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ORIGINAL ARTICLE

Fernanda Gonçalves Salum · Karen Cherubini · Maria Antonia Zancanaro de Figueiredo · Milton de Oliveira Amado · Eloni Terezinha Rodrigues · Liliane Soares Yurgel

Histologic evaluation of effect of thiocyanate topical application on hamster cheek pouch

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Abstract The present study was conducted to investigate the effect of topical thiocyanate (SCN^-) application on hamster cheek pouch mucosa. Sixty-six hamsters were divided into two groups. The test substance containing potassium thiocyanate was applied on the hamsters' cheek pouches at 55 mg/kg dosages. Cheek pouches were grossly examined after 12 weeks. Histometric evaluation included height measurements from the epithelium without stratum corneum, stratum corneum, and full epithelium. Clear cells frequency was compared between groups. Results were analyzed using Student's *t* test. The experimental group showed a lower height of the stratum corneum (p=0.035) and higher frequency of clear cells (p<0.001). There was no height difference for the epithelium without stratum corneum (p=0.677) and full epithelium (p=0.904) between groups. SCN^- promotes no gross alterations on hamsters' cheek pouch and does not induce dysplastic features at the epithelium or inflammation at the connective tissue. The ion promotes histologic alterations on mucosa that need to be investigated in further studies.

Keywords Thiocyanates · Topical administration · Mouth mucosa · Epithelium · Connective tissue · *Mesocricetus*

F. G. Salum · K. Cherubini · M. A. Z. de Figueiredo · L. S. Yurgel
Department of Stomatology, São Lucas Hospital, PUCRS, Porto Alegre, Brazil

M. de Oliveira Amado
Department of Veterinary Medicine, Universidade Federal de Pelotas, Pelotas, Brazil

E. T. Rodrigues
Department of Pharmacology, São Lucas Hospital, PUCRS, Porto Alegre, Brazil

F. G. Salum
Hospital São Lucas, PUCRS, Avenida Ipiranga 6690, Sala 231 90610-000, Porto Alegre, Rio Grande do Sul, Brazil

F. G. Salum (✉) · K. Cherubini · M. A. Z. de Figueiredo · M. de Oliveira Amado · E. T. Rodrigues
Stomatology and Buco-Maxilo-Facial Cancer Prevention Unit, São Lucas Hospital, Pontifícia Universidade Católica do Rio Grande do Sul (PUCRS), Porto Alegre, Brazil
e-mail: fesalum@terra.com.br
Tel.: +51-33203254

Introduction

The best understood and well-researched function of the thiocyanate ion (SCN^-) in the saliva is its participation in the salivary peroxidase enzyme system [10, 19]. Reduction in plaque and gingival index has been obtained by using a dentifrice containing SCN^- and H_2O_2. The effects observed in the reduction of the gingival index were attributed to the intensification of the salivary peroxidase system and, also, to the influence of SCN^- in the modulation of tissue response [11, 12]. Studies have shown that SCN^- facilitates the migration of cations to the interior of cells, performing ion channel modulation of cell membranes [8, 16]. In addition to that, Kramer et al. [7] and Weuffen et al. [17] demonstrated that, when applied to animal subjects, SCN^- intensifies hair growth.

Smoking inhibits the recurrent aphthous ulceration (RAU) through an unknown mechanism, although it has been suggested that this inhibition derives from an accretion in the stratum corneum of the buccal mucosa [15]. On the other hand, smokers present increased salivary concentrations of SCN^-, when compared to nonsmokers [3, 4]. Taking the properties attributed to SCN^- into consideration, it has been proposed that it participates in the mechanism through which smoking inhibits RAU via the intensification of the proliferative activity and the accretion in the stratum corneum of the buccal mucosa [3]. Concentrations of the ion in the saliva higher than in the other bodily fluids, the evidence of significantly augmented SCN^- salivary concentrations in smokers, and this ion's effect on the stimulation of cell proliferative activity encourage the idea that its participation in the peroxidase system is not the sole function of thiocyanate in the buccal

cavity. The present study proposes to investigate the histologic effects promoted by SCN^- when topically applied on hamster cheek pouches.

