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Experimental Oral Pathology

Mitochondrial volume densities in the smokeless tobacco-treated hamster cheek pouch epithelium

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OBJECTIVES: To compare the morphological changes and quantitative distribution of mitochondria in the hamster cheek pouch (HCP) epithelium treated with smokeless tobacco (ST).

MATERIALS AND METHODS: Archives of experimental material from previously published studies (Ashrafi et al., 1992) were utilized. Animals in experimental group received moist 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 microscopy study. Volume densities of mitochondria were assessed by morphometry.

MAIN OUTCOME MEASURES: Mitochondrial volume densities in the two groups, experimental *vs* control.

RESULTS: In both control and experimental groups mitochondria were concentrated between the nucleus and basal cell plasma membrane. A decrease in the mean mitochondrial volume density (Vvmit) was observed from the basal layer to the more superficial layers in both groups. The experimental HCP displayed more mitochondria than control, and the granular epithelial cell layer in experimental group showed significantly a higher mean Vvmit than the control group ($P = 0.03$). It was concluded that greater numbers of mitochondria were retained in ST-treated granular cells of the hyperplastic epithelia than in the normal epithelium.

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Keywords: hamster cheek pouch epithelium; smokeless tobacco; mitochondria; mitochondrial volume density; granular cells

Introduction

Earlier reports of decrease in the prevalence of cigarette smoking among US adults were associated with increased consumption of smokeless tobacco (Marcus *et al*, 1989; Rouse, 1989; USDHHS, 1990).The decline in smoking prevalence appears to have slowed during the late 1990s (CDC, 2001). Recently, however, was the report of increase in smoking by adolescents during the 1990s, as well as a decline in use of smokeless tobacco (ST) (Smith and Fiore, 1999), although ST use among white males, especially adolescents remains very high (Marcus *et al*, 1989; Epps, Manley and Glynn, 1995; Smith and Fiore, 1999). The resurgence in the use and appeal of ST coincided with new evidence regarding the ill-effects of tobacco use. The use of all forms of ST (chewing tobacco, snuff) by young males assume a major public health significance because of the association of ST with oral leukoplakia and oral cancer (USDHHS, 1986; Winn 1988), occurring at the site of placement of the snuff. Furthermore, oral mucosal inflammation has been reported in habitual ST users (Gao *et al*, 1996), while cytological studies of oral mucosal cells have reported hyperkeratosis and dysplasic changes (Goral *et al*, 1999). Similarly, decrease in cell size and increase in nuclear diameter has been documented in both dysplasia and oral cancer in Asian ST users (Ramesh *et al*, 1999). Worawongvasu *et al* (1991) showed that the daily application of snuff to hamster cheek pouch (HCP) epithelium for 6 months resulted in hyperplastic and hyperkeratotic conditions. Later Ashrafi *et al* (1992) showed that prolonged exposure (24 months) of HCP epithelium to snuff produced wider intercellular spaces, numerous shorter desmosomes, and many thin filament bundles. Lindal *et al* (1981) showed that mitochondria tend to be found where energy is most needed, a function that is closely related to the number and integrity of mitochondria (Ernster and Schatz, 1981). It has been shown that ST contains carcinogens that are activated by cytochrome p450 enzymes in mitochondria to trigger cellular changes (Bagchi, Bagchi and Stohs, 1996; Koide *et al*, 1999; Liu, Trimarchi and Keefe, 2000; Nair and Bartsch, 2001).

Therefore the aim of this investigation was to study the changes in volume densities and distribution of mitochondria in hyperplastic epithelium of HCP epithelium after 24 months treatment with ST. Understanding

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the volume densities distribution of mitochondria in pathological cells may reveal another approach to the detection of precancerous oral conditions.

