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ORIGINAL ARTICLE

Smokeless tobacco-induced lamellar body abnormalities

MD Colvard[1], SH Ashrafi[1], OK Alonge[1], GA Cordell[1,2]

[1]*Department of Oral Medicine and Diagnostic Sciences, College of Dentistry;* [2]*Department of Medicinal Chemistry and Pharmacognosy, College of Pharmacy, University of Illinois at Chicago, Chicago, IL, USA*

OBJECTIVES: To compare the morphological changes and quantitative distribution of lamellar bodies (Lb) (membrane coating granules) in the hamster cheek pouch epithelium with smokeless tobacco (ST).

MATERIALS AND METHODS: Archives of experimental material from previously published studies [S. Ashrafi, A. Das, R. Worawongvasu, B. Mehdinejad and J. Waterhouse (1992) *Scanning Microscopy* 6: 183] were utilized. Animals in experimental group received most ST (snuff) in their right pouch, 5 days weekly, for 24 months, while no snuff was given to control group. After 24 months, the epithelial tissues were processed for electron microscopic study. Volume densities of Lb were assessed by morphometry.

MAIN OUTCOME MEASURES: Densities of Lb in the two groups, experimental *vs* control.

RESULTS: In the control, Lb extruded their contents into the intercellular spaces of upper granular layers and in between the last granular cell layers and keratin layers to form a permeability barrier. Conversely, in the smokeless tobacco-treated epithelium, the majority of the Lb that were formed remained inside and accumulated within the granular cells, without extruding their contents into the intercellular spaces to form a lipid compound permeability barrier.

CONCLUSIONS: Commercial alkaline ST may have contributed to the abnormal accumulation of Lb in the granular cell layer and affected the extrusion process of Lb to form an incomplete permeability barrier in the oral epithelium.

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Keywords: ethnomedicine; smokeless tobacco; lamellar bodies; co-evolution; adaptation

Correspondence: Dr Michael Colvard, 801 South Paulina, College of Dentistry, University of Illinois at Chicago, Chicago, IL 60612-7227, USA. Tel: 312 996 7480, Fax: 312 355 2688, E-mail: colvard@uic.edu
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Introduction

Humans, for millennia, have placed various plant and plant-based products within the oral cavity, as a way to deliver chemical compounds for various stimulant, medicinal, and hallucinogenic effects (Sullivan and Hagen, 2002). The oral cavity utilizes biochemical and physiologic systems to cope with exposures to exogenous foreign substances such as bacteria, natural products, synthetic chemicals, toxins, and virii. The oral mucosa also allows masticated substances to diffuse rapidly into the blood stream bypassing first-pass metabolism (Sullivan and Hagen, 2002). The robustness of the tissues of the oral cavity to withstand natural and synthetic substances is due principally to its unique co-evolutionary (Wink, 1998) and functional nature (Wink, 1993). The oral cavity is lined entirely by a stratified squamous epithelium supported by a connective tissue lamina propria (Wertz and Squier, 1991). Areas of the mouth subject to extreme friction, thermal challenge, and mastication, such as the palate and gingiva, evolved to include thickened cornified or keratinized layers that resemble the epidermis (Squier and Hill, 1989).

Oral mucosal permeability to natural and synthetic compounds, as well as resistance to diffusion of these compounds, is primarily associated with the lipids of the outer layers of the oral mucosal tissues (Wertz and Squier, 1991; Wertz *et al*, 1996; Fartasch, 2004). Normal oral pH ranges from 6 to 7.0. Exogenous compounds (natural and synthetic) of different pH present different diffusion characteristics (Cordell, 1981; Chen *et al*, 1999). Oral mucosal lamellar bodies (Lb), play a central role in the trans-cellular and para-cellular diffusion pathways (Squier and Ronney, 1976; Chen *et al*, 1999). Oral Lb arise within the stratum spinosum of keratinized oral epithelium, migrate through the stratum granulosum, and structurally disintegrate as they discharge their lipid-based contents into the inter-cellular spaces through exocytosis (Ashrafi *et al*, 1977; Hayward, 1979; Schmitz and Muller, 1991). The migration of oral Lb, filled with the necessary molecular precursors required for the formation of the intercellular permeability barrier, and extrusion of lamellar body contents, is consistent with lamellar body barrier