Materials and methods

The protocol of this study was approved by the Scientific and Ethical Committee of Pontifícia Universidade Católica do Rio Grande do Sul's Dentistry School. The sample comprised 66 Syrian golden hamsters (*Mesocricetus auratus*), 8-week-old adult males, randomly distributed into an experimental group (n=34) and a control group (n=32). The animal subjects received solid food and were given water ad libitum.

Thiocyanate and vehicle To carry the potassium thiocyanate (KSCN), sodium starch glycolate (Explosol) was employed. This vehicle was manipulated at a concentration of 6%, by adding PVP (polyvinylpyrrolidone) 0.5%, nipagin 0.1%, nipazol 0.02%, and warm water. The mix was shaken until a gel-like viscosity was obtained; moment at which the KSCN, concentrated at 1.67% (0.172 mol/l), was added to the test substance.

Topical administration The test and control substances were topically applied to the left cheek pouch of the animal subjects, via a needleless hypodermic syringe. KSCN was administered to the experimental group at daily dosages of 55 mg/kg of the animals' body weight. To the control group, the gel, without the addition of KSCN, was applied in the exact same way as to the experimental group. After each administration, the animal subjects were deprived of water and food intake for 1 h. The administrations were conducted daily for 12 weeks.

Euthanasia On the 85th day after the beginning of the experiment, the animal subjects were killed through the administration of lidocaine at 2% on the nape of the neck, according to LD_{50}.

Gross examination After the euthanasia, the left cheek pouch of the animal subjects was everted for visual inspection.

Specimen acquisition The left cheek pouch of the animal subjects was dissected as close to its base as possible. The specimens were fixated in a solution of formaldehyde at 10% for 24 h, and then subjected to histological processing for embedding in paraffin. Each pouch originated six tissue fragments which were included in two blocks. A 5 μm-thick tissue section, cut at a right angle to the epithelial surface, was obtained from each block. Those sections were stained with hematoxylin and eosin (HE).

Histologic evaluation The histologic evaluation was performed by one sole examiner, calibrated and blinded as to the group each slide belonged to. Eighteen fields per pouch were captured, via an optical microscope Olympus AX 70, connected to an Olympus U-PMTVC digital image capture system. The images were enlarged 400×.

The histometric evaluation was performed through the use of an Image-Pro Plus version 4.0 software for digitalized image analysis. Measurements of the height of the epithelium without stratum corneum and stratum corneum were conducted. Full epithelium was obtained by adding the results from the two aforementioned variables. The measurements were obtained by tracing a perpendicular line to the epithelial layers under investigation. In each field analyzed, three measurements of each variable were conducted; the average of the three measurements, provided by the software itself, was recorded (Figs. 1 and 2).

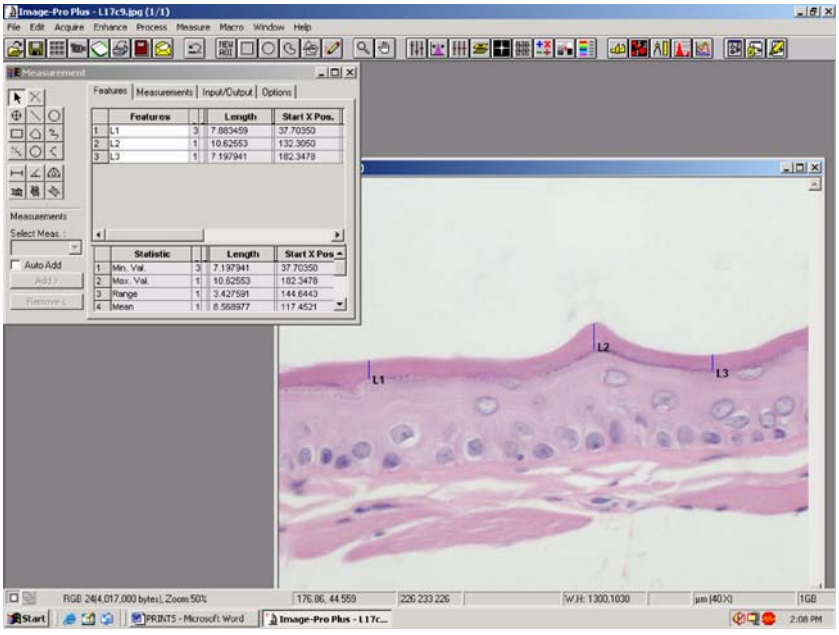

Fig. 1 Histometric analysis of epithelium: measurement of stratum corneum (HE, 400×)

