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The Effect of Vitamin C on the Hamster Cheek Pouch Treated With the Water Soluble Carcinogen 4-nitroquinoline-1-oxide (4NQO)

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Vitamin C is an essential nutrient whose protective influence in carcinogenesis has been reported frequently, suggesting that vitamin C inhibits the formation of some carcinogens and decreases the incidence and delays the neoplastic lesions. However, the mechanisms by which this occurs are unknown. In this study, the water soluble carcinogen 4-nitroquinoline-1-oxide (4NQO) has been used to induce a high yield of tumours in the oral cavity either singly or in combination with tobacco. Since the mucosa of rats is less susceptible to carcinogens than the hamster cheek pouch, the hamster cheek pouch has been used to study the influence of vitamin C on 4NQO-induced oral malignancy. The aim of this study was to determine whether topically applied vitamin C had an effect on the oral carcinogenesis induced by application of 4NQO. Similarly, an attempt was made to study the modulating effect of vitamin C on the histopathological and ultrastructural changes during the neoplastic process in the hamster. Vitamin C appeared to delay tumour induction and had other protective effects against neoplasia. Copyright © 1996 Elsevier Science Ltd

Keywords: vitamin C, 4NQO (water soluble), tobacco hamster cheek pouch, experimental carcinomas

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INTRODUCTION

The tumour induction in the oral cavity of various species by environmental and chemical carcinogens has been frequently reported [1–6]. The high incidence of oral precancer and cancer in the Indian subcontinent and South-East Asia has been stressed very often and has been attributed to the chewing of tobacco either by itself or as a part of the betel quid. The use of tobacco has been cited as a cause of oral cancer [1, 2, 7, 8]. Numerous vitamins and their analogues have been shown to be capable of reversing epithelial lesions induced by carcinogens [1, 2, 5, 6, 9]. Vitamin C is an antioxidant, enhancing immunity and is claimed, thereby inhibiting certain types of tumours. The protective role of vitamin C in carcinogenesis is controversial, although, epidemiological data show that consuming a large amount of vitamin C is associated with a lower risk of certain cancers. However, the use of vitamin C in immunotherapy and in combination with chemotherapy in malignancies needs much more study in order to evaluate its potential significance.

In 1973, Wallenius and Lekholm described the induction of squamous cell carcinomas of the palatal mucosa of rats by the repeated application of 4-nitroquinoline-1-oxide (4NQO). Later the carcinogenic potential of 4NQO was demonstrated in a number of investigations in mice, rats and hamsters [4, 10, 11].

The present investigation was designed to determine the effect of vitamin C applied topically to the hamster cheek pouch treated with 4NQO and tobacco extract, singly and in combination.

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MATERIALS AND METHODS

Test substances

Tobacco (Nicotina tabacum). The tobacco used is the "Jada Jarda" grown in Gujarat and sold commercially in a powder form. The finely powdered tobacco was subjected to an aqueous extraction using the procedure of Guttenplan [12]. Phosphate buffer (pH 7.4) (10 mM/g of tobacco) was added and extraction was carried out at room temperature (30°C for 24 h). The extracted material was centrifuged at 18,000 rpm for 15 min and the supernatant lyophilised to dryness. The lyophilised extract was solubilised in propane-1-2-diol (PD) to a final concentration of 0.42 mg of original tobacco.

4-nitroquinoline-N-oxide (4NQO). 4NQO and vitamin C (ascorbic acid) were obtained from Sigma Chemical Co. (St Louis, Missouri, U.S.A.) and stored in sealed containers at 20°C. A fresh solution of 0.5% w/v concentration in PD was stored at 5°C as was a 50 mg/ml solution of vitamin C in

deionised water. Five hundred micrograms of vitamin C were applied to the individual cheek pouch and fresh solution was prepared each time.