function in skin (Schmitz and Muller, 1991; Squier, 1991). Maintenance of the permeability barrier is completely dependent on the secretion of the lipid and hydrolytic enzyme contents of oral Lb. Furthermore, permeability barrier integrity is dependent on the postsecretory processing of these polar lipids within Lb into non-polar lipids (Elias *et al*, 1998; Mauro *et al*, 1998). These lipid compounds subsequently extrude into the intra-cellular space between epithelial cells, providing a permeability barrier and fostering breakdown of the intra-cellular connections between epithelial cells with subsequent epithelial shedding.

Current research demonstrated that the daily application of smokeless tobacco (ST) to cheek epithelium fosters the development of a hyperplastic and hyperkeratotic oral epithelium in hamsters (Worawongvasu *et al*, 1991; Ashrafi *et al*, 1992, 1997), in Sprague–Dawley rats (Hirsch *et al*, 1983), and in humans (Tomar *et al*, 1997; Payne *et al*, 1998; Martin *et al*, 1999). Long-term studies (Ashrafi *et al*, 1992) reported that prolonged ST exposure (24 months) on the hamster cheek pouch (HCP) epithelium produced wider intercellular spaces, numerous shorter desmosomes, many thin tonofilament bundles, and increased basal laminin production and keratinization (Ashrafi *et al*, 1997). Alonge *et al* (2003) reported higher numbers of mitochondria (when compared with controls) in HCP epithelium exposed to ST for 24 months.

The continued concern over the contribution and role of ST in the development of oral hyperkeratosis and cancer has led to numerous efforts at discovering and determining the pharmacognostic and patho-biological mechanisms, that ST may make toward the development of oral hyperkeratosis and permeability barrier disruption. Therefore, the present research project was designed to investigate the effects of ST on the formation and distribution of Lb in oral epithelium of HCP epithelium. Transmission electron microscopy was used to qualitatively and quantitatively compare control and experimental HCP epithelial tissues exposed to ST for 24 consecutive months. Statistical methods were used to quantify the numbers of Lb and their distribution in the granular cell layers of both control and experimental hamster check pouch epithelial tissues.

Materials and methods

The archives of the experimental derivatives from the previous studies were utilized. Details of the experimental design were described by Ashrafi *et al* (1992) and Alonge *et al* (2003). Originally, 32 male Syrian golden hamsters, 12–14 weeks old, were used in the study. Two grams of commercial moist snuff (SKOAL) were placed into the cheek pouch of the 16 smokeless tobacco-treated (STT) (experimental) animals 5 days a week for 24 months. Sixteen negative control animals did not receive any snuff, but did receive mechanical stimulation of the pouch epithelium without delivery of snuff in the area of placement. These hamsters did receive oral mucosal cheek pouch tissue manipulation from the same glass syringe, without delivery of tobacco. After 24 months of experiment, animals were killed with ether inhalation. The cheek pouch epithelial tissues were obtained via standard oral surgical biopsy technique for histo-pathological analysis and processing for electron microscopy. The HCP epithelial tissues from six experimental and six control animals were selected for the present study. The tissues were fixed immediately in Karnosky fixative (Karnosky, 1965) after removal from the pouch. After formaldehyde–glutaraldehyde fixation they were postfixed in osmium tetroxide and embedded in Araldite. Sixty to seventy nanometer thin sections of tissue samples from different treated and control animals were obtained to demonstrate the basal, spinous, and granular cell layers of the HCP epithelium. The thin sections of tissue samples were mounted on copper grids and stained with uranyl acetate and lead citrate for electron microscopic examination. The tissue sections from six STT and six control animals were randomly selected for electron microscopic examination. A total of 60 experimental and 60 control oral epithelial tissue micrographs of different animals were used for the analysis of morphology and cell/volume density distribution of Lb.