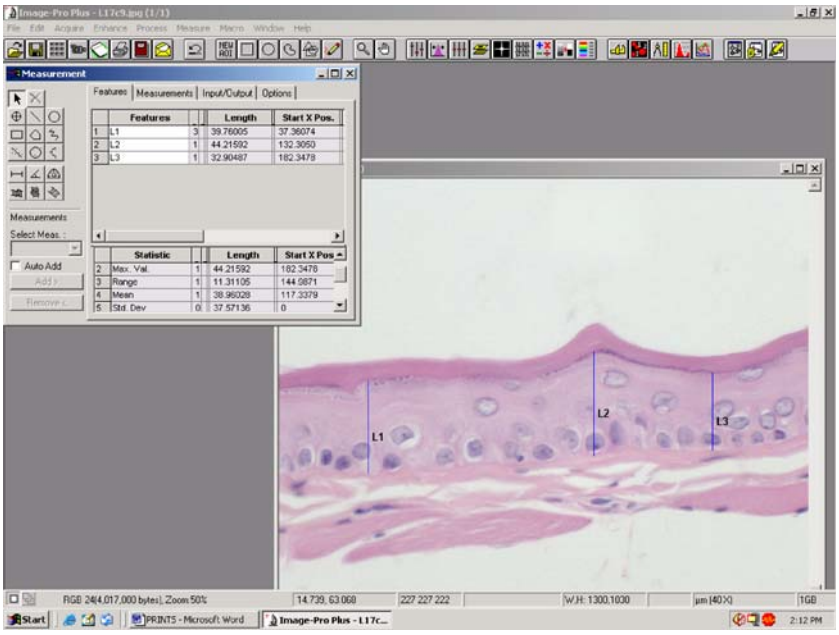

Fig. 2 Histometric analysis of epithelium: measurement of the other epithelial strata (HE, 400×)

In the epithelial tissue, the following alterations in cell morphology were classified as present or absent: (a) basal cells polarity loss; (b) basilar hyperplasia; (c) increase in nuclear-cytoplasmic ratio; (d) tear drop-shaped epithelial projections; (e) irregular epithelial stratification; (f) increase in the number of mitoses or presence of atypical mitoses; (g) mitoses in the superficial half of the epithelium; (h) cell pleomorphism; (i) nuclear hyperchromatism; (j) enlarged nucleoli; (l) cell cohesion loss; (m) individual keratinization or keratinization of groups of cells in the spinous layer [18]. Depending on the alterations, the dysplasia was classified as light, when up to two histologic alterations were present; moderate, in the presence of three to four alterations; and as severe dysplasia, if five or more alterations were observed [2]. The histologic evaluation also included, in the epithelial tissue, the quantification of clear cells. The clear cells (Fig. 3) were defined according to the following criteria: lower volume than that of the adjacent keratinocytes, pycnotic, and hyperchromatic nucleus, and presence of clear perinuclear halo. In the underlying connective tissue, the presence of hyperemia, edema, inflammatory infiltrate, and fibroplasia was evaluated.

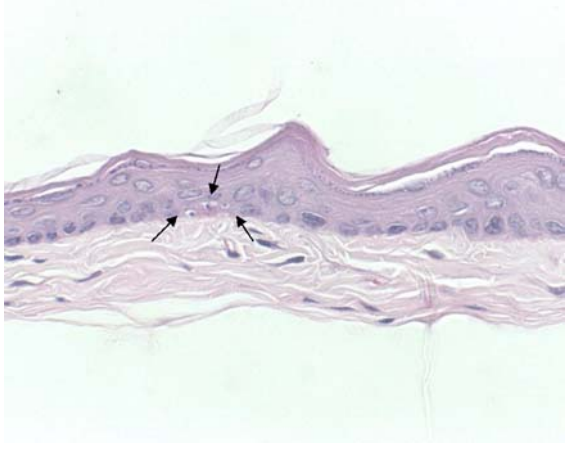

Fig. 3 Three clear cells in the basal layer of epithelium (HE, 400×)

Data analysis The results obtained were analyzed via descriptive statistics and Student's *t* test ($\alpha \leq 0.05$).