Fifty-six inbred Golden Syrian hamsters (*Mesocricetus auratus*) at an average weight of 40–50 g at the start of the experiment were used in this study. (The design of the experiment and histopathological changes are shown in Table 1.) The animals were divided into control and experimental groups. Control groups: I, untreated control; II, solvent (PD) control; III, vitamin C (0.25 mg) control; IV, tobacco control (0.42 mg). Experimental groups: V, 4NQO (0.1 mg); VI, tobacco (0.42 mg) + vitamin C (0.25 mg); VII, 4NQO (0.1 mg) + vitamin C (0.25 mg); VIII, 4NQO (0.1 mg) + tobacco (0.42 mg); IX, 4NQO (0.1 mg) + tobacco (0.42 mg) + vitamin C (0.25 mg).

Animals were kept on standard laboratory diet and water *ad libitum*. The cheek pouches were initially cleaned with cotton swabs and the test substances were topically applied with a Bioscience push button pipette inside the cheek pouch, three times a week until the tumours were induced. The cheek pouch was held with forceps for about 2 min after application to ensure that the test substances were not lost, but were absorbed into the cheek pouch epithelium. Experimental animals along with respective controls were sacrificed when the tumour reached approximately 1 cm in size. The pouches were examined visually, excised and cut into two portions for histopathological and ultrastructural observations. Histopathological observations were carried out on 6 μm thick paraffin embedded sections, stained with haematoxylin and eosin. Ultrastructural observations were done on 600–800 Å thin araldite embedded sections of glutaraldehyde–osmium tetroxide fixed tissue, double stained with uranyal acetate and lead citrate. Sections were scanned under Zeiss EM-109 electron microscope.

RESULTS

Macroscopic observations

All animals appeared to be in good health, as evidenced by subjective assessment of their physical mobility and a good fur coat in lustre until the end of the experiment, when the 4NQO treatment animals tended to be lighter in body weight than the controls. The animals treated with tobacco and tobacco + vitamin C showed altered cheek pouches to a lesser degree than those animals treated with 4 NQO plus test substances. By 12 or 16 weeks most of the animals of groups V, VII, VIII and IX showed thickened, rough and inflamed white reddish cheek pouches with small nodules further developed into papillomas or tumours (~ 1 cm) at 24–26 weeks. A significant delay in the tumour induction and reduction in the size and numbers of papillomas and tumours were observed in the animals of groups IV, VI, VII and IX exposed to tobacco, 4NQO singly and in combination with vitamin C. The summary of the papillomas/tumour development is shown in Table 2.

Histopathological observations

Table 1 summarises the histopathological observations of control and experimental animals. Control animals showed mild hyperplasia with hyperorthokeratosis (Fig. 1) and parakeratosis in a few animals. Tobacco and tobacco + vitamin C treated animals showed dysplasia, inflammatory exudate and lymphocytic proliferation in a few animals, while in the animals of the experimental groups VI, VIII and IX, there was marked to massive dysplasia with inflammatory exudate and lymphocytic proliferation (Fig. 2). In the papillary epidermoid carcinoma there were dysplastic changes, mainly altered nuclear cytoplasmic ratio, nuclear pleomorphism/hypertrophy, lack of stratification of spinous cells; basal cell hyperplasia, loss of polarity of basal cells (Fig. 3), numerous mitotic figures, etc. Tumour bearing animals showed well differentiated squamous cell carcinomas with severe dysplastic changes (Fig. 4) and a number of pearls of keratinisation (Fig. 5) which were minimal in the animals treated with 4NQO + vitamin C and 4NQO + tobacco + vitamin C (Fig. 6).

Ultrastructural observations

The basal lamina of the animals of all control groups I, II, III and IV was well defined, intact and continuous, with an unbroken basal lamina studded with numerous hemidesmosomes (Fig. 7). Epithelial cells of all strata were well preserved and healthy with well developed intact desmosomes. Upper spinous cells showed an increase in tonofilaments, pleomorphic keratohyaline and membrane coating granules (Fig. 8). In tumour bearing experimental animals of groups V, VII, VIII and IX, the basal lamina is discontinuous, fragmented, diffused and broken with a decrease of hemidesmosomes with occasional invaginating cytoplasmic basal cell processes (Fig. 9). There were occasionally cytoplasmic processes pinched off from the basal cells and lying freely in the subepithelium (Fig. 10). There were large widened intercellular spaces lined with plasma membrane interdigitations and often containing microvilli (Fig. 11), clusters of broken desmosomes (Fig. 12) and the cells of inflammatory lymphocytes (Fig. 13). The spinous cells consisted of disorientated and randomly scattered tonofilaments, numerous pleomorphic keratohyaline and membrane coating granules and occasionally mitotic cells (Fig. 14). Fragmented and vacuolated mitochondria, dilated rough endoplasmic reticulum and profiles of golgi complexes were also seen in the animals of the above groups. The above changes are very much minimised in the animals of groups IV and VI.

