

Ultra structural study of the rat cheek epithelium treated with Neem extract

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Abstract: The purpose of this study was to investigate the effects of neem extract (*Azadirachta indica* A. Juss) on the ultrastructure of the rat oral epithelium, because neem extract has been added in the tooth paste as an anti-plaque-forming substance in Asian countries. The non-toxic dose of 2000 mg/kg body weight of Neem extract (NBE) was applied daily to the surface of buccal epithelium for four weeks and controls did not receive Neem extract. After four weeks cheek epithelial tissues were excised and processed for light microscopy, scanning and transmission electron microscopy. Light microscopy did not show significant differences between NBE-treated and control epithelium. Difference between control and treated rats weight was non-significant. Moreover, time period was also non-significant. Irregular cell surfaces were noticed when compared to control specimens when examined by scanning electron microscopy. Under transmission electron microscopy, wider intercellular spaces were observed in the treated epithelial spinous cellular layers when compared to control. Further, more keratohyalin granules were present in experimental granular cells. It was concluded that present study showed differences between Neem-treated and control in epithelial tissues but these structural differences may not be related to adverse side effects of the Neem extract.

Keywords: Rat, cheek epithelium, neem extract (NBE), light, scanning, transmission microscopy.

INTRODUCTION

The widespread use of the Neem “chewing stick” as an oral hygiene device in certain Asian and African countries has raised the question that it may possess some anti-plaque properties. Dentists in areas where it is in use have noted its effectiveness in preventing certain plaque-related dental diseases (Elvin-Lewis, 1980). From the data presented, it is apparent that the Neem plant contains components, which can inhibit some oral *Streptococci*, which influence plaque formation (Wolinsky *et al.*, 1996). Neem extracts have generally been found safe for mammals and the environment and no toxicity was observed in albino rats and mice fed with Nimbidin (Neem extract) at doses up to 2000 mg/kg body weight (Pillai and Santhakumari, 1984a,b). Ding *et al.*, (2014) reported that human cell culture and HCP data showed that phytochemicals extracted from avocado exhibit anticancer activity by inhibiting cancer cell proliferation and progression. However, substances extracted from the Neem tree have been added into a commercial dentifrice in India known as Neem toothpaste, and into tooth powders in Pakistan called Neemodent (Elvin-Lewis, 1980). Based upon their wide occurrence and their potential application as antiplaque agents, this study was undertaken to investigate the effects of *Azadirachta indica* (Neem) berries extract on the ultrastructure of the oral epithelium of Sprague Dawley rats using 2000 mg per kg as this dose was found to be non-toxic to rats (Pillai and Santhakumari, 1984a,b).

MATERIALS AND METHODS

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Chemical

Crude extract (NBE) of air-dried ripe berries kernel of Neem *Azadirachta indica* A. Juss was obtained from HEJ Research Institute of Chemistry, University of Karachi. The procedure of extraction was as follows. Seeds of Neem (*Azadirachta* A. Juss) were separated from the fruits manually. The seeds after removing the hard covering were soaked in Ethanol and extracted repeatedly (x5) at room temperature. The extract solvent was removed under reduced pressure Tariq *et al.*, (2001).

Animal

Male white Sprague Dawley rats of approximately 200-225 grams weight were obtained from Harlan laboratories, (Indianapolis, Indiana) and they were housed two per cage and given dry pellets rat food (Harlan Tekland, Madison, Wisconsin) and water ad libitum. There were four replicates for each time period (weight) and the same number for control.

A Neem extract, (NBE), dose of 0.25ml approximately 2000 mg/kg body weight was applied on the surface of right and the left buccal epithelium of the experimental animals once a day for 4 weeks, and nothing was placed into the buccal cheeks of the control.