Materials and methods

The details of experimental design and tissue processing procedure for electron microscopy have been described previously (Ashrafi *et al*, 1992). Briefly, 32 male Syrian golden hamsters 12–14 weeks old were used in this study; 24 animals were in experimental group, while eight were in control group. Two grams of commercial moist snuff (SKOAL) were placed into the right buccal pouch of experimental animals for 5 days week^{-1} for 24 months. The control group animals did not receive snuff in their pouches. After 24 months of the experimentation, the animals were killed with ether inhalation. The oral mucosa specimens from the buccal pouches of control and experimental animals were obtained and processed for routine electron microscopy. The tissue blocks of the specimens were embedded in Araldite, then trimmed, and thick sections of about 0.5–1 μM were cut to determine orientation of the specimens. Tissue blocks sectioned perpendicular to the epithelial surface were selected and thin sections of 60–70 nm, showing basal, spinous and granular cell layers were obtained. The three strata of epithelium were defined as follows: cells of the basal layer were those in direct contact with the basal lamina complex. Cells of the granular layer contained electron-dense keratohyalin granules. Cells between these two defined layers were considered to belong to the spinous layer. The sections were mounted on copper grids, and stained with uranyl acetate and lead citrate.

Sampling for transmission electron microscope
From the archives of previously reported experimental studies (Ashrafi *et al*, 1992), tissues belonging to five control and five experimental animals were randomly selected for this study. One block was then randomly selected from a total of five blocks per animal per group. Four micrographs per cell layer were obtained at ×9100 magnification and printed at ×22 000. The sections were examined with a Phillips 301A transmission electron microscope (Philips Electronic Instruments, Inc., Mount Vernon, NY) at 60 kV. A total of 120 micrographs, 60 experimental and 60 control oral epithelial micrographs were obtained for the analysis of volume density of mitochondria (Vvmit).

Morphometric analysis
Morphometric analysis of mitochondria was carried out using the method of stereological point counting. A coherent quadratic double test lattice composed of parallel lines separated by 1 cm running at right angles to each other was superimposed over each micrograph. The numbers of points overlying each mitochondrion (Pmit), and the numbers of points overlying each epithelial cytoplasm (Pcyt), were counted. Vvmit was estimated according to the relationship described by Weibel (1969): Vv = Pp.

Thus the mean Vvmit per epithelial cell layer, per group was determined from the pooled Ppmit. The Student's *t*-test was used to determine the significance of differences between the experimental and control groups. Differences were considered to be significant at $P = 0.05$.

Results

The gross examination of the ST-treated HCP epithelium for 24 months showed evidence of leukoplakia, while the control epithelium appeared normal. Light microscopy of the ST-treated pouch epithelium indicated hyperplastic and hyperkeratotic stratified squamous epithelium. The granular cell layers were more prominent in experimental specimen than in the control group. The connective tissue consisted of dense fibrous tissue and contained some inflammatory cells. Electron microscopic examination of ST-treated epithelium also showed hyperplastic and hyperkeratotic changes (Figure 1). Widened intercellular spaces, numerous shorter desmosomes, many thin filaments bundles and several keratohyalin granules were seen in experimental than in control epithelium. Qualitative and quantitative differences were observed between the mitochondria of control and ST-treated epithelial cell layers of the HCP.

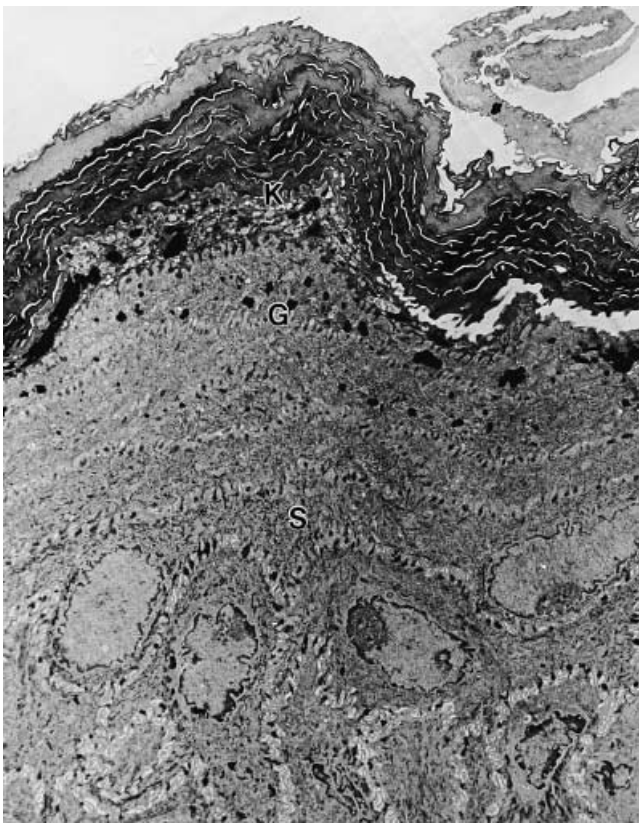

Figure 1 Transmission electron micrograph of snuff-treated hamster cheek pouch epithelium showing hyperplasia and hyperkeratosis. Spinous cells (S), granular cells (G) and hyperkeratinised cell (K). ×2800