DISCUSSION

In this study, the delay in the induction of tumours in animals exposed to vitamin C was comparable to that reported in the hamster cheek pouch injected with Bacillus Calmette-Guerin (BCG) and levamisole [13, 14], 13-*cis*-retinoic acid and excess of vitamin A palmilate [5, 8, 15, 16]. The mechanism of influence of vitamin C in this phenomenon is not known. Immunoenhancing agents such as BCG and levamisole are also capable of inhibiting oral carcinogenesis in the 9,10-dimethyl benz(a)anthracene (DMBA) treated hamster cheek pouch [13, 14]. It is quite conceivable that enhanced immune responses due to vitamin C and antioxidant and immunocompetent properties of vitamin C delay the tumour induction. This study confirms the recent reports which claim delaying and inhibiting action of vitamin C in experimental carcinogenesis [6, 17, 18]. This hypothesis is strengthened by the observation of marked to massive lymphocytic proliferation in the epithelium of vitamin C treated animals of groups VI, VIII and IX with 4NQO and tobacco. A mild to marked epithelial hyperplasia, hyperorthokeratosis and parakeratosis seen in the animals of groups I, II, III and IV are non-specific

Table 1. Summary of the epithelial response (histopathological changes)

Group	Type of treatment	No. of animals	Duration in weeks	Hyperplasia	Hyperorthokeratosis	Hyperparakeratosis	Dysplasia	Inflammatory exudate	Lymphocytic proliferation
Control									
I	Untreated control	4	2–26	+	—	—	—	—	—
II	Solvent control (PD)	4	2–26	+	+	—	—	—	—
III	Vitamin C control	4	2–26	+	+	—	—	—	—
IV	Tobacco control	4	2–26	++ to +++	++ to +++	++	++	+	+
Experimental									
V	4NQO	8	2–26	+++ to ++++	+++ to ++++	+++	++++	+++	+++
VI	Tobacco + vitamin C	8	2–26	++	++	++	++	++	++++
VII	4NQO + vitamin C	8	2–26	+++	++ to +++	+++	++	++	++++
VIII	4NQO + tobacco	8	2–26	++++	++++	+++	++++	++++	+++
IX	4NQO + tobacco + vitamin C	8	2–26	+++	+++	++	+++	+++	++++

+ = mild; ++ = moderate; +++ = marked; ++++ = massive.

Table 2. Summary of the induction of papillomas and tumour (macroscopic and microscopic observations)

Group	Type and treatment	Number of animals	Papilloma or nodules	Tumour	Type of tumours
IV	Tobacco	4	3.00±1.00 3/4	—	Papilloma (75%)
V	4NQO	8	—	1.29±0.76	Well differentiated squamous cell carcinoma (100%). About 1 cm in size and 1 or 2 in number
VI	Tobacco + vitamin C	8	1.25±0.50 4/8	—	Papilloma (50%)
VII	4NQO + vitamin C	8	1.80±0.45 5/8	1.00±0.00 3/8*	*Papillary epidermoid carcinomas (37.5%) and papilloma (62.5%)
VIII	4NQO + tobacco	8	—	2.43±0.53 8/8	Well differentiated squamous cell carcinoma (100%). Larger than 1 cm in size and considerably more than 4–5 in number
IX	4NQO + tobacco + vitamin C	8	3.00±0.7 5/8	2.67±0.58 3/8*	*Papillary epidermoid carcinomas (37.5%) and papillomas (62.5%)

Fig. 1. Mild epithelial hyperplasia and hyperkeratosis seen in the animal treated with vitamin C: Group III (H and E, ×300).

