QUANTITATIVE STUDY OF AGNOR IN LINGUAL MUCOSAL CELLS OF BALB/C MICE SUBMITTED TO THE 4-NQO CARCINOGEN: THE EFFECT OF VITAMIN E

QUANTIFICAÇÃO DAS AGNOR EM CÉLULAS DA MUCOSA LINGUAL DE CAMUNDONGOS BALB/C SUBMETIDOS AO 4-NQO: EFEITO DA VITAMINA E

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RESUMO

Animal models have shown to be important in understanding multifactorial diseases with complex mechanisms and multiple molecular events as in oral squamous cell carcinoma. 4-NQO exhibits a potent carcinogenic effect in various animal species, producing DNA mutations, altering base pairs, provoking deletions and chromosome aberrations. Micronutrients, among them, vitamin E may inactivate free radicals and reactive metabolites of oxygen, which are potent causes of DNA damage. The aim of the present study was to evaluate the impact of vitamin E on the index of cellular proliferation, through the AgNOR technique, in the lingual mucous of BALB/C mice submitted to 4-NQO carcinogen. BALB/C male mice (112) were divided into: group 1: treated with topical applications of 0.5% 4-NQO, group 2: treated with 4-NQO and 800 IU of vitamin E, group 3: treated with 800 IU of vitamin E; group 4: untreated (control). Seven animals from each group were put down at intervals of 8, 16, 20, and 24 weeks. No statistically significant differences were observed among the different groups or among the various time intervals. It suggests that the cellular proliferation did not increase with the time of application or that it was not reflected in the

Key words: *4-NQO, experimental carcinogenesis, AgNOR, tongue*

INTRODUCTION

Chemical carcinogenesis involves a number of steps: activation and metabolism of the carcinogens, susceptibility of the cell and tissue targets, capacity for DNA repair and immunological response[1]. Animal models have been shown to be important in understanding multifactorial diseases, which have complex mechanisms and depend on multiple molecular events as in oral squamous cell

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AgNOR counts. Thus, it was not possible to evaluate the effect of vitamin E in these indices.

carcinoma[2]. The most common, and probably most reproducible model was first developed by Wallenius and Lekholm[3] and involves the topical application of 4-nitroquinoline-1-oxide (4-NQO) on the palate of mice. 4-NQO exhibits a potent carcinogenic effect in various animal species, due to its rapid conversion to 4-hydroaminoquinoline -1-oxide (4-HAQO)[4]. The final metabolite acts producing DNA mutations, altering base pairs, provoking deletions as well as inducing chromosome aberrations[5, 6].

Many studies have evaluated the action of micronutrients as agents of prevention and therapy in cancer, which include carotenoids, lycopene, retinoids, b-carotene, lycopene and a-tocopherol[7]. A diet rich in vegetables and fruits is related to a lower risk of various types of cancer[8], including oral cancer[9]. The anti-carcinogenic activity of these micronutrients includes an increase of immunological surveillance, activation of tumour suppressor genes and deactivation of oncogenes, which contributes to a reduction in angiogenic tumour activity[10]. Micronutrients as vitamin E may inactivate free radicals and reactive metabolites of oxygen which are potent causes of DNA damage and result in genetic mutation[11]. This potent anti-oxidant captures free radicals and therefore inhibits the lipid peroxidation of cellular membranes contributing to prevent carcinogenesis[7].

AgNOR are NOR-associated argyrophilic acid proteins and their number in tumour cells is directly related to the speed of cellular proliferation[12, 13, 14]. This technique has become useful as a possible tool in histopathology diagnosis although it still produces conflicting results. Experimental studies in tongue carcinoma in rats, suggest that the average number of AgNOR reflects the histological level in the carcinogenic process[15].

The aim of the present study was to evaluate the impact of vitamin E on the index of cellular proliferation, through the AgNOR technique, in the lingual mucosa of BALB/C mice submitted to 4-NQO carcinogen.