Morphometric analysis of the Lb was done using stereological point counting methodology. A test lattice of 1 cm^2 with crossing points was superimposed over each micrograph. The number of crossing test points on lamellar bodies (P Lb) and the number of points falling on the top of the cells (P cell) were counted. The last granular layer immediately adjacent and closest to the keratin layer was designated as K-1. The next granular layer to K-1 was designated as K-2, etc. The granular layer furthest away from the keratin layer was called K-3. The mean of P Lb and P cell points was counted. Student *t*-test was used to find the significance of differences between the experimental and control groups. The differences were considered significant if P-value ≥ 0.05.

Results

The STT HCP epithelium showed hyperplastic and hyperkeratotic stratified squamous epithelium during light microscopic examination, as described previously (Ashrafi *et al*, 1992). The granular cell layers were more prominent in the experimental specimens than in the control groups. Qualitative differences were observed between the Lb of STT and control epithelial cell layers.

Distribution of lamellar bodies

The Lb were noticed in the spinous cell layers of controls, as well as in STT HCP epithelium. In the lower spinous cell layers, Lb were distributed in both the STT and in the control epithelium. In a few, variations in the internal structure of Lb were noticed. There appeared to be two types of Lb – structural variations consisting of ovoid lamellate forms and circular vesicular forms of different sizes (Fig. 1). The number of Lb was increased from lower to upper spinous layer and into the granular cell layers in both experimental and control groups. This increase was more evident in granular cells of the STT

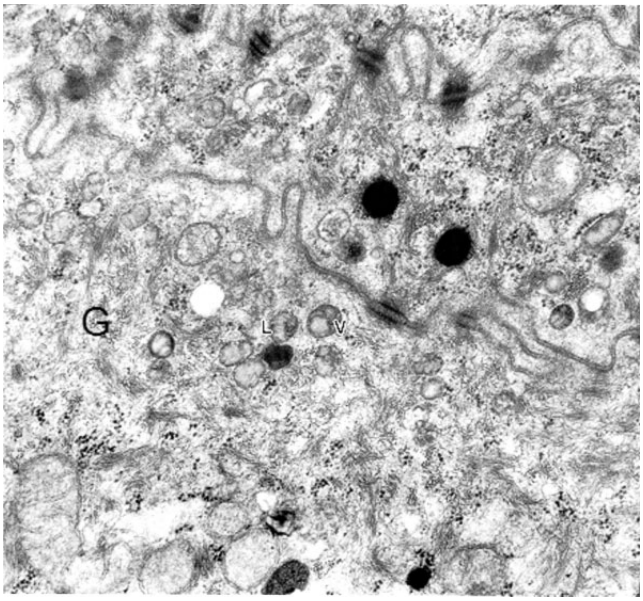

Figure 1 Transmission electron micrograph of control hamster cheek pouch epithelium showing structural variations of lamellar bodies (membrane coating granules) consisting of ovoid lamellate (L) forms and circular vesicular (V) forms divided into compartments with vesicle-like structure inside the limiting membrane. 47 500×