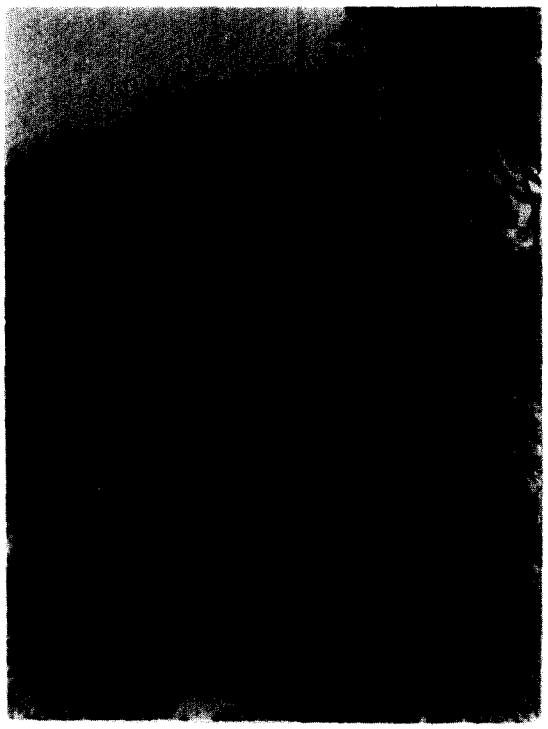

Fig. 2. Epithelium shows marked to massive dysplasia, lack of stratification, nuclear hyper-chromatism, cell vaculation, invagination of epithelium with inflammatory exudate and lymphocytic proliferation: Groups VI, VIII, IX (H and E, ×180).

phenomena which occur even after exposure to the external environment [19] and is probably due to the trauma induced by storage of food and aging, and considered to be routine pathological change.

Epithelial dysplasia is recognised by the presence of a number of criteria such as irregular stratification, basal layer hyperplasia, loss of polarity of basal cells, increased nuclear cytoplasmic ratio, nuclear hypertrophy, inter/intracellular spaces, etc. [7]. Epithelial dysplastic changes are seen in the oral cavity of various rodent species treated with carcinogens and betel quid ingredients [1, 4–6], and progression of these changes to oral malignancy with carcinogens have been reported frequently [5, 8, 13, 16, 19, 20]. In the present study, it was demonstrated that epithelial dysplasia preceded tumour development as evidenced by the fact that by 24–26 weeks of treatment of 4NQO singly and in combination with tobacco, 100% of the animals had developed squamous cell carcinomas. The papillary epidermoid carcinomas (37.5%) developed in animals treated with the test substances along with carcinogens.

Although an inflammatory exudate was observed in all experimentally induced tumours, lymphocytic proliferation was dense in animals of groups VI, VII, and IX. This supports the suggestion that chemoprevention of squamous cell, papillary epidermoid carcinoma and squamous cell carcinoma by vitamin C, could be due to its immunoenhancing property and it may be speculated that the antioxidant and immunocompetent properties of vitamin C modify the mechanism of action of the carcinogen. On the other hand, the enhanced immunological surveillance and detection of potential neoplastic cells may also prevent the carcinogenic process. Therefore, it will be important to determine in future studies whether vitamin C interferes sufficiently with neoplastic transformation to delay the progression of neoplastic disease.