Tissue preparation

At the end of the experiment, the animals were killed by ether inhalation. A 5 to 8 mm² piece of cheek oral mucosa was excised from each animal. The tissue was further divided into several small pieces, which were fixed into 2% glutaraldehyde in 0.1M phosphate buffer pH 7.2. The specimens were processed for examination by light,

transmission and scanning electron microscopy. For transmission electron microscopy (TEM) studies, the tissues were further trimmed into blocks of approximately 1mm³ and fixed again in 2% glutaraldehyde prepared in 0.1 M phosphate buffer (pH 7.2) at 4°C for 2 hours. The fixed tissues were washed in 0.1M-phosphate buffer overnight and postfixed in 1% osmium tetroxide for 2 hours. The specimens were then dehydrated in ascending series of ethyl alcohol and propylene oxide. Tissues were embedded in Araldite. Ultra-thin sections 65-70nm were cut, mounted on copper grids, stained with uranyl acetate and lead citrate. The stained sections were examined with a Philips 301A transmission electron microscope at 60 kV for observing ultra structural features. The 0.5um thick Araldite embedded sections of tissue were used for light microscopic examination and for orientation to select the areas for TEM examination.

For the scanning electron microscope (SEM) studies, tissues were fixed for 2 hours in 2% glutaraldehyde made in 0.1M phosphate buffer (pH7.2) at 4°C and then transferred to 0.1M phosphate buffer for washing overnight. Subsequently, the specimens were dehydrated in ascending grades of ethanol and dried using a Denton critical point drying system (Denton Vacuum, Inc., Moorestown, New Jersey) tissue specimens were then mounted on aluminum stubs, coated with gold-palladium and surface topography was examined using a Joel 35C SEM operated at 18kV with a tilt angle of 39 degrees. One-way ANOVA was performed by complete randomized design (table 1).

Table 1: ANOVA table for control and treated rats (weight)

Source	SS	Df	MS	F	p
Time	46.75	4	11.91	0.096	0.980
Treatment	12.1	1	12.10	0.097	0.756
Interaction	10.65	4	2.66	0.021	0.999
Error	3713.5	30	123.78	-	-
Total	3783.9	39	-	-	-

LSD_(time) = 11.36 LSD_(treatment) = 7.185

RESULTS

Gross observations

After 4 weeks treatment with Neem extract, the rat cheek mucosa appeared normal and there were no differences between the experimental and control specimens. The experimental rats showed no weight loss and the increase in weight was normal.

Light microscopic observations

Control

The cheek mucosa consisted of a uniform thick ortho-keratinized stratified squamous epithelium (fig. 1a). The keratin layer consisted of non-nucleated cells. Granular

cell layers were prominent and contained keratohyalin granules. No dysplastic changes of the epithelium were observed. The interface between the basal cell layer and the lamina propria displaced broad based rete ridges.

Experimental

The cheek mucosa consisted of an ortho-keratinized stratified squamous epithelium (fig. 1b). The keratin layer showed non-nucleated cells with distinguishable cell boundaries. The granular cells were more prominent than those of the control group and also contained numerous deeply basophilic keratohyalin granules. No dysplastic changes were observed.



Fig. 1a: Photomicrograph from control rat showing nucleated (NE) and keratinized (KE) epithelium. X320

Few mitotic figs. were seen in the basal cell layer. A mild inflammatory cell reaction was noticed in the tissue region. Few macrophages components were aligned near the basal lamina in the connective tissue region.

Scanning electron microscopic observations

The control cheek mucosa at low magnification showed a smooth surface with folds. The individual cell boundaries

were distinct (fig. 2a). A few desquamating cells were seen. The cell outlines were polygonal and the surface displayed a mosaic-like pattern.

The experimental cheek mucosa at low magnification showed an irregular surface as compared to the controls (fig. 2b). More desquamating cells than control specimens were seen. The margins of the individual cells were clearly defined.