MATERIAL AND METHODS

A hundred twelve BALB/C male mice were divided into the following groups: A:group 1: treated with topical applications of 0.5% 4-NQO (SIGMA Chemical Co. St Louis, Missouri, USA) in propylene glycol, three times a week on the mucosa of the palate; B: group 2: treated with 4-NQO

and 800 IU of vitamin E (a-tocopherol –succinate acid) (SIGMA Chemical Co. St Louis Missouri, USA); C: group 3: treated with 800 IU of vitamin E; D: group 4: untreated (control). The mice were maintained with water ad libidum. Seven animals from each group were sacrificed at intervals of 8, 16, 20, and 24 weeks to evaluate clinically and histologically the palate and tongue. The tongue was removed, hemi-sectioned, and a fragment fixed in 10% buffered formalin and routinely processed for paraffin blocks. Two adjacent 3 µm sections were obtained from each block for hematoxylin and eosin (HE) staining and the other for a silver staining technique[16,17]. The hydrated sections were submitted to antigenic recuperation in a 10mM (ph 6) solution of citric acid, in a microwave oven for two 5 minutes cycles and incubated in a freshly prepared solution formulated by dissolving 2g gelatin in 1% aqueous formic acid and added to two parts of 50% aqueous silver nitrate. The samples were then washed and dehydrated in baths of increasing concentrations of alcohol, clarified in xylol and mounted with Permount ® (FISHEWR SCIENTIFIC, USA). AgNOR distinctive dots were evaluated in 200 basal epithelial and supra-basal cells of each case at a magnification of 1000X, under immersion. The Kruskall-Wallis test was used and results were considered statistically significant when P< 0.05.

Ethical approval was obtained from the Scientific and Ethical Committee in Animal Experimentation (CETEA) of Minas Gerais Federal University.

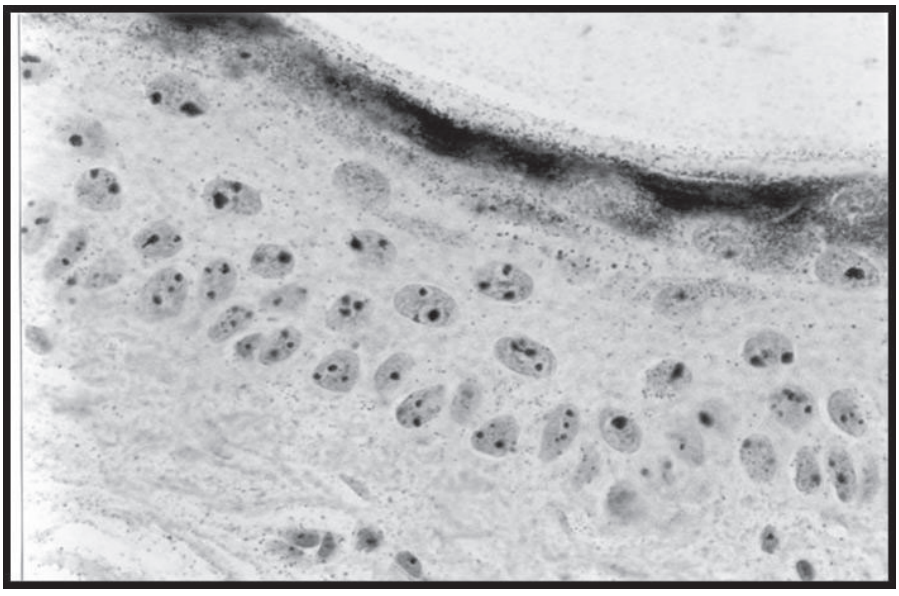

Figure 1. *Visualization of AgNOR dots in epithelial cells of lingual mucosa. Silver staining. Immersion 1000X.*

RESULTS

Squamous cell carcinomas or macroscopic morphological alterations and histological changes, in HE, were not observed in the tongue. Areas of discrete acanthosis were observed in the groups that received vitamin E, regardless the period of the treatment. AgNOR were identified as black or brown individual intranuclear dots (FIG. 1). The average numbers of AgNOR are summarized in Table 1. No statistically significant differences were found when the groups were compared to each other, or between the various groups at the same time period of treatment.