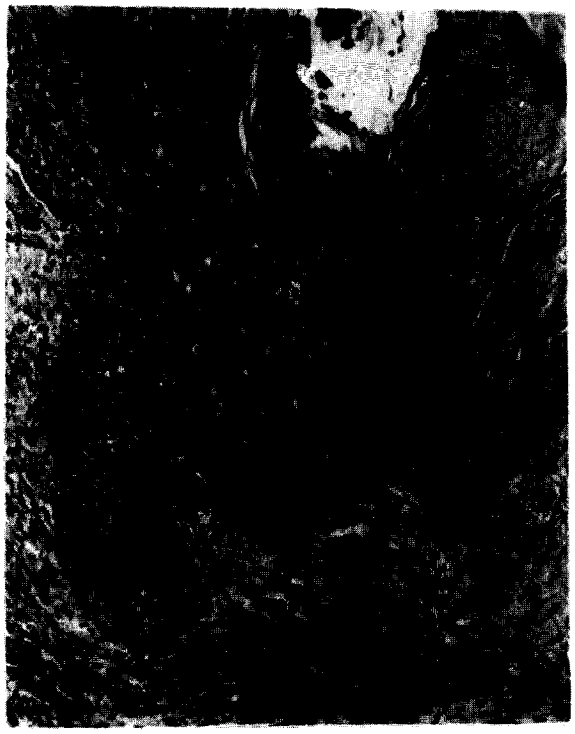

Fig. 3. Papillary epidermoid carcinoma showing hyperplasia, hyperorthokeratosis with dysplastic change mainly altered nuclear cytoplasmic ratio, nuclear pleomorphism, loss of polarity of basal cells, numerous mitotic figures and cell vaculation in the animal treated with 4NQO + tobacco: Group VIII (H and E, × 180).

Fig. 4. Tumour bearing animal treated with 4NQO shows well differentiated marked invasive squamous cell carcinoma with severe dysplastic changes: Group V (H and E, × 300).

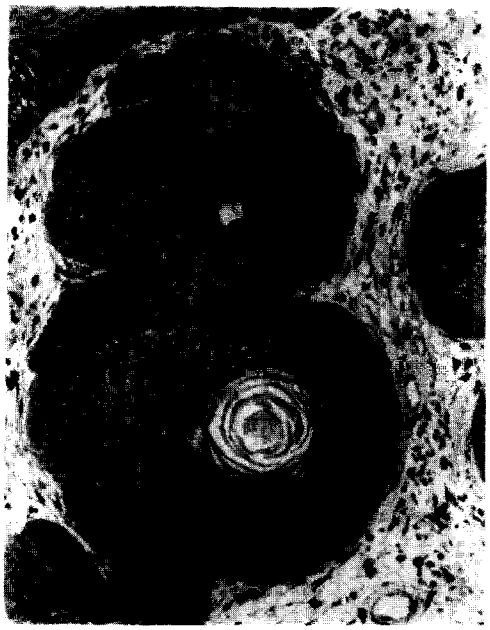

Fig. 5. Number of pearls of keratinisation are seen in the small well differentiated "squamous cell carcinoma" in the animals treated with 4NQO + tobacco + vitamin C: Group IX (H and E, × 300).

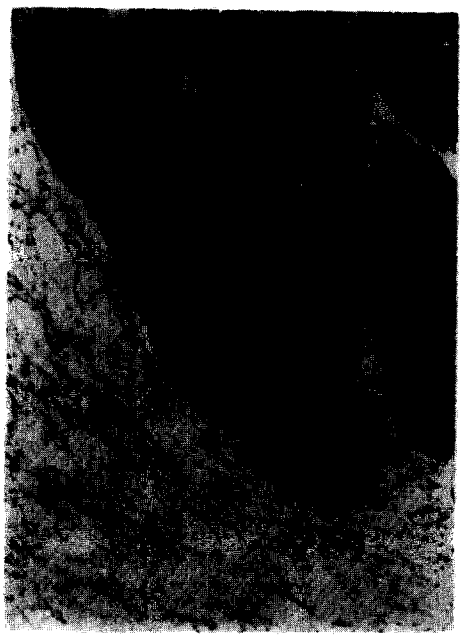

Fig. 6. Small epidermal carcinoma shows epithelial hyperplasia and marked dysplastic changes with inflammatory exudate in the animal treated with 4NQO followed by tobacco and vitamin C: Group IX (H and E, × 180).