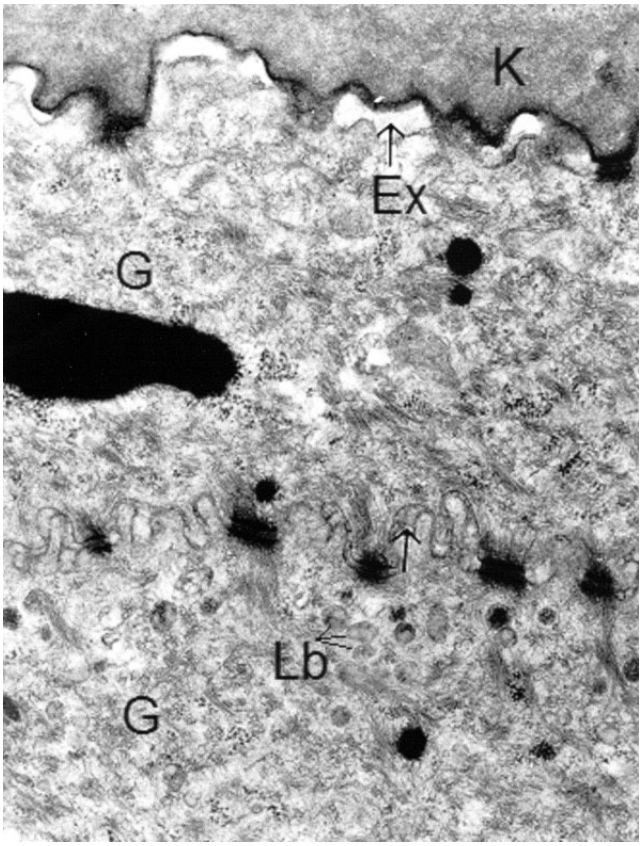

Figure 2 Control cheek pouch epithelium showing lamellar bodies (Lb) extruding (Ex) lamellar contents into the intercellular spaces (arrow) between strantum granulosum (G) cell layer and stratum corneum keratin (K) cell layer. 37 500×

specimens than in the control. In contrast to experimental samples, Lb in the control started aligning earlier and in parallel to upper plasma membranes of the granular cell layers as the cells started moving toward the keratinized region (Fig. 2).

In the control group, Lb started extruding their contents into the inter-cellular spaces of the granular layer and this process was completed at the junction of the last granular and first keratin cell layers. The majority of the Lb in the STT samples were noted to occupy the last granular cell layers. Some were extruding their contents into the intercellular spaces manifesting a patchy filling appearance (Figure 3).

Quantitative analysis of lamellar bodies

The structural alterations observed in the Lb were not statistically significant, therefore the quantitative analysis of all Lb was done by using the point counting method to calculate volume density in granular cells in both the control and STT specimens. The concentration means of Lb in the control granular cell layers K-1, K-2, and K-3 were 3.1, 4.0, and 1.3, respectively, and in STT cells K-1, K-2, and K-3 were 8.5, 7.4, and 6.3 respectively (Fig. 4). Therefore, the estimated maximum concentration of Lb was found in STT K-1 and the greatest difference between control and experimental specimens was also noted in K-1 granular cells (P value ≤ 0.0005).

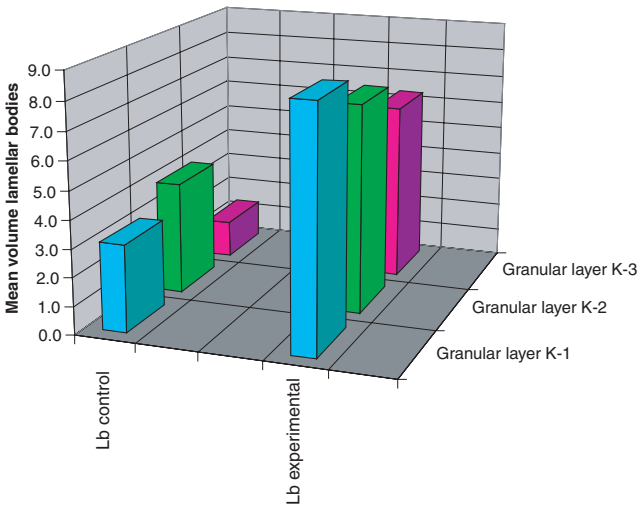

Figure 3 Graph y-axis showing mean volumes of lamellar bodies (Lb) within the three epithelial granular layers. Mean Lb volumes (Con = 3.1 and Exp = 8.5) within the granular layer K-1 are closest to the stratum corneum. Mean Lb volumes (con = 1.3 and Exp 6.3) within the granular layer K-3 are furthest from the stratum corneum