TABLE 1. *Mean numbers of AgNOR in the groups studied at different periods of time*

Time of sacrifice	Experimental group	Mean ± SD
8 weeks	Control	2.36±0.07
	4-NQO	2.10±0.21
	4-NQO+ vit E	2.32±0.22
	Vitamin E	2.51±0.02
16 weeks	Control	2.32±0.14
	4-NQO	2.35±0.22
	4-NQO+ vit E	2.32±0.10
	Vitamin E	2.28±0.29
20 weeks	Control	2.35±0.04
	4-NQO	2.25±0.15
	4-NQO+ vit E	2.36±0.11
	Vitamin E	2.41±0.14
24 weeks	Control	2.20±0.02
	4-NQO	2.14±0.11
	4-NQO+ vit E	2.15±0.33
	Vitamin E	2.18±0.15

Vit E: Vitamin E; 4-NQO: 4-nitroquinoline-1-oxide

DISCUSSION

An understanding of the carcinogenic process in the oral cavity is impaired, in part, by the difficulty in establishing an adequate in vivo experimental model that faithfully reproduces the conditions involved in human oral carcinogenesis. Experiments in cell culture may often be efficient, but they require greater in vivo extrapolation[18].

Since the work of Wallenius and Lekholm [3] studies have used 4-NQO in rats and mice with the purpose of reproducing an experimental model of oral cancer. 4-NQO is a potent carcinogen, which undergoes rapid conversion to 4-HAQO[6]. Studies using topical applications of 4-NQO to the palate of animals have observed that the tongue also undergoes cellular alterations, including the formation of carcinoma and therefore behaves in a similar way to the primary site (palate). This is probably due to lingual contact with the carcinogen[19]. von Pressentin et al.[2], studying the higher selectivity of 4-NQO for the tongue of transgenic lacZ mice, in relation to other oral sites, emphasize the greater presence of reductases in the tongue, which are necessary for carcinogenic effect. Therefore investigation of possible alterations in the tongues of the animals studied was included even though the primary site of application of 4-NQO was the palate.

Many studies have associated the low consumption of fruit and vegetables with an increased risk of cancer including oral cancer[10]. Antioxidants are the probable factors conferring protection, which are involved in the destruction of free radicals and therefore offering protection from damage to DNA. Many epidemiological and experimental studies have shown the effect of vitamin E in reducing the risk of cancer through its anti-oxidant effect[20] or reducing the tumour mass, inhibiting angiogenesis and the expression of TGF-α[21].

In our study, neither squamous cell carcinomas nor macroscopic and histological morphological alterations were observed in the tongue. It is important to remember that initiated cells are morphologically unaltered and may remain latent for an undetermined period of time[22]. Studies point out that the quantity of AgNOR protein is associated with the speed of cellular division[23] and therefore as an index of cellular proliferation. However, there were no differences in the indices of cellular proliferation between the various groups, both by type and by time period of treatment. These differences may really not have occurred, according to the averages of AgNOR found. However, if there were differences between the groups they were not reflected in the AgNOR counting.

The present study has given continuity to the investigations of Fonseca (2003)[24] who used the

experimental model in the palate of BALB/C mice to evaluate the action of the carcinogen 4-NQO and the role of vitamin E in the carcinogenic process. The greatest number of AgNOR was observed in the animals submitted to 4-NQO when compared to the group submitted to 4-NQO+vitamin E, and the numbers of AgNOR were less in the groups submitted to only Vitamin E. Thus, vitamin E appeared to act as an important antioxidant and the indices of cellular proliferation by Ki67 immunostaining and AgNOR counting suggested an inhibitory effect of vitamin E on these indices.

Some authors[25] were not able to determine the progression from normal mucosa to dysplasia and microinvasive carcinoma through AgNOR counting, which was possible by morphometric analysis. Their results are in agreement with previous studies[12,14] that suggest the superiority of the morphometric analytic method. Considering the fact that counting was done manually in our study, often crowded points or isolated points of AgNOR totally or partially superimposed were not distinguishable one from the other and were therefore considered as a single point, making this method subjective and questionably reproducible. Other authors have considered the AgNOR technique limited or usefulness for diagnosis[26].

Nevertheless, our results diverge from those of Fonseca (2003)[24] that used the same methodology and animal model but which found the AgNOR index to be directly proportional to the type of treatment received and the time of application of the carcinogen. Route of administration and concentration of carcinogen and varying specificity of oral tissues at different anatomical sites may be responsible for these different results. It may be supposed, that the quantity of carcinogen into contact with the tongue, which was not the primary site of application, was not sufficient to provoke tissue changes. The hyperortokeratinization of the epithelium observed in the HE samples, may have impaired the penetration and degradation of the carcinogen. However, as various authors have shown alterations in the mucosa of the tongue, using the same methodology[19], we believe that this factor does not have an important role in the absence of alterations.