Results

The cheek pouches did not present alterations in color or tissue texture in any of the animals of either the control group or the experimental group.

The histometric evaluation data were analyzed according to the average of the 18 fields from each animal subject (Table 1). The figures obtained regarding the height of the epithelium without stratum corneum did not present a statistically significant difference between the control and experimental groups (Student's *t* test, $p=0.677$). The height of the stratum corneum presented a statistically significant difference between the two groups evaluated, with the experimental group presenting a lower average to that of the control group (Student's *t* test, $p=0.035$). Full epithelium did not differ significantly between the control and experimental groups (Student's *t* test, $p=0.904$). Of the 612 fields evaluated from the experimental group, 12 presented basilar hyperplasia. A similar result was observed in the control group, which presented that alteration in 14 of the 576 fields analyzed. To compare the frequency of clear cells between the two groups, the total amount of the 18 fields analyzed was utilized for each animal. Later, the average of each group was calculated (Table 2). The average frequency of clear cells was significantly higher in the experimental group, when compared to the control group (Student's *t* test, $p \leq 0.001$). The connective tissue did not present hyperemia, edema, inflammatory infiltrate, or fibroplasia in any of the 1,188 fields analyzed.

Table 1 Average and standard deviation of height of epithelium without stratum corneum, height of stratum corneum, and full epithelium of hamster cheek pouch subjected to topical application of thiocyanate and control group

Variables	Group				p*
	Control		Experimental		
	Average (μm)	Standard deviation	Average (μm)	Standard deviation	
Epithelium without stratum corneum	34.21	3.06	34.57	3.75	0.677
Stratum corneum	6.40	0.75	5.95	0.93	0.035
Full epithelium	40.61	3.26	40.52	3.76	0.904

*Student's t test for independent samples, significant difference if $p \leq 0.05$

Discussion

The higher concentrations of SCN^- in the saliva when compared to the other bodily fluids, the even higher concentrations in the saliva of smokers [3, 4] and the lack of knowledge on possible effects of thiocyanate on the buccal mucosa aroused the interest to carry out this investigation.

At the gross examination, the cheek pouches of the animals presented pale pink coloration and slightly rugose texture, consistent with the normal condition of the tissue [14], indicating that the thiocyanate ion was not able to promote macroscopic alterations in the tissue.

Based on the inverse correlation, there is between smoking and RAU, Cherubini's study [3] compared the buccal environment of smoking, nonsmoking, and lesion-carrying individuals. Although they did not prove the existence of a link between the concentrations of salivary thiocyanate and the occurrence of RAU, the results of the study do not discard the possibility of thiocyanate participating in the mechanism of lesion inhibition in smokers. Hoogendoorn and Piessens [5] reported an improvement in the clinical picture of RAU in patients who used a potassium thiocyanate-based collutory. The hypothesis that such an action derives from an increment in the stratum corneum or in the other epithelial strata, however, has been refuted by the findings in the present study.

In the histometric evaluation, the height of the stratum corneum showed significantly lower figures in the experimental group than in the control group. Weuffen et al. [17] suggest that SCN^- exercises an influence on keratin hydrophilia. In the experimental group, the test substance may have elevated the hydrosolubility of that layer and solubilized cellular surface proteins, resulting in keratotic tissue scaling. The molecular mechanisms involved in this keratolytic effect and the clinical relevance of this finding need to be clarified. Because SCN^- stimulates tissues with fast proliferative activity [6, 7, 11, 12, 17], it was expected that it would promote an increase in the height of epithelial tissue and stratum corneum. There being stimulation of cellular proliferative activity, there should be an increase in the number of cells, or epithelial hyperplasia and, consequently, the height of the epithelium should suffer an increment. However, there was no significant difference between the groups in terms of the variables' height of the epithelium without stratum corneum and full epithelium. Cellular proliferative activity should, even so, be investigated by future studies through other methods, such as the comparison of the number of mitoses between the groups.

