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The effect of protocatechuic acid on the tongue mucosa in rats exposed to N-nitrosomethylbenzylamine

Wpływ kwasu protokatechowego na błonę śluzową języka u szczurów poddanych działaniu *N*-nitrozometylobenzyloaminy

INTRODUCTION

Oral carcinoma is a common term used for all the epidermal-origin neoplasm located in the oral cavity. Histologically, about 90% of them are squamous cell carcinomas of tongue and lips. Less frequently pathological process originates from the floor of the mouth, mucosa of the cheek, gingiva or palate [4]. Despite relatively easy diagnosis and well-established pre-cancerous lesions (leukoplakia, erythroplakia, oral submucous fibrosis), its worldwide incidence is high, since 640,000 new cases being revealed each year. The highest morbidity and mortality rate is noted in Southern Asia, India, China and Brazil [6, 16]. The most important risk factors are tobacco smoking and smokeless tobacco use, alcohol consumption, including chronic application of alcohol-containing mouthwashes, as well as human papillomavirus type 16 or 18 infections [6]. In many Asian countries betel quid (paam) chewing is also strongly related to that carcinoma. Only in India where such practice is common, the oral carcinoma represents about 40% of all malignancies, compared to 4% in the Great Britain and 8% in USA [16].

To better understand biology of the tumor and to evaluate new chemopreventive agents, a few standardized experimental animal models have been established [3]. Natural plant phenol – protocatechuic acid (PCA; 3,4-dihydroxybenzoic acid) displayed a protective effect in various rodent studies, including oral tumorigenesis induced by 4-nitroquinoline 1-oxide (4-NQO) [12]. In the current study the effectiveness of PCA is evaluated in rats exposed to different carcinogen – N-nitrosomethylbenzylamine (NMBA), usually used for esophageal tumor experiments. The richest

source of PCA is an oil of acai Palm (Euterpe oleracea) [5]. High amounts are also present in many fresh fruits and vegetables [1, 12]. It is worth to mention that doses of PCA applied in the study, i.e. 1000 and 2000 ppm are respectively 8 and 16 times higher than daily human consumption, since 10 g of lettuce or strawberry contains about 10-40 mg PCA/100 g.

MATERIAL AND METHODS

The experiment was carried out according to the National Institute of Health Guidelines for the care and use of laboratory animals and to European Council Directive on 24.11.1986 for Care and Use of Laboratory Animals (86/609/EEC), and approved by the Local Ethics Committee. Adult male Wistar CRL:(WI)WUBR rats were used for the study. NMBA (NARD, Japan) was applied subcutaneously between 3-8 weeks of the experiment (0.5 mg/kg/dose; 3 doses/week) (Fig. 1). PCA (Fluka Chemica, Switzerland) was administered with a daily diet in two doses 1000 (group NPX7, NPX24) or 2000 ppm (NPY7, NPY24) for the first 7 (NPX7, NPY7) or 24 (NPX24, NPY24) weeks of the experiment (groups NPX7, NPY7). The platted food was prepared by Agropol (Motycz, Poland). In group N rats were exposed exclusively to carcinogen and were kept on the basic diet.

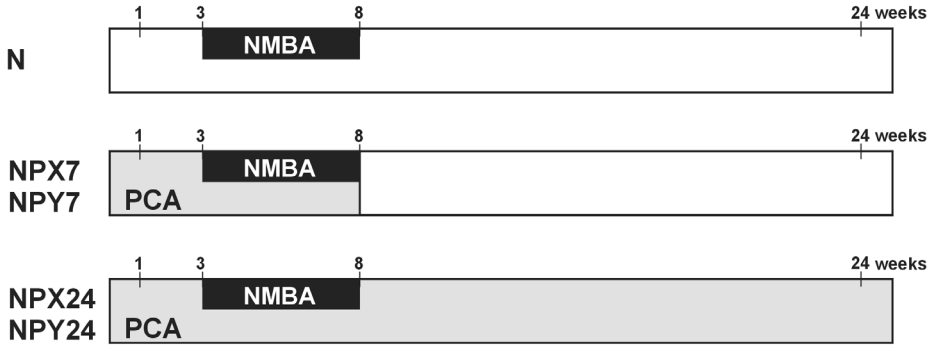


Figure 1. Protocol of the experiment. NMBA – N-nitrosomethylbenzylamine (N; 0.5 mg/kg 3 times per week), PCA – protocatechuic acid (P) in dose 1000 (X) or 2000 ppm (Y).