Differences in the mutagenic susceptibility or in the capacity of mucosal cells of the tongue to activate

metabolites may be determinant factors in the response to this carcinogen, in comparison to mucosal cells of the palate. Some authors suggest that the lack of local specific reactivity to the carcinogen is more related to the susceptibility of the specie animal than to an inherent factor of the carcinogen[27]. Metabolic activation and detoxification routes of this and other carcinogens are, probably, the major source of inter-individual variation in susceptibility to cancer, being determined, at least in part, through the degree of expression or activity of enzymes involved in the activation of carcinogen[28]. The carcinogen 4-NQO is rapidly metabolized undergoing a reduction to 4-HAQO, through reductases. We can suppose that the BALB/C mice show a reduced or even absent capacity to metabolize this carcinogen in the tongue, due to the low activity of the reductases, which is in agreement with the assertion that the distribution of these enzymes within the oral cavity of rats is co-related with the distribution of carcinomas induced by 4-NQO, and that this activity is greatest in the tongue[2]. Previous study has shown differences of susceptibility and resistance of particular strains of animals[29]. It is possible to propose that BALB/C mice may have dominant genes that offer them cellular protection, principally in the lingual mucosa.

Observations, in vitro, that epithelial cells were resistant to the action of 4-NQO, when compared to fibroblasts, suggest a co-relation with the activity of GST (glutathione-S-transferase), which was significantly greater in lines of epithelial cells[18]. This susceptibility and resistance may be seen in humans where not all individuals exposed to potent carcinogens, such as tobacco, develop cancer. It may be suggested, that greater activity of GST in the lingual mucosa cells may offer resistance to 4-NQO in BALB/C mice. Future investigations are necessary to clarify this hypothesis.

Observation of areas of acanthosis in groups that received vitamin E are in agreement with those of Fonseca[24] which suggest that this vitamin has a role in the differentiation of epithelial cells. Moreover the fact that these areas were morphologically similar in all groups, suggests that this effect is not time dependent.

Subsequent studies must be amplified and encouraged in the experimental induction of oral cancer in order to understand the stages, as well as the protective effect of micronutrients.

CONCLUSIONS

There was no significant difference in the number of AgNOR when considering the time of application of the carcinogen, suggesting that the cellular proliferation did not increase with the time of application or that it was not reflected in the AgNOR average counts. Thus, it was not possible to evaluate the inhibitory effect of vitamin E in the indices of cellular proliferation. Areas of acanthosis observed in the HE samples suggest the role of vitamin E in cellular differentiation.

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RESUMO

Modelos animais têm se mostrado importantes para o entendimento de doenças multifatoriais com mecanismos complexos e eventos moleculares múltiplos como o carcinoma de células escamosas de boca. O 4-NQO exibe potente efeito carcinogênico em várias espécies animais produzindo mutações no DNA, alterando pares de bases, promovendo deleções e aberrações cromossômicas. Micronutrientes como a vitamina E podem inativar radicais livres e metabólitos reativos de oxigênio, que são potentes causas de dano ao DNA. O objetivo do presente estudo foi avaliar o impacto da vitamina E no índice de proliferação celular através da técnica de AgNOR, na mucosa lingual de camundongos BALB/C submetidos ao carcinógeno 4-NQO. Cento e doze camundongos BALB/C machos foram divididos em: grupo 1 – tratados com aplicações tópicas de 4-NQO 0,5%; grupo 2 – tratados com 4-NQO e 800 IU de vitamina E; grupo 3- tratados com 800 IU de vitamina E; grupo 4 – controle. Sete animais de cada grupo foram sacrificados em intervalos de 8, 16, 20 e 24 semanas. Não foram observadas diferenças estatisticamente significantes entre os diferentes grupos ou entre os diferentes intervalos de tempo, sugerindo que o índice de proliferação celular não aumenta com o tempo de aplicação do carcinógeno ou que não tenha sido refletido pela quantificação de AgNORs. Não foi possível, dessa forma, avaliar o efeito da vitamina E nesse índice.

Descritores: *4-NQO, carcinogênese experimental, AgNOR, língua*

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