Qualitative observation of mitochondria
Control group

Mitochondria were normal looking in basal cells. The outer membrane of the mitochondria was enclosing the inner membrane. The lamellar cristae were connected to the inner membrane and the mitochondrial matrix was translucent (Figure 2). Mitochondria were more concentrated in the proximal portion of the basal cell between nucleus and basal cell plasma membrane, and in the distal portion between nucleus and plasma membrane of adjacent cell. In the basal cell, the majority of mitochondria displayed circular shape while dumb-bell and J-shapes were occasionally seen. In the spinous layer, mitochondria looked similar to those of the basal layer, although the circular and rod shapes appeared to be more common in the spinous cells. The numbers of mitochondria were reduced in granular cell layers. Except for a few J-shaped, most of the mitochondria were circular in shape. In the upper granular cell layer, mitochondria showed signs of degeneration, devoid of cristae and matrix was translucent.

Snuff-treated group

The mitochondria in the basal layer cells of snuff-treated HCP epithelium were dispersed and densely distributed throughout the cytoplasm. The majority of mitochondria were elliptical in shape, and other shapes of mitochondria as seen in the control group were also observed. The spinous and granular layers showed circular, rod, J- and dumb-bell-shaped profiles of

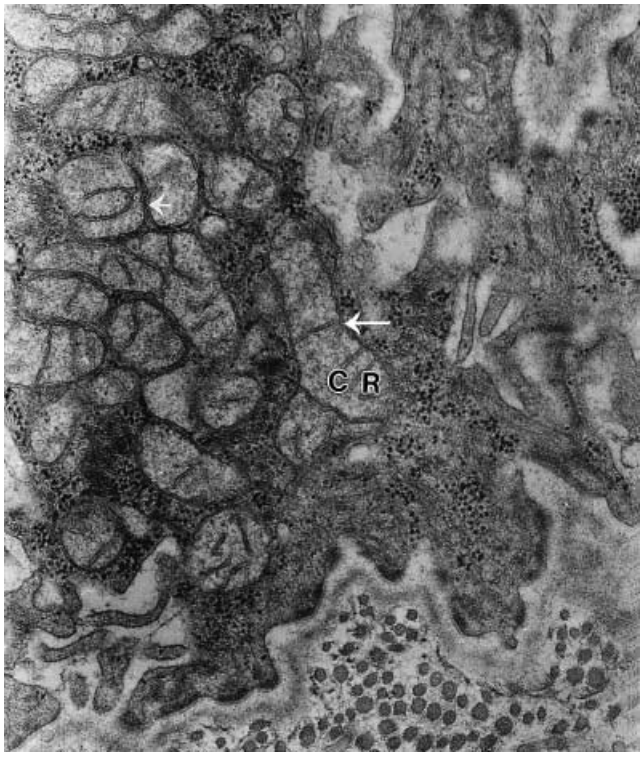

Figure 2 A portion of a control hamster cheek pouch epithelial basal cell showing mitochondrial outer membrane (arrow), inner membrane (arrow head) and electron lucent cristae (CR). ×22 500