After 24 weeks, rats were sacrificed and grossly examined during autopsy. The incidence (percentage of rats with lesions), multiplicity (number of lesions/rat) and localization of lesions on the tongue were noted. Furthermore, formalin-fixed and paraffin-embedded samples from the organ were stained routinely with hematoxylin and eosin and than evaluated microscopically.

The obtained numerical data was presented using: arithmetical mean (M), minimal-maximal values (min-max) and standard deviation (SD). Differences were analyzed by ANOVA Kruskal-Wallis test. The 0.05 confidence level ($p<0.05$) was used as criteria of significance.

RESULTS

In most rats exposed to NMBA multiple lesions were grossly seen on the dorsal surface of the tongue (Fig. 2). They were randomly distributed; however larger lesions were more common in the intermolar prominence. They varied from small slightly elevated white plaques to big pedunculated or sessile exophytic tumors with irregular papillary surface. In all groups kept on the diet supplemented by PCA the multiplicity (number of lesions/rat) were lower than in exclusively NMBA-exposed group (Tab. 1). The lowest number of gross lesions was noted in NPY24 group ($p<0.05$).

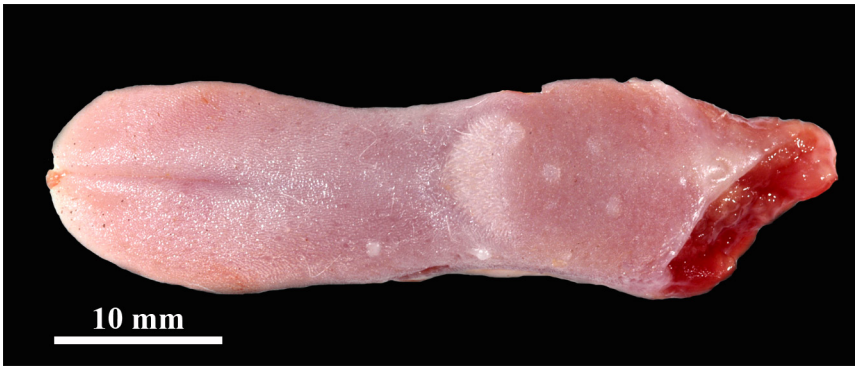


Figure 2. Multiple whitish plaques on the dorsal surface of the tongue in the carcinogen-exposed rat (NPX7 group).

Table 1. Incidence and multiplicity of gross lesions on the tongue in the carcinogen-exposed groups.

	n1	n2	%	min	max	M	SD	ANOVA
N	17	17	100.00	1.00	7.00	4.24	1.82	H = 9.57 p = 0.0483
NPX7	13	12	92.31	1.00	8.00	3.83	2.48	
NPY7	15	14	93.33	1.00	8.00	3.64	2.53	
NPX24	14	11	78.57	1.00	7.00	3.18	2.23	
NPY24	12	11	91.67	1.00	3.00	1.73	0.90	

n1 – number of rats at the end of the experiment; n2 – number of rats with lesions at the end of the experiment; (%) – percentage of rats with lesions at the end of the experiment (incidence); M – mean number of lesions/rat at the end of the experiment (multiplicity)

On microscopic examination the most common lesion was focal simple, acanthotic or papillary hyperplasia of the keratinized squamous epithelium of the tongue mucosa (Fig. 3A; Tab. 2). It was usually accompanied by prominent hyperkeratosis. Differences between multiplicities of hyperplasia in selected five rats from each group were insignificant (Tab.2). Low-grade dysplasia of the epithelium was noted sporadically (Tab. 3). In some animals squamous cell papillomas was observed with stromal edema and inflammatory infiltration or rarely with reactive epithelial changes (Fig. 3B; Tab. 3). Squamous cell carcinomas were not revealed.