Discussion

The present study supported the earlier, qualitative observations reported by Ashrafi *et al* (1992) that chronic use of snuff induced oral leukoplakia in HCP epithelium. It also correlated previous studies demonstrating thickened keratin layers, as determined by Worawongvasu *et al* (1991). This study also confirmed

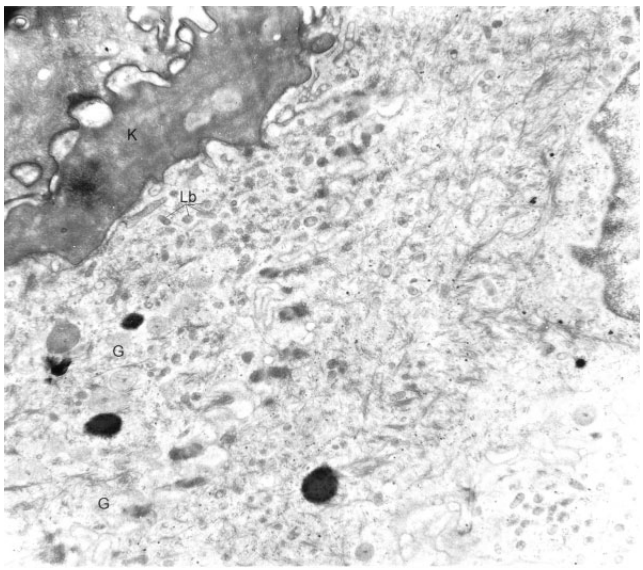

Figure 4 Smokeless tobacco-treated (STT) hamster cheek pouch epithelium showing majority of lamellar bodies (Lb) present in the last granular layer closest to the stratum corneum or K-1. 37 500×

the related findings by Hirsch and Thilander (1981) and Hirsch and Johansson (1983) demonstrating keratin layer thickening in snuff-induced lesions in rat oral epithelium, with changes similar to human oral leukoplakia as reported by Frithiof *et al* (1983), respectively. In conjunction with increased keratin production, wider intercellular spaces were observed in the STT epithelial samples (Kandarkar *et al*, 1991; Ashrafi *et al*, 1992; Grasso and Mann, 1998). The presentation of hyperkeratotic lesions in this study mimicked the hyperkeratotic leukoplakia lesions noted in humans (Frithiof *et al*, 1983; Ibrahim *et al*, 1998). Acanthosis of HCP epithelium treated with ST was also reported by Summerlin *et al* (1992).

The initial appearance of the membrane coating granules (or Lb) in the lower spinous cell layer of STT hamster cheek epithelium (HCE) supported the findings of Ashrafi *et al* (1992) in zinc-deficient rat cheek epithelium. The mixed population of Lb noticed in STT HCE was also observed by Ashrafi and Said-AL-Naief (1996) in zinc deficiency and by Braun *et al* (1990) in non-keratinized sulcular epithelium. Morphologically different lamellar granules in human epidermis were shown by using osmium tetroxide and ruthenium tetroxide fixed methods by Swartzendruber *et al* (1995). Ishida-Yamamoto *et al* (2004) provided evidence that different content markers are distributed in a separate population of LB molecules in a compartmentalized fashion. Immuno-electron microscopy studies regarding LB are difficult to interpret as there is no definitive marker for LB localization. Definitive LB localization is based on their internal membrane structure stained with osmium tetroxide or ruthenium tetroxide. Present EM study have shown two morphological different types of LB. One oblong type filled with lipid lamellae and the other containing small vesicles inside. We suggest that future investigations of LB should be directed to study the variety of lamellar-body associated antigens assembling the different cargos in compartmentalized fashion.