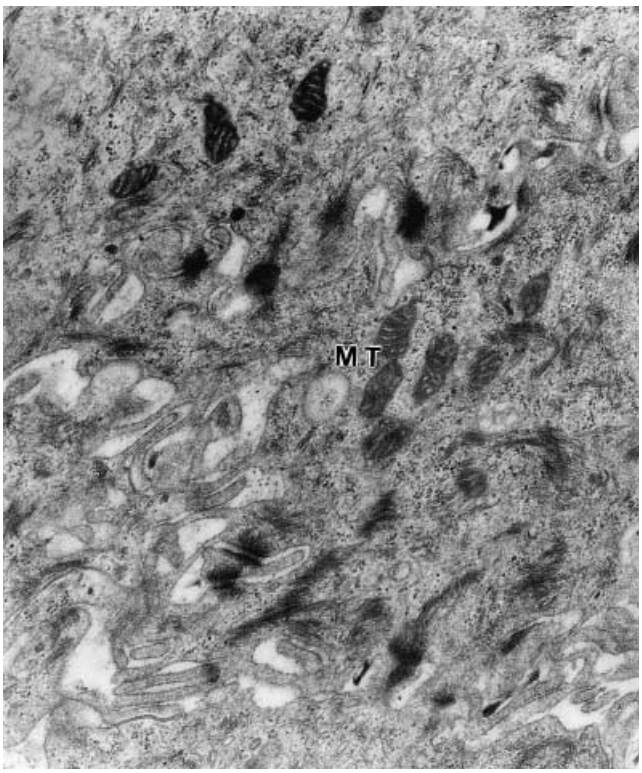

Figure 3 Snuff-treated spinous epithelial cells showing mitochondria (MT) with densely packed cristae. ×18 500

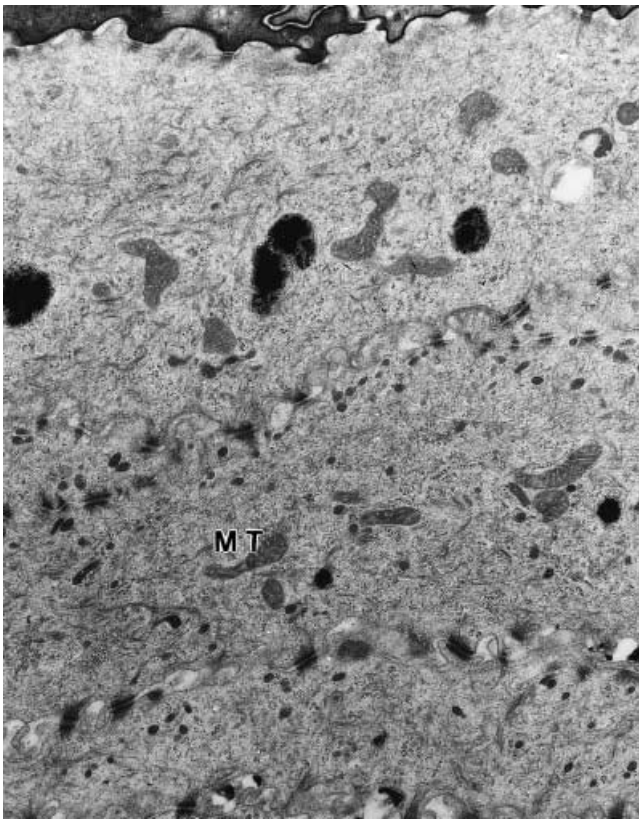

Figure 4 Snuff-treated granular epithelial cells showing mitochondria (MT) with electron dense cristae. ×18 500

Table 1 Comparison of mitochondrial volume densities in control and ST groups

	Mean mitochondria volume density (Vv) ± SD				
Layer	*Control group (C)*	*Snuff-treated group (E)*	*Percentage difference (C–E/C%)*	*t Statistic*	*P-value*
Basal	0.062 ± 0.017	0.061 ± 0.026	0.001/0.062 = 1.61%	0.1175	0.45
Spinous	0.041 ± 0.015	0.048 ± 0.020	0.007/0.041 = −17.07%	1.1888	0.12
Granular	0.029 ± 0.009	0.038 ± 0.019	0.009/0.029 = −31.03%	1.8999	0.03

Basal layer: $P > 0.05$; spinous layer: $P > 0.05$; Granular layer: $P = 0.03$.

mitochondria. The majority of mitochondria in upper spinous (Figure 3) and granular layer (Figure 4) cells did not show signs of degeneration as was noticed in control. The mitochondria contained densely packed cristae, with majority being lamellated. The number of mitochondria in spinous and granular cell layers appeared to be greater compared with the control group.

Quantitative analysis of mitochondria

Using morphometric analysis, a decrease in the mean Vvmit was seen with upward progression from the basal layer to the more superficial layers in both control and experimental groups (Table 1).

In the control group, the basal cells had the highest mean mitochondrial density (0.062), while the granular cells had the smallest mean Vvmit, at 0.029 (Table 1).