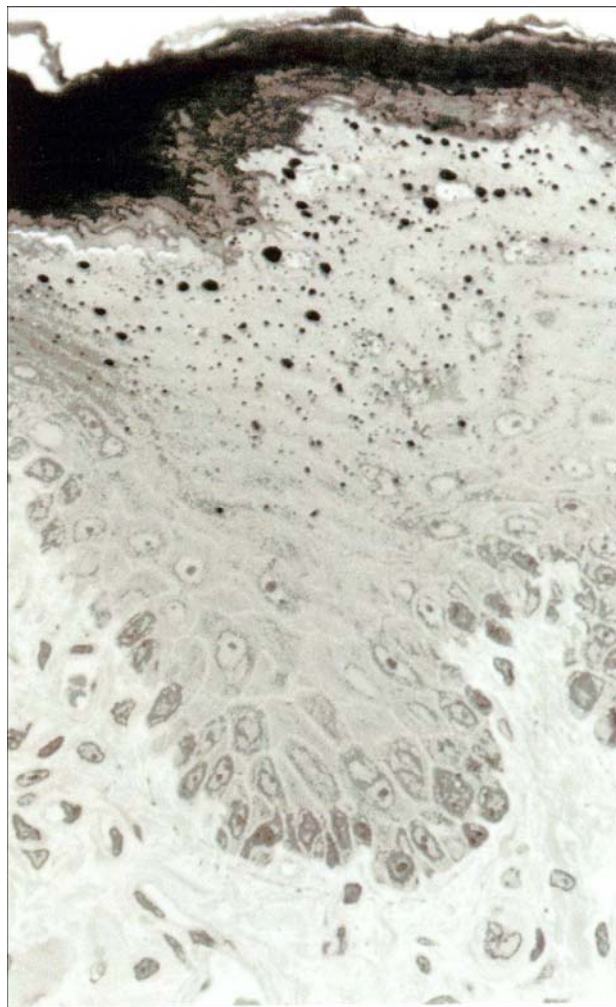


Fig. 1b: Photomicrograph from Neem extract treated rat illustrating buccal mucosa, showing increase in thickness of (NE) and (KE). Keratohyalin granules (KHG) are more prominent. X320

Transmission electron microscopic observations

Control group

The stratified squamous epithelium of the rat cheek epithelium consisted of basal cell, spinous cell, granular cell and keratinized cell layers. The basal cells were bounded by a continuous basal lamina zone. Distinct lamina lucida and lamina densa were parallel to the plasma membrane of the basal cell and separated the underlying connective tissue from basal cells. The basal

cell nuclei showed aggregates of prominent heterochromatin associated with the nucleolus (fig. 3). Few profiles of rough endoplasmic reticulum were seen and ribosomes were distributed throughout the cytoplasm. Mitochondria and thick bundles of tonofilaments were seen throughout the cell. The adjacent basal cells were separated by narrow intercellular spaces. The spinous cell layer consisted of several layers. Heterochromatin in the spinous cell nuclei was not prominent, and tonofilament bundles were shorter. Intercellular spaces were narrower like in the basal cell layer (fig. 4). The granular cell spanned 2-3 layers. The cells had elongated nuclei, few mitochondria and several membrane-coating granules. Elongated or circular keratohyalin granules (KHGs) were present. Shorter tonofilament bundles and few desmosomes were observed as compared with spinous cell layer. The ortho-keratinized epithelial cell layer was electron dense.



Fig. 2a: A scanning electron micrograph of the surface of rat buccal mucosa in 4week control. The surface is thrown into folds. There are a few scattered desquamating epithelial cells (arrows). The individual cell boundaries are distinct. X150

Experimental group

The cheek epithelium of the NBE-treated showed wider intercellular spaces than controls. Compared with controls, the basal cells showed few dilated rough endoplasmic reticulum, shorter desmosomes with narrow attachment plates. The tonofilament bundles were thin. The nuclei displayed a mix of heterochromatin and euchromatin. The widened intercellular spaces were filled with micro villous projections from the plasma membranes. Membranous processes of the basal cells projected into the underlying connective tissue zone. The spinous cells showed widened intercellular spaces and thin tonofilament bundles attached to desmosomes (fig. 5). The granular cells of the treated epithelium showed more keratohyalin granules (KHGs) than the controls. The early ortho-keratinized cell layers were less electron dense as compared to the superficial layers. Some of the cells in the early layers still contained cytoplasmic organelles, especially KHGs and degenerated nuclei (fig. 6).