The ultrastructural changes seen in this study match the pathological lesions seen in humans and experimental oral cancer. These changes are seen predominantly in the animals of groups V, VII, VIII and IX, but not very noticeable in the animals of control groups I, II, III, IV and experimental animals of groups V and VII. Only a few studies are available on ultrastructural morphology of hamster cheek pouch

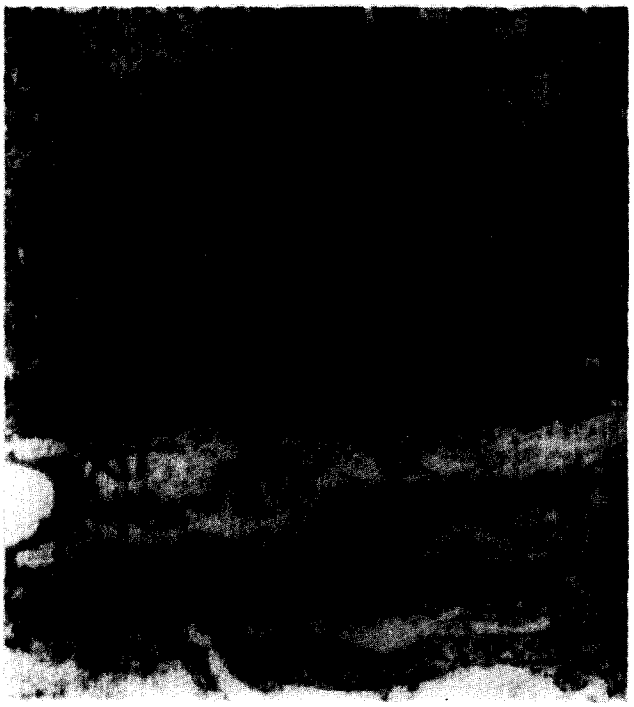

Fig. 7. Electron micrograph showing well-defined intact, continuous basal lamina (BL) with numerous desmosomes (× 10 500).

Fig. 8. Electron micrograph showing numerous keratohyalin (KG) and membrane coating granules (MCG) and parts of tonofilaments (TF) (× 25 200).

Fig. 9. Electron micrograph showing discontinuous, fragmented broken basal lamina (BL) with hemidesmosomes (HD) (× 27 000).

Fig. 10. Electron micrograph showing cytoplasmic processes (CP) pinched off from basal cell surrounded by basal lamina (BL) lying freely in the underlying subepithelium (× 14 600).

epithelium in chemically induced carcinogenesis [4–6, 8, 16, 21, 22]. The submicroscopic changes seen in this study, mainly widened intercellular spaces filled with microvilli, loss of intercellular contacts, clustering of broken desmosomes in the intercellular spaces, etc., are also observed in chemically induced oral malignancy [4, 6, 21, 23, 24], in oral leucoplakia [25], various acanthotic abnormalities [26] and epidermal hyperplasia in skin [27]. These observations agree with the pathological lesions seen in the human precancerous and cancerous oral mucosa.

A discontinuous, fragmented and diffused basal lamina with

Fig. 11. Electron micrograph showing widened intracellular spaces (ICS) filled with microvilli (MV) (× 10 500).

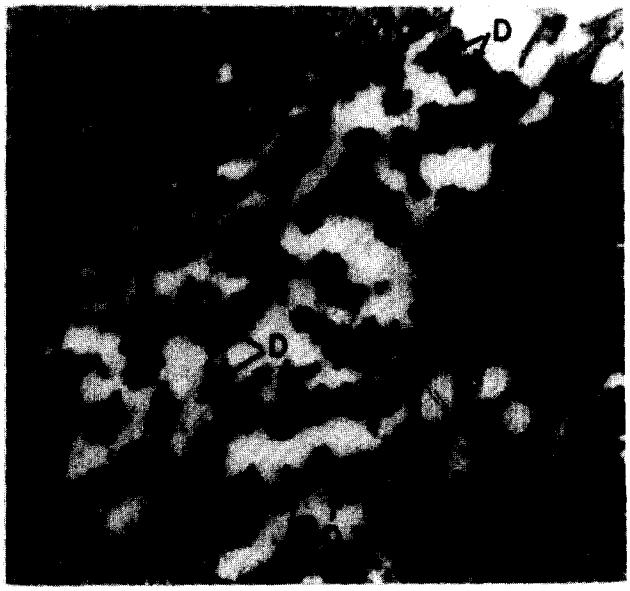

Fig. 12. Electron micrograph showing intracellular spaces filled with broken desmosomes (D) (× 10 500).