In the snuff-treated group, the highest mean mitochondrial density of 0.061 was recorded among the basal cells, while the granular cells had lower mean mitochondrial volume density of 0.038, but higher than in the control group (Table 1).

There were no statistical significant differences in Vvmit between control and experimental groups except in the granular layer ($P = 0.03$) (Table 1).

Discussion

Hamster cheek pouch mucosa has been used extensively as an experimental model (Salley 1954; Morris 1961; Chen, Johnson and Squier, 1994; Koide *et al*, 1999; Shklar 1999). Worawongvasu *et al* (1991) and Ashrafi *et al* (1992) also used this model to investigate the effect of snuff on the epithelium at light and electron microscopic levels. Earlier observations suggested that the HCP epithelium was suitable to investigate qualitative and quantitative distribution of mitochondria in snuff-treated epithelium.

The density gradient of mitochondria decreased successively from basal to granular cell layers in both snuff-treated and control epithelium (Table 1). Similar volume density decrease of mitochondria was reported from basal to granular layers in the normal human oral gingival epithelium (Schroeder and Munzel-Pedrazolli, 1970), and buccal mucosa (Landay and Schroeder, 1977). Decreasing mitochondrial volume density gradient from basal to granular layer in pathological conditions has also been described in the HCP epithelium (White and Gohari, 1983). As far as we know this study was the longest experimental period (24 months) to expose the HCP epithelium to ST. This produced hyperplastic and hyperkeratotic changes reported earlier, but did not show any cancerous lesions in the epithelium. A higher mean mitochondrial volume density in the granular cell layer is reported here.

Smokeless tobacco is reported to contain high levels of carcinogens notably tobacco specific nitrosamines (TSNs) (USDHHS, 1986; Mattson and Winn, 1989; Hecht 1996 and 1997). Cytochrome p450 (CYP) related enzymes are known to be involved in metabolic activation of tobacco carcinogens (Koide *et al* 1999; Nair and Bartsch, 2001). TSNAs in ST are converted to DNA reactive metabolites by CYP isoforms enzymes which accumulate preferentially in mitochondria (Hecht 1996; Bear and Teel, 2000; Bartsch *et al*, 2000; Scully, Field and Tanzawa, 2000; Nair and Bartsch, 2001). The conversion of carcinogens by CYP to DNA reactive metabolites might cause an increase in reactive oxygen species (ROS). The increased ROS could lead to a significant decrease in mitochondrial respiration and oxidative phosphorylation (Bagchi *et al*, 1996). Increase in ROS is associated with mitochondrial membrane permeability (Castedo *et al*, 2002).

Changes in mitochondrial membrane function has been identified as an essential stage in apoptosis or programmed cell death (PCD). Furthermore, ROS has been reported as a mediator in PCD (Petit, 2001). Mitochondria have been implicated in both PCD and necrosis. PCD is a regulated mechanism that is responsible for elimination of aged, damaged or useless cells from the tissues. In several pathological conditions such as in cancer, there is a resistance to PCD by the cancerous cells (Petit, 2001). Identifying changes in mitochondria could be used to detect apoptosis at an early stage (Petit, 2001; Castedo *et al*, 2002).

The cause of mitochondrial proliferation in ST-treated HCP epithelium is currently unknown. Although it is difficult to correlate morphological observation to functional changes, mitochondrial proliferation in ST-treated epithelial cells may be to compensate for a decline in ATP level. The difference in mitochondrial volume density between control and ST groups may therefore indicate differences in aerobic respiration especially in granular cell layers, and the presence of excessive number of mitochondria in the granular cells may be to compensate for the reduced metabolic function in hyperplastic lesions.

The limitation of the study was that the model did not include sham treatment of the control pouch. Addition-

ally, limitations of stereological technique to measure volume density from two dimensional images include (1) mitochondria of similar shape could produce heterogenous profiles or shapes when sectioned and (2) mitochondria have complicated shapes, sectioning may result in erroneous shapes. (3) Only average estimates are produced, and (4) if points are too close together, one may be easily ignored unlike if points are distant from each other.

The present study is an exploratory investigation. Further additional research is needed in quantifying and correlating mitochondrial morphological and biochemical changes in pathological conditions, especially in neoplasia. It was concluded that ST treatment of HCP epithelium for 24 months produced hyperplastic and hyperkeratotic condition and the number of mitochondria was increased in the granular cells.

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