SCN^- originates, also, from the detoxification of hydrocyanic acid present in the smoke of tobacco combustion [9]. Even though the carcinogenic potential of tobacco to the buccal mucosa is well established, possible effects of direct SCN^- administration on the latter are ignored. Thus, in the morphological evaluation, the possibility that the test substance induces the development of dysplasia was investigated. The only observed alteration was basilar hyperplasia, which is associated to a higher proliferative activity and inherently characteristic of hamster pouch epithelium, having been observed in some histological fields [14].

When the effects of SCN^- in the hair growth of the animal subjects were investigated, studies of 4, 7, 10, and 16 weeks were conducted [7, 17]. However, it is possible that the 12-week SCN^- administration period may have not been enough to induce dysplasia. The verification that SCN^- did not promote development of dysplastic features in hamster pouch mucosa encourages further investigation of its influence on the buccal mucosa of animal subjects, employing different ion dosages for longer periods of time.

Also investigated, in the connective tissue, were acute and chronic inflammatory alterations that might have been

Table 2 Average and standard deviation of clear cells frequency in epithelial tissue of hamster cheek pouch subjected to topical application of thiocyanate and control group

Cells	Group				p*
	Control		Experimental		
	Average	Standard deviation	Average	Standard deviation	
Clear	3.81	2.19	7.35	4.72	<0.001

*Student's t test for independent samples, significant difference if $p \leq 0.05$

induced by the administration of the test substance. No alteration was observed in the connective tissues of animals of either group. Therefore, neither SCN^- nor the mechanical manipulation performed resulted in damage to that tissue. The stratum corneum, even reduced as it was in the experimental group, may have shielded the underlying connective tissue against the thiocyanate-based induction of alterations.

The average frequency of clear cells was significantly higher in the experimental group than in the control group. It is possible that the clear cells are Langerhans cells and that its higher frequency represents increase of oral mucosa immune activity induced by thiocyanate. However, future studies employing immunohistochemistry techniques are necessary for the classification of clear cells.

The presence of salivary SCN^- and the activity of the salivary peroxidase system in hamsters were demonstrated by Saji et al. [13]. However, the lack of saliva in deeper portions of the hamster pouch may have been a limitation to the present study because it confers different aspects to this environmental site when compared to the human buccal mucosa, possibly generating the unequal SCN^- effects on these two environments. SCN^- administration, therefore, needs to be investigated on other experimental sites, which are actually bathed in saliva, because it is possible that the activity of the ion depends on solutes and on the nature of bodily fluids, in which the ion exchange is facilitated by the environment.

The mechanism of action of thiocyanate still requires more detailed investigations, but its biological effects are enzyme activation, such as Na^+, K^+-ATPase, modulation of cellular membrane permeability, and stimulation of cellular proliferation in tissues with fast turnover [16]. The participation in the oral peroxidase system seems to be the most important function of the ion in the mouth because, besides the production of hypothiocyanate, oxygen radicals are reduced, which protects the mucosa from the deleterious effects of those substances.

This study has suggested the possibility that SCN^- acts directly on the buccal mucosa, promoting the intensification of epithelial proliferative activity and keratin increment. However, these alterations were not observed at gross and histological evaluations. On the other hand, the increase in clear cells frequency and the decrease in height of the stratum corneum were promoted by the administration of the ion. That keratolytic effect observed in the experimental group, although mild, may be associated to the increase in keratin hydrophilia, a mechanism that should be investigated by new studies. No dysplastic features were observed in the epithelial tissue, or inflammatory in the connective one, that could have been induced by the ion. Nevertheless, it is possible that, in saliva-bathed sites, thiocyanate produces effects on the mucosa diverse from those observed in the present study, as a consequence of the interaction with elements in the environment and of the stimulation to carry elements to the cells [1, 16]. The influence of this ion in stimulating proliferation in fast turnover tissues needs to be investigated in new studies.

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