The accumulation of Lb in the last granular layers of STT HCE was more pronounced than majority of the LB seen in the control group. The majority of the Lb did not appear to extrude their contents into intercellular spaces in STT HCE as they generally do in control to form permeability (Ashrafi *et al*, 1980; Squier, 1991; Whittle *et al*, 1997). Nicotine in STT HCE may have inhibited Lb to extrude their lamellar lipid contents into the intercellular spaces between the granular cell layers and keratin cell layers. Similar observations were reported by Ashrafi and Said-AL-Naief (1996) during zinc deficiency in rat cheek epithelium, and Palungwachira *et al* (1992) in keratoderma hereditaria. Schmuth *et al* (2001) showed that permeability barrier abnormality in epidermolytic hyperkeratosis could be related to abnormal lamellar body secretion. Kelsell *et al* (2005) reported abnormal granule formation in harlequin ichthyosis (HI) epidermis and suggested that the lack of the ABCA 12 gene in granules may have caused epidermal barrier defect in HI.

Statistically, significantly increased differences were found in the number of Lb in all granular epithelial layers of STT epithelium compared with the non-treated control epithelium, K-1 $P < 0.0005$, K-2 $P < 0.0025$, and K-3 $P < 0.0025$. Other investigators have noted that the relative number of Lb was increased in several cutaneous disorders. Hashimoto and Lever (1966) noted that patients with psoriasis showed an abnormally large number of Lb.

The pH of commercially prepared ST is artificially elevated from pH 6.0 to the range of pH 7.0–8.4 during the processing of ST (Henningfield *et al*, 1995; Henningfield *et al*, 1997; Tomar and Henningfield, 1997). Brunnemann *et al* (2002) demonstrated that only 1% of nicotine is present as a free base when ST was of pH 5.84. When ST was of pH 7.99 the free base form of nicotine was 59%, or a 580-fold increase.

Chen *et al* (1999) demonstrated that significant nicotine absorption (by passive diffusion) occurred in the oral mucosa of pigs when exposed to ST products at pH 8.8. Significantly less nicotine absorption occurs with less than neutral pH ST. Thus, the permeability of nicotine varies as a function of pH modulation. The same group also found that neutral nicotine permeability was proportional to the occupied volume of membrane coating granule lamelli in the intercellular space of oral mucosa. Mauro *et al* (1998) reported an increased accumulation of Lb in hairless skin treated with neutral or alkaline pH buffer solutions. But the alkaline environment inhibited lipid secretion processing of Lb to form a permeability barrier at the junction of the stratum granulosum and stratum corneum interface. Similarly, in the present investigation, HCP epithelium treated with commercial ST with elevated (alkaline) pH may be preventing the complete extrusion of the contents of all Lb into intercellular spaces. This decrease in the lipid extrusion of Lb into the

intercellular spaces will form an incomplete permeability barrier. Thus, the presence of more Lb within the granular cells of STT epithelium, may be attributed to alkaline pH of ST, which may have inhibited Lb to extrude their contents into intercellular spaces. This may have also led to the continuous accumulation of epithelial cells, and the subsequent formation of hyperkeratosis in STT HCE (Brunnemann *et al*, 2002). Fluhr *et al* (2004) found that high pH created barrier abnormality in neonatal stratum corneum. This delayed recovery in barrier formation was due to incompletely extruded lipid membranes into intercellular spaces of stratum corneum.

It may be concluded that commercial alkaline pH of ST may have contributed to the abnormal accumulation of Lb in granular cells and disrupted the extrusion process of the Lb to form an incomplete permeability barrier in the oral epithelium. No evidence of oral mitotic activity related to carcinogenesis was observed in this study. Further research is needed to determine whether this response is an example of a true oral mucosal pathology or is an example of co-evolutionary adaptation of the oral mucosa to tobacco products.

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