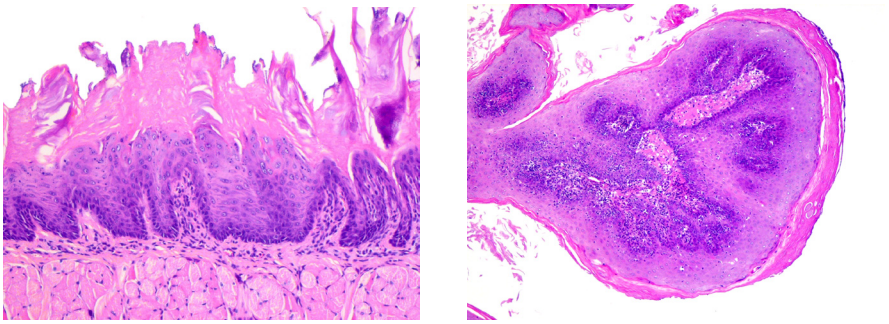
Table 2. Multiplicity of hyperplasia on the tongue of selected rats in the carcinogen-exposed groups.

	n	min	max	M	SD	ANOVA
N	5	4.00	8.00	5.80	1.79	H = 5.09 p = 0.2781
NPX7	5	2.00	6.00	4.00	1.58	
NPY7	5	1.00	5.00	2.80	2.05	
NPX24	5	2.00	6.00	4.20	1.48	
NPY24	5	1.00	6.00	3.60	2.07	

M – mean number of hyperplasia/rat at the end of the experiment (multiplicity)

Table 3. Number and percentage of dysplasia and squamous papilloma on the tongue of selected rats in the carcinogen-exposed groups.

	dysplasia		papilloma	
	n	%	n	%
N	2	40.00	4	80.00
NPX7	2	40.00	2	40.00
NPY7	2	40.00	3	60.00
NPY24	2	40.00	3	60.00
NPX24	0	0.00	2	40.00



A B

Figure 3. Hyperplasia and hyperkeratosis of the epithelium (A), and squamous papilloma (B) of the tongue in the carcinogen-exposed rat (A – NPY7 group; B – NPX24 group; hematoxylin and eosin, objective magn. A – 10x, B – 5x).

DISCUSSION

Obtained results indicate the dose- and time-dependent chemoprevention activity of PCA in the experimentally-induced oral tumorigenesis, since incidence of gross lesions was lower in groups treated by the compound, especially in higher dose, compared to exclusively NMBA-exposed animals. Moreover, insignificant changes were reported in rats receiving PCA for seven weeks. It is worth to mention that, in spite of oral and esophageal mucosal lesions, a significant increase of the body weight in group exposed to carcinogen and PCA for 24 weeks was observed. The food

and water intake, liver and kidney weight differed insignificantly in groups consuming diet rich in PCA when compared to exclusively NMBA-exposed group [10].

Such data is similar to previous experiments with PCA in which oral lesions were induced by 4-NQO in Fisher 344 rats [9, 13]. Both studies have different protocol, however similar outcomes. In the first one conducted by Tanaka et al. [13], PCA (500, 1000 and 2000 ppm) was administered in the initiation[1] and postinitiation[2] phase of carcinogenesis. Unlike our data a significant decrease of the body weight gain was reported in groups treated with PCA (1000, 2000 ppm) in the postinitiation phase, while decrease of liver weight was also found in groups receiving the lowest dose of PCA (500 ppm) in the initiation phase. Most lesions were seen on the dorsal surface of the tongue. Since 4-NQO is more specific carcinogen for oral mucosa, Tanaka et al. [13] were able to report a high incidence of the squamous cell carcinoma of the tongue – 58% in the carcinogen-exposed control group (11/19 – 11 cases from 19 exposed rats). The neoplasm was sporadically observed in groups dosed with PCA during the initiation (500 ppm: 5%, 1/20) and postinitiation phase (500 ppm: 5%, 1/20; 1000 ppm: 11%, 2/20). The incidence of squamous papilloma was similar in all the examined groups exposed to carcinogen. The number of hyperplasia and dysplasia in exclusively 4-NQO-exposed animals was 89 and 58%, respectively. PCA significantly decreased the incidence of both preneoplasmatic lesions in all groups treated with the compound and similar to our study the effect was dose-dependent.