Fig. 13. Electron micrograph showing lymphocytic invagination (× 10 500).

Fig. 14. Electron micrograph showing mitotic cell in the upper spinous cell layer (× 10 500).

diminished lamina densa and decreasing hemidesmosomes with microinvasion of the basal cell cytoplasmic processes in the subepithelium were observed in tumours developed by carcinogen [2, 5, 6, 8, 16, 20]. Cytoplasmic processes of basal cells and basal cell invagination are predominantly seen in the experimental tumour bearing animals. This non-specific alteration is reported in the chronic inflammation seen in tumours induced by 4NQO and 4NQO + tobacco. This is mainly accompanied by damaged, discontinuous and diffused basal lamina [4–6, 15, 16, 20]. A fundamental feature of carcinogenesis appears to be the failure of the neoplastic epithelium to maintain a basal lamina at its interface with the underlying subepithelial connective tissue. This unknown function is exemplified at focal regions of hyperactivity by basal cell invagination and damaged, discontinuous and diffused basal lamina densa in squamous cell carcinomas. The changes described above are very prominent in the tumour bearing animals treated with 4NQO and 4NQO + tobacco and minimised in animals treated with 4NQO + tobacco + vitamin C.

In studies of human oral leucoplakia and hyperkeratinised oral mucosa, increased pleomorphic keratohyaline and membrane coating granules are often accompanied by randomly scattered disoriented tonofilaments in the process of keratinisation [2, 16, 25]. Their significance to the process which culminates in cornification of stratified squamous epithelia is reflected in their absence in unkeratinised epithelia and increase in hyperorthokeratosis. This change is seen in the animals of all experimental groups treated with 4NQO and tobacco singly and in combination with vitamin C.

In conclusion, the histopathological and ultrastructural observations suggest that: (1) vitamin C delays tumour induction and reduces the size and number of tumours and induces the papillomas in the hamster cheek pouch. (2) 4NQO water soluble carcinogen treated cheek pouch developed well differentiated squamous cell carcinomas at 24–26 weeks, whereas with vitamin C in combination with 4NQO and tobacco papillary epidermoid carcinomas developed with less invasion in some animals. (3) Vitamin C restricts the progression of the initiated cells to frank malignancy and delays or inhibits the tumour cell proliferation in the subepithelium. (4) There is clear indication that vitamin C does play a definite role in the modification of growth and inhibition and delay of tumour induction in these experiments conducted on the effect of vitamin C on oral and other cancers appears a rewarding area of investigation.