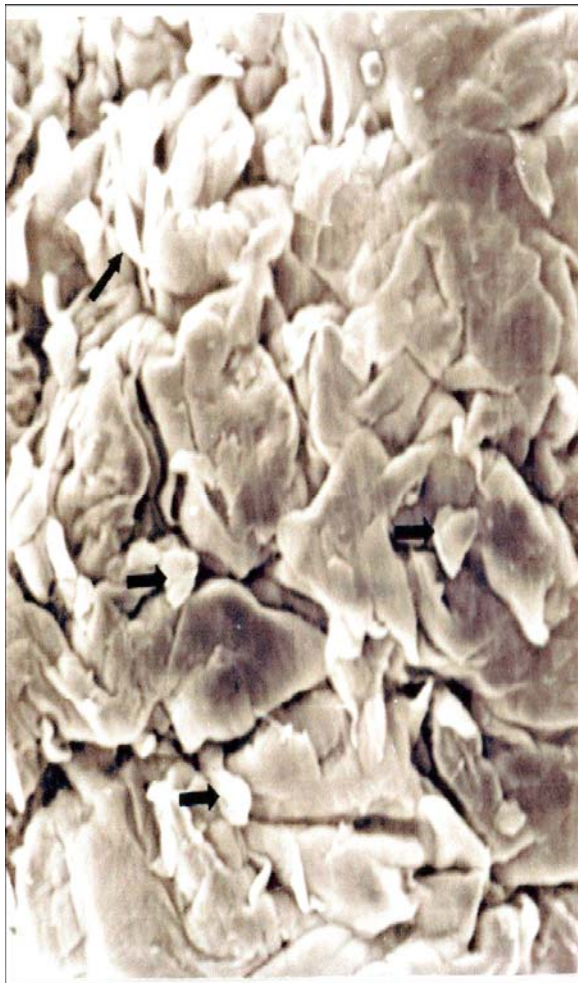


Fig. 2b: A scanning electron micrograph of the surface of rat buccal mucosa in 4 week at low magnification. The surface of the epithelium is more irregular than in the control specimens. Several desquamating cells (arrow) are also seen. X150

Control and treatment weights were found to be non-significant ($F=0.097$, $P=0.756$). Time was also found to be non-significant ($F=0.096$, $P=0.98$) (table 1).

DISCUSSION

The cheek epithelium showed no difference in the number of mitotic fig. between the animals treated with Neem Extract (NBE) and the controls. The mitotic cells were randomly distributed throughout the germinative compartment. They were not seen to occur in clusters, as described by (Harald *et al.*, 1972). In the present study, the use of 2000 mg/kg dose for four weeks showed few ultrastructural changes in the cheek epithelium. The number of basal cell processes present in treated epithelium extending into the connective tissue was not seen in control. Intercellular spaces in the spinous layer were widened in treated epithelium as compared with control. This finding is similar to that of hamster cheek pouch epithelium developed in response to carcinogens (Hassanin and Ashrafi, 1988). Ashrafi *et al.*, 1992 also reported an increased of basal cell processes in snuff-treated oral epithelium. The early keratinized cells were less electron dense, which could be a manifestation of altered keratinization process.

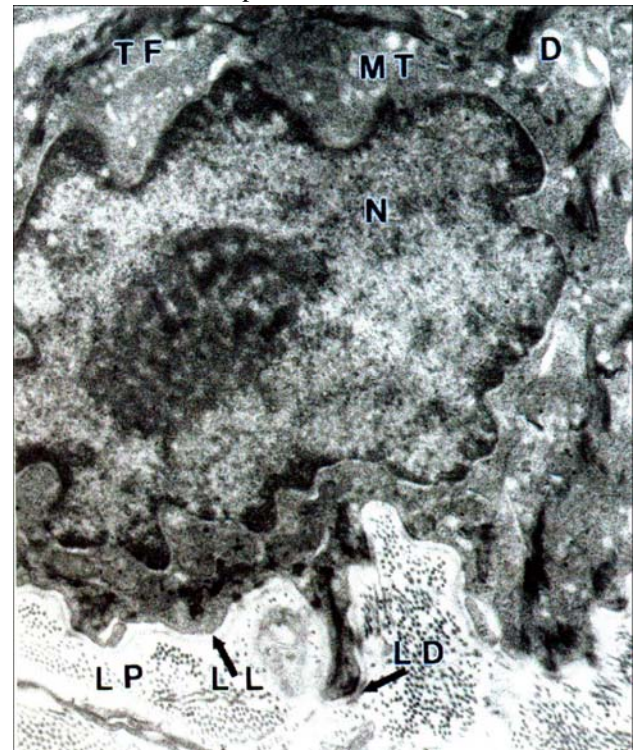


Fig. 3: Transmission electron micrograph of a control rat stratified squamous epithelium showing a basal cell. The basal lamina region shows hemidesmosomes, lamina densa (LD) and the lamina lucida (LL). Bundles of collagen fibrils are present in the lamina propria (LP). Ribosomes, mitochondria (MT) and tonofilament bundles (TF), large nucleus (N) with heterochromatin and desmosomes (D) are present in the basal cell. X7, 100

Smooth surface areas without micro ridges on the cell surfaces were not noticed in NBE-treated epithelium in comparison to the control. Similar changes in snuff-treated hamster cheek pouch epithelium were also reported by Ashrafi *et al.* (1992). Moreover, Nagini (2009) suggested that squamous cells carcinomas (SCCs) induced by 7, 12 dimethylbenz [a] anthracene (DMBA) in the Hamster buccal pouch (HBP) reiterate many of the features observed in human OSCC's. like human oral carcinogenesis, HBP carcinogenesis is a multistep process that involves sequential progression from hyperplasia to invasive carcinoma through varying degree of dysplasia.

The present study confirmed specific differences between the normal and NBE-treated rat cheek epithelium. Although (Pillai and Santhakumari, 1984), reported no toxic effect in albino rat at a dose of 2000 mg/kg body weight. Yet, the present observations using the same dose showed some structural differences between the experimental and controls in oral epithelium at the electron microscopic level. This study does not provide for inferences that these structural differences may be related to adverse effects of neem extract (NBE). Accordingly further dose-related studies are needed to provide a sound biological rationale for the choice of a safe dose of NBE that can be added to toothpaste.

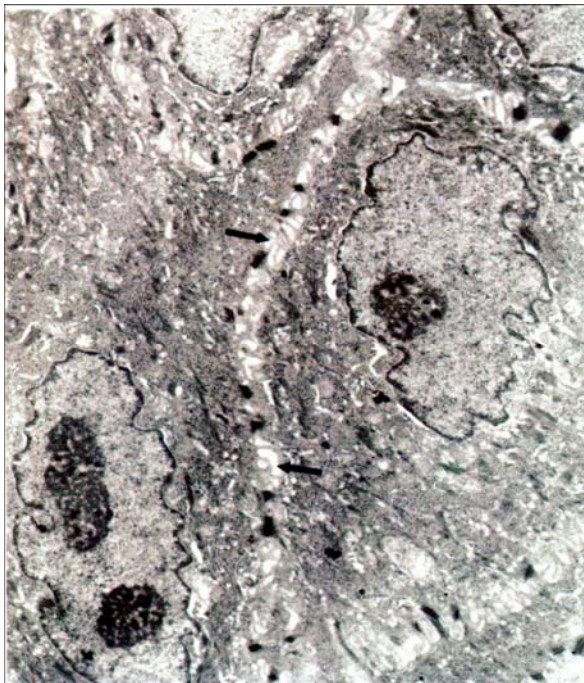


Fig. 4: Electron micrograph from control animal epithelium showing spinous cells with narrow intercellular spaces (arrow). X4, 500

The present study also agreed with (Balasenthi *et al.*, 1999; Subapriya *et al.*, 2004) and (Subapriya and Nagini, 2005) who reported that neem extract suppressed buccal pouch carcinogenesis.

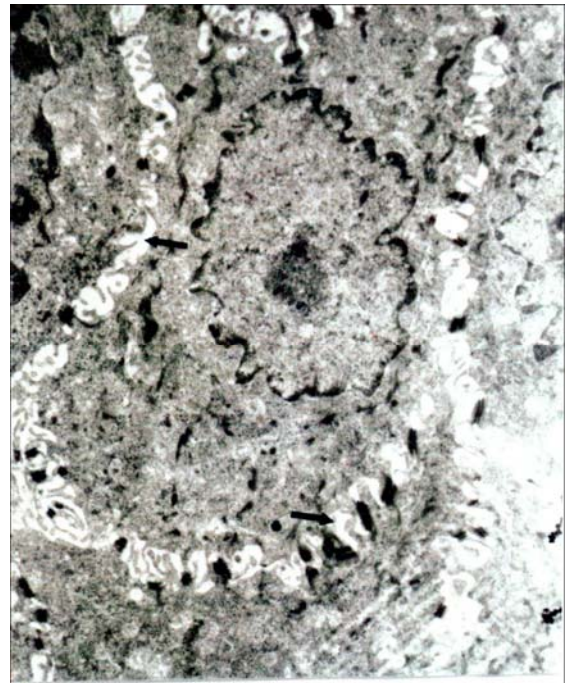


Fig. 5: Electron micrograph from experimental animal epithelium showing spinous cells with broader intercellular spaces (arrow). X4, 500

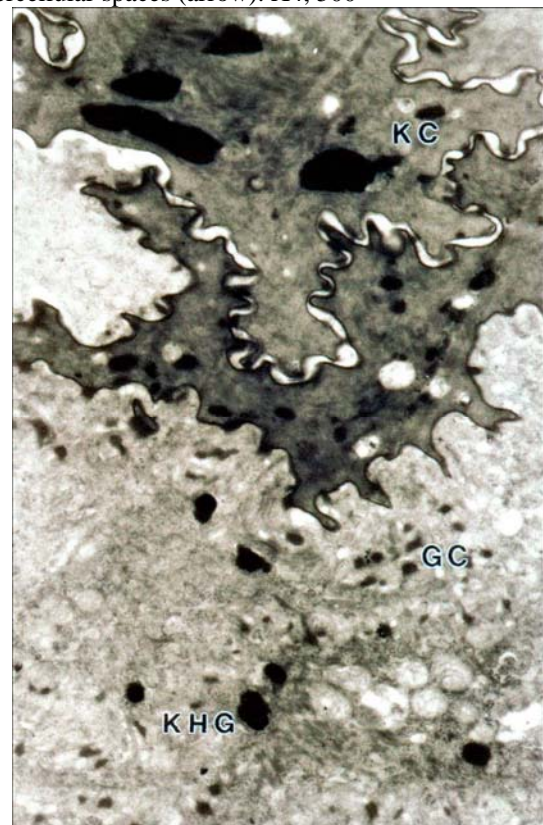


Fig. 6: Electron micrograph from experimental animal epithelium showing granular cell (GC) and keratinized cells (KC). Note presence of prominent number of keratohylin granules (KHG) in GC and degenerating KHG in KC. X7, 100.

ACKNOWLEDGEMENTS

We are indebted to Dr. Aidan Stephens, Acting Head, Department of Oral Medicine and Diagnostic Sciences and Dr. Robert Scapino, Acting Head, Department of Oral Biology for providing the Departmental facilities during the progress of the study. We would like to thank Isabel Stoncius for printing the illustrations and Amir-Robert Tarik for typing the manuscript. This study was supported in part by the Council for International Exchange of Scholars, Washington, DC, under the Fulbright Scholar Program for M. Arshad Azmi.

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