Partly similar data was also reported by Suzuki et al. [9]; however 4-NQO was administered for four initial weeks with or without an additional fourth cycle of two weeks exposure with two carcinogen-free weeks interval from 18 to 32 weeks of the study. PCA (2000 ppm) was given in the diet at week 16th and continued until the end of the experiment. The PCA treatment significantly increased liver weight and decreased the number of the squamous cell carcinoma of the tongue that incidence drop from 47% (7/15) in groups in which the carcinogenesis was accelerated with an additional exposure to 4-NQO to 10% (2/20). Interestingly, carcinoma did not develop in any rats at week 16 and 24. Similar to Tanaka et al. [13] the incidence of papilloma was similar in both carcinogen-exposed groups with (32%, 5/15) or without the acceleration (16% - 3/19) and among animals obtaining PCA (10%, 2/20). However, the total incidence of hyperplasia and dysplasia in groups dosed with PCA was high – 100 (20/20) and 90% (18/20), respectively. In the first case, it was similar for the animals with accelerated carcinogen exposure (100%, 15/15) or significantly increased when compared with exclusively 4-NQO-exposed rats for the first weeks of the study (68%, 13/19). Similar data was reported for dysplasia – 100% (15/15) and 58% (11/19), respectively.

The current and above mentioned reports [9, 13], as well as other models of experimentally induced urinary bladder [2], colonic [14], hepatic [15] and esophageal [10] tumorigenesis, proved that PCA is an effective chemoprevention agent during both initiation and postinitiation phases. It was postulated that such activity is associated with antioxidative and antiproliferative properties of PCA [9, 10, 13]. Furthermore, modulation of the activity of enzymes involved in carcinogen metabolism

[1] One week before carcinogen administration, though out seven weeks of 4-NQO exposure and week after the carcinogen dosage was terminated (1-10 weeks of the study protocol).

[2] From 10th to 32nd week of the study protocol.

(i.e., cytochrome P450 isoenzymes), inhibition of DNA adducts formation and expression of gene engaged in proliferation, apoptosis and angiogenesis are also important [10, 12].

It should be mentioned that similar chemoprevention activity in experimental oral carcinogenesis was also revealed for many natural or synthetic agents including caffeic, chlorogenic, ellagic and ferulic acids, resweratrol, hesperidin, curcumin, garcinol, indole-3-carbinol, diallyl sulfide, green tea polyphenols, Zeng Sheng Ping (mixture of six traditional Chinese herbs) as well as piroxicam, nimesulid or troglitazone [3,7,8,11].

In conclusion, it could be stressed that PCA is a chemoprevention agent in experimental oral tumorigenesis in rats. However, additional studies with different laboratory animals and other preclinical observations are desirable before the results are transferred to human practice. It could be also stressed that the NMBA is not a potent carcinogen for the oral mucosa due to the low incidence of neoplasm. In such studies a more effective substance, like 4-NQO should be used.

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ABSTRACT

The effect of natural plant-derived phenol – protocatechuic acid on lesions induced by carcinogen – N-nitrosomethylbenzylamine in the squamous epithelium of the tongue was investigated in rats. Carcinogen was applied for 3-8 weeks of the experiment (0.5 mg/kg/dose; 3 doses/week). Protocatechuic acid was administered with a daily diet in two doses 1000 or 2000 ppm for first 7 or 24 weeks of the experiment. Incidence and multiplicity of gross lesions on the dorsal surface of the tongue was the highest in rats exposed exclusively to carcinogen. Multiplicity of lesions was significantly lower in the group fed with diet containing protocatechuic acid in higher dose for the whole experiment. The most frequent lