboratories, Nice, France. The effect of nicotine on epithelial cytokine production was assessed using commercially available assay kits (R&D systems). This was done using the enzyme linked immuno-sorbent assay. **Result:** In this study there was evidence that after 5 minutes treatment on un-inflamed mucosa with nicotine at 10 mm concentration GM-CSF release decreased, and also after 24 hours treatment with nicotine at 10mM concentration GM-CSF release increased. TNF-α increased release of pro-inflammatory cytokines, IL-6, IL-8, and also GM-CSF from the model mucosa after 24 hours, but had no effect on the release of IL-1α., IL-6, IL-8, and GM-CSF after 5 minutes and 24 hours respectively. **Conclusion:** In conclusion at all the concentrations used in this experiment, nicotine had no effect on the TNF-α stimulated tissue and un-inflamed mucosa and had no significant effect on cytokine release including IL-1α., IL-6, IL-8, and GM-CSF after 5 minutes and 24 hours respectively.

Keywords: Nicotine, oral mucosa, cytokine

INTRODUCTION

One third of the world's adult population is affected by the use of tobacco products. Tobacco is to be considered as the main cause of early death and morbidity, and it has been suggested that it may cause up to 10 million deaths by year.[1] It is well established that tobacco plays a significant role in many diseases such as chronic obstructive pulmonary diseases, cardiovascular diseases, and several forms of cancer, in particular, cancers of the lung, oropharynx, larynx, and oesophagus, leading to either death or disability.[2-4] People consume different types of tobacco products which can be smoked, chewed or sniffed.[5] These include products that are smoked such as cigars, cigarettes, pipe tobacco and roll- your-own or consumed smokeless as chewing tobacco and snuff.[6]

Nicotine is an alkaloid, which only exists in tobacco plants called *Nicotina tabacum*, and accounts for 95% of its total alkaloid content.[7,8] The side-effects associated with the smoking of tobacco are based on a dose-response relationship to heavy smoking, and time duration.[3] Tobacco also acts as the delivery system for the nicotine inside the body and its use is harmful and dangerous from a health perspective.[9]

Oral lesions and conditions associated with tobacco use include oral precancerous lesions such as leukoplakia, erythroplakia and smokeless tobacco keratosis, oral cancers such as squamous cell carcinoma of the tongue, floor of the mouth, lip and gingival, also verrucous carcinomas of the buccal mucosa gingival and alveolar ridge.[10] Excessive consumption of tobacco has also been associated with other lesions within the oral cavity such as tooth stains, abrasions, smoker's melanosis, acute necrotising ulcerative gingivitis, burns and keratotic patches, nicotinic stomatitis, peri-implantitis and other periodontal conditions including increased plaque and calculus depositions, gingival recession and alveolar bone loss.[11]

IL-1 is produced mainly by the activated mononuclear phagocyte and has two principal forms including IL-1α and IL-1β. However, other cell types also secrete IL-1, such as epithelial cells (keratinocytes) and endothelial cell, and thus provide potential local sources of macrophage-rich infiltrate. IL-1 stimulates T-cells to produce IL-2, IL-4, IL-6, interferon gamma (IFN-γ) and colony stimulating factors and increases IL-2 receptor expression on T-cells.[12]

IL-6 plays an important role in host response to injury and infection, and also increases the secretion of acute phase proteins by the liver, enhances B and T-cell activation and proliferation and stimulates keratinocyte proliferation.[13]

IL-8 is secreted by variety of cells including polymorphonuclear neutrophils, monocytes and macrophages, but also by fibroblasts and keratinocytes, it is considered as a potent cytokine for neutrophils. Major function of IL-8 is to induce directional migration for neutrophils and T-lymphocytes in inflammatory responses due to its chemo attractant activity for these

cells.[14] IL-8 is also suggested to be involved in epithelial repair, inducing keratinocyte proliferation and migration. It has been concluded that oral keratinocytes can secrete IL-8 following cytokine stimulation.[15]

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is an immunomodulator of hematopoietic cells and is associated with keratinocyte proliferation, and also induces regenerative differentiaition in the epidermis.[16] GM-CSF is a glycoprotein that stimulates the production of granulocytes, macrophages, and white blood cells. It has been concluded that GM-CSF production increased when stabilizing polymer gelatin was added to plant suspension cultures.[17] One (1 μM) Nicotine increased GM-CSF release from cultured human bronchial epithelial cells; however the stimulatory effect was attenuated or even lost with high agonist concentrations 100 μM nicotine suggesting receptor desensitization.[18] Application of nicotine derived N-nitrosamine to U937 human macrophages resulted in modulation of GM-CSF release, and this modulation of GM-CSF is suggested to be associated with immunosuppression in smokers which may lead to the inhibition of immune functions and development of lung cancers in humans.[19]

Nicotine is associated with a variety of lesions within the oral cavity.[9] It is suggested that nicotine might be associated with the pathogenesis of oral white pre-malignant lesions.[20] Carcinogens found in the tobacco smoke are associated with the developing of oral diseases and cancer.[21] Nicotine can contribute to cancer aetiology if it is nitrosated to form carcinogenic tobacco-specific nitrosamines.[22] In vivo studies showed that when 0.216 M of nicotine is applied topically to the oral mucosa for a period of two hours leads to alterations within the epithelium like acantholysis and nuclear shrinkage.[23]

Nicotine agents by acting on nicotine acetylcholine receptors, directly modulates the stimulated release of calcitonin gene related peptide (CGRP). This modulation can contribute to inflammatory processes within the oral cavity.[24] As oral keratinocytes, are the first cells in contact with tobacco components, thus keratinocyte inflammation has been stated as a critical step in tumor promotion.[25]

Oral keratinocytes exposed to nicotine at 0.1 μM, 10 μM and 1 mM concentrations for 4, 24, and 48 hours respectively, produced various inflammatory mediators, including PGE2 and IL-1. It has been concluded that gingival oral keratinocytes produced interlukin-1 at 1 mM dose, and this elevated levels of IL-1 may have implications in tobacco-induced lesions, given the central role played by IL-1 in tissue response to injury.[26]

The aim of this study, therefore, was thus to establish the effect of nicotine on an *in vitro* reconstituted model oral mucosa, the effect of treatment with this compound was measured in terms of cytokine production.

MATERIAL AND METHODS

This observational study was conducted at Department of Oral Pathology, Barts, and London Queen Mary School of Medicine and Dentistry Queen Mary, University of London. Various concentrations of nicotine were used during the experiment. Working solutions (10 μM, 100 μM, 1 mM, and 10 mM) of nicotine were prepared from a 2.5 M stock solution (Sigma, UK). The working solutions were diluted in phosphate buffered saline immediately before use.

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 (Rupniak *et al* 1985).[27] The cells were seeded (8×10^5 cells/Cm^2) on a 0.5 Cm^2 inert polycarbonate membrane and cultivated in a defined medium for 14 days at the air- liquid interface (Rosdy and Clauss 1990).[28] The resulting culture formed a stratified epithelium with 5–7 cell layers devoid of stratum corneum. Skin Ethic Laboratories also supplied maintenance medium (MCDB 153 containing 5 μg/ml insulin and 1.5 mM Ca^{++}).

The effect of nicotine on epithelial cytokine production was assessed using commercially available assay kits (R&D systems). This was done using the enzyme linked immunosorbent assay. The appropriate kit by DUOSET was taken out of the fridge and its contents were allowed to come to room temperature and the levels of cytokine was measured following the manufacturers protocol and the concentration of cytokine released by the epithelial cultures during the treatment period expressed as ρg/ml. The purpose of the Elisa experiments was to determine whether the TR-146 cell line responded or not over the increasing periods of time in terms of producing the cytokines IL-1α, IL-6, IL-8, and GM-CSF respectively.

The results were analysed using an independent student's *t*-test. Results were considered significant with $p<0.05$.

RESULTS

Values of cytokines level released by epithelial cells on application of various concentrations of nicotine (n=5) after 5 minutes and 24 hours on un-inflamed tissue are shown in Table-1 and 2.

Values of cytokines level released by epithelial cells on application of TNF-α are tabulated as Table-3. Values of cytokines level released by epithelial cells on application of various concentrations of nicotine (n=4) after 5 minutes and 24 hours on inflamed tissue are shown in Table-4 and 5.

Cytokine released from inflamed mucosa TNF-α increased release of pro-inflammatory cytokines, IL-6, IL-8, and also GM-CSF from the model mucosa after 24 hours, but had no effect on the release of IL-1α.

Table-1: Cytokine release after 5 minutes nicotine treatment on un-inflamed tissue

	Cytokine IL-1		Cytokine IL-6		Cytokine IL-8		GM-CSF	
Treatment	ρg/ml	SD	ρg/ml	SD	ρg/ml	SD	ρg/ml	SD
10 µM	3.15	3.23	6.33	2.59	702.26	142.01	10.24	1.79
100 µM	3.13	2.48	4.55	1.49	496.03	79.56	7.44	0.30
1 mM	2.55	1.84	4.32	0.95	485.08	126.83	8.49	1.60
10 mM	4.96	0.81	4.36	2.53	574.63	206.46	5.15	0.81
PBS	2.79	2.59	4.32	2.61	370.96	111.44	10.40	0.26

Table-2: Cytokine release after 24 hours nicotine treatment on un-inflammed tissue

	Cytokine IL-1		Cytokine IL-6		Cytokine IL-8		GM-CSF	
Treatment	ρg/ml	SD	ρg/ml	SD	ρg/ml	SD	ρg/ml	SD
10 µM	5.05	3.06	10.56	7.12	1227.09	394.24	38.42	12.24
100 µM	3.01	1.91	11.67	14.86	1034.1	353.03	28.37	4.65
1 mM	2.7	1.47	5.11	2.23	956.66	298.20	34.95	5.98
10 mM	7.06	1.77	11.66	10.02	1280.20	253.48	56.78	6.93
PBS	4.28	4.75	4.63	1.29	892.95	183.62	21.44	2.56

Table-3: Effect of TNF α on cytokine release

Treatment	IL-1	IL-6	IL-8	GM-CSF
TNF-α	4.44±1.6	33.8±11	2927±236	40.8±3.8
Untreated	4.3±4.6	4.6±1.3	892±183	21.4±2.5

Table-4: Cytokine release after 5 minutes nicotine treatment on inflammed tissue

	Cytokine IL-1		Cytokine IL-6		Cytokine IL-8		GM-CSF	
Treatment	ρg/ml	SD	ρg/ml	SD	ρg/ml	SD	ρg/ml	SD
10 µM	7.12	1.39	27.28	8.51	2576.02	176.57	12.38	3.94
100 µM	5.09	1.60	22.47	12.71	2216.19	178.14	9.35	1.55
1 mM	6.24	2.30	32.33	31.00	1998.80	594.81	6.10	2.77
10 mM	5.97	2.14	46.97	15.26	2468.84	424.98	10.85	1.49
PBS	7.00	2.61	24.37	15.10	2174.78	566.97	8.91	2.61

Table-5: Cytokine release after 24 hours nicotine treatment on inflamed tissue

	Cytokine IL-1		Cytokine IL-6		Cytokine IL-8		GM-CSF	
Treatment	ρg/ml	SD	ρg/ml	SD	ρg/ml	SD	ρg/ml	SD
10 µM	7.90	1.64	22.29	9.42	2969.89	245.32	38.36	8.39
100 µM	5.18	1.07	32.23	7.25	3130.47	231.46	40.64	1.62
1 mM	4.18	1.40	38.07	21.77	2657.56	110.83	44.31	5.17
10 mM	3.89	1.98	32.39	8.14	2626.02	253.48	29.14	11.01
PBS	4.40	1.59	33.81	11.00	2927.26	236.82	40.88	3.55

DISCUSSION

The aim of this study was to investigate the effect of nicotine on an un-inflamed and TNF-α stimulated reconstituted oral mucosa. The epithelial model allowed us to consider the effect of nicotine on an epithelial layer in the absence of any influence from mesenchyme. Stratified cultures were treated for 5 minutes and 24 hours respectively. Immunological studies were conducted to establish whether nicotine had an inflammatory or irritant action on the '*in vitro*' stratified epithelium.

However the treatment with nicotine on cytokine study provided us with some results, it was suggested that GM-CSF release decreased at 10mM nicotine dose after 5 minutes treatment of un-inflamed mucosa when compared to control (PBS), but it was observed that after 24 hours, GM-CSF release increased from un-inflamed tissue when compared to control (PBS) respectively. Nicotine had no significant effect on the release of other cytokines investigated in this study at all concentrations used.

There is an induction of inflammatory response as soon as cells are exposed to any toxic compound. In relation to this response expression of cell surface adhesion molecules, receptors and cytokine release is modified by these cells. In an *in vitro* study when mucosa exposed to 10 µM, and 100 µM of nicotine concentration it increases the expression of differentiation-specific proteins or marker such as K13, involucrin and profilaggrin/filaggrin.[20] This suggest that this abnormal differentiation might be associated with the pathogenesis of oral white premalignant lesions, such as oral leukoplakia which is considered as a common sequela from the use of tobacco in the oral cavity.[29]

Although little is known about the involvement of oral kerationcytes in inflammatory oral disease, recent studies suggested that oral keratinocytes may produce and release a number of cytokines, and also have the potential to initiate an inflammatory response.[15,30]

This is the first study to investigate the effect of nicotine by using an *in vitro* reconstituted stratified

squamous epithelium in context of cytokine release. Nicotine has the ability to produce inflammatory response in the oral mucosa (Dussor *et al*).[24] As oral keratinocytes represents the cells that first encounter any toxic agent like nicotine, thus nicotine induced abnormal alteration of the mucosal keratinocyte may contribute to the development of cancer, and also keratinocyte inflammation has been linked to tumour promotion (Barker *et al*).[25]

In this experiment, nicotine had no effect on IL-1α release at all the concentrations used over 24 hours from both un-inflamed and TNF-α stimulated inflamed tissue, this is of suggestive that IL-1α was not produced in response to nicotine on keratinocytes and would have been possible that IL-1α had bound to IL-1α receptors on the cell that have synthesised it.[31] This in turn could lead to autocrine and paracrine activation of the keratinocytes. Whether this occurs in response to nicotine requires further investigation.

Other evidence does support the existence of a role for IL-1α in oral disease in relation to nicotine induced toxicity. It has been concluded with the fact that IL-1α released from epidermis accelerates the tumour development.[32] In another study shown that IL-1α release by the oral keratinocytes in response to nicotine is associated with tobacco-induced lesions, given the central role played by IL-1α in tissue response to injury[26], subsequently increased IL-1α secretion by the cells had also linked nicotine to tissue destruction.[33]

IL-6 had been also reported to be associated with head and neck cancer. It has been concluded that IL-6 is expressed in oral squamous cell carcinoma.[34] In another study stated that periodontitis and cancer onset in oropharyngeal carcinoma is due to the up regulation of cyclooxygenase and inflammation via IL-6 release.[35]

IL-6 release was also not affected by nicotine from the model mucosa however there is evidence that 1nM nicotine stimulated interlukin-6 (IL-6) secretion in human gingival fibroblasts. In a study concluded that a synergistic effect in the up regulation of IL-6 was seen with combined treatment of 1 mM nicotine and *E. Coli*, LPS (lipopolysacchride) or *P. gingivalis* LPS at 24 hour time point respectively, this environment, enriched with bacterial LPS and nicotine, regulated HGF inflammatory mediator production.[36]

IL-8 was selected for investigation based on the evidence that IL-8 had been released by the oral keratinocytes.[15] However IL-8 release was also not affected by nicotine at any of the concentrations used.

The results from IL-1α, IL-6, and IL-8 release suggest that these cytokines may not be relevant in tobacco related diseases at the nicotine concentrations used and for the time period for which the culture models were exposed to the nicotine. The previous studies do not support this conclusion, thus there may be a potential difficulty that had limited the importance of the findings in this study. This may reflect that *in vivo*, mucosa adjacent to nicotine is not a monolayer but a stratified squamous epithelium and therefore nicotine must permeate through the superficial layers of the epithelium and cross the permeability barrier to exert its effect.

In a study confirmed that GM-CSF was secreted by human gingival keratinocytes.[37] This evidence supported our intention to investigate the release of GM-CSF from reconstituted oral mucosa. Furthermore, GM-CSF had been previously linked to tumour progression and metastasis formation. Gabri *et al* (1999).[38] It has been concluded that GM-CSF expression was associated with highly invasive and metastatic mammary carcinoma in mice[38]. Furthermore the decreased production of GM-CSF linked with inflammation and squamous cell carcinoma of the oral cavity.[39]

In a previous *in vitro* study showed that increased GM-CSF expression by the oral epithelial cells could generate effective immunity in immuno-compromised patients against infections like oral candidiasis, that this effect may be due to keratinocyte proliferation and degenerative differentiation of the epidermis via GM-CSF.[16]

The results for GM-CSF secretion although not statistically significant suggest that GM-CSF secretion decreased at 10 mM of nicotine concentration after 5 minutes from the un-inflamed mucosa, and also GM-CSF release increased at 10 mM of nicotine concentration after 24 hours from the un-inflamed mucosa. The significance of this effect is not known. In light of these results it would be necessary to repeat this study and furthermore increase the sample size to establish whether the change in GM-CSF release was statistically significant.

It is also important to note that our experiment was limited to the application of nicotine that is for 5 minutes and 24 hours respectively. Therefore any changes in the timing of nicotine treatment might alter the result of this experiment. Furthermore, our *in vitro* stratified model was grown using transformed oral keratinocytes (TR146) who may not respond in the same manner as cells *in vivo*.

In conclusion, there was no effect on IL-1α, IL-6, IL-8 release, but GM-CSF showed variable pattern with the highest doses of nicotine, short exposure to nicotine caused it to decrease whereas prolonged exposure caused it to increase.

CONCLUSION

The concentrations used in this experiment, nicotine had no effect on the TNF-α stimulated tissue and un-inflamed mucosa and had no significant effect on cytokine release including IL-1α., IL-6, IL-8, and GM-CSF after 5 minutes and 24 hours respectively.

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