1. Kandarkar SV, Sirsat SM. Changes in vitamin A conditioned hamster cheek pouch epithelium on exposure to commercial shell lime (calcium-hydroxide) and tobacco—I. Optical histopathology. *J Oral Pathol* 1977, **6**, 191–202.
2. Kandarkar SV, Sirsat SM. Changes in vitamin A conditioned hamster cheek pouch epithelium on exposure to commercial shell lime (calcium-hydroxide) and tobacco—II. Ultrastructure. *Indian J Cancer* 1978, **15**, 14–19.
3. Potdar PD, Kandarkar SV, Sirsat SM. Light and electron microscopic study of hamster cheek pouch treated with 9,10-dimethyl-1-2-benzanthracene and retinoic acid. *Ann Acad Med (Singapore)* 1989, **18**, 523–527.
4. Kandarkar SV, Reade PC. Effect of topical vitamin C on palatal oral mucosal carcinogenesis using 4-nitroquinoline-1-oxide. *J Biol Buccale* 1991, **19**, 199–204.
5. Kandarkar SV, Sirsat SM. Periodic histopathological and ultrastructural changes of excess of vitamin A on oral carcinogenesis. *Indian J Exp Biol* 1990, **28**, 10–17.
6. Potdar PD, Kandarkar SV, Sirsat SM. Modulation by vitamin C of tumour incidence and inhibition in oral carcinogenesis. *Func Develop Morphol* 1992, **2**, 167–172.
7. Pindborg JJ. Oral precancerous conditions in South-East Asia. *Int Dent J* 1965, **15**, 190–199.
8. Kandarkar SV, Hasgekar NH, Sirsat SM. Optical and ultrastructural pathology of vitamin A pretreated hamster cheek pouch-exposed to lime [$Ca(OH)_2$] and tobacco over life span. *Neoplasma* 1981, **28**, 728–737.
9. Galatthaar BE, Hornig DH, Moser U. The role of ascorbic acid in carcinogenesis. In Poirier LA, Newberne PM, Michael WP, eds. *Advance in Experimental Medicine and Biology*. New York, Plenum, 1986, 357–377.
10. Eveson JW, MacDonald DG. Effects of the water-soluble carcinogen 4-nitroquinoline-*n*-oxide on hamster lingual mucosa. *Oral Surg* 1977, **44**, 600–605.
11. Steidler NE, Reader PC. Initiation and promotion of experimental oral mucosal carcinogenesis. *J Oral Pathol* 1986, **15**, 43–47.
12. Guttenplan JB. Mutagenic activity in smokeless tobacco products sold in the USA. *Carcinogenesis* 1987a, **8**, 741–743.
13. Giunta JT, Reif AE, Shklar G. Bacillus Calmette-Guerin and antilymphocyte serum in carcinogenesis: effects of hamster cheek pouch. *Arch Pathol* 1974, **98**, 237–240.
14. Eisenberg E, Shklar G. Lamisole and hamster pouch carcinogenesis. *Oral Surg* 1977, **43**, 562–571.
15. Shklar G, Schwartz J, Grau D, Trickler D, Wallae KD. Inhibition of hamster cheek pouch carcinogenesis by 13-*cis* retinoic acid. *Oral Surg* 1980, **50**, 45–50.
16. Kandarkar SV, Sirsat SM. Influence of excess of retinoid on DMBA carcinogenesis. *Neoplasma* 1983, **30**, 43–50.
17. Counsell JN, Hornig DH, eds. *Vitamin C (Ascorbic Acid)*. London, Applied Science, 1981.
18. Dounham WB, Zucker Kandl E, Reynolds R, *et al.* Effects of intake of L-ascorbic acid on the incidence of dermal neoplasms induced in mice by ultraviolet light. *Proc Natl Acad Sci. USA* 1982, **79**, 7532–7536.
19. Sirsat SM, Kandarkar SV. Histological changes in the oral mucosa of the wistar treated with commercial lime (calcium-hydroxide)—An optical and submicroscopic study. *Br J Cancer*, 1968, **22**, 303–315.
20. Potdar PD, Kandarkar SV, Sirsat SM. Effect of DMBA and retinyl acetate on ultrastructural morphology of hamster cheek pouch. *Indian J Exp Biol* 1988, **26**, 205–210.
21. Marefat MP, Albrigh JT, Shklar G. Ultrastructural alterations in experimental lingual leukoplakia and carcinoma. *Oral Surg* 1979, **47**, 334–342.
22. Kandarkar SV, Potdar PD, Sirsat SM. Dose response effect of retinyl acetate on DMBA induced carcinogenesis in the hamster cheek pouch. *Neoplasma* 1984, **31**, 415–421,
23. White FH, Gohari K. A qualitative ultrastructural study of the intercellular spaces between epithelial cells treated *in vivo* with DMBA. *J Oral Pathol* 1984, **23**, 231–234.
24. Kandarkar SV, Sirsat SM. Periodic histopathological and ultrastructural changes of excess of vitamin A on oral carcinogenesis. *Indian J Exp Biol* 1990, **28**, 10–17.
25. Sirsat SM, Daftary NA. Keratinisation patterns in the human oral mucosa in relation to oral habits and malignancy—II. Ultrastructure. *Indian J Cancer* 1874, **2**, 13–27.
26. Silverman S Jr. Ultrastructural studies of oral mucosa—I. Comparison of normal and hyperkeratotic human buccal epithelium. *J Dent Res* 1967, **46**, 1433–1443.
27. Bhisey RA, Sirsat SM. Ultrastructural analysis of epidermoid hyperplasia induced by multiple 12-*O*-tetradecanoyl phorbal-13-acetate (TPA) treatment of mouse skin. *Tumori* 1986, **72**, 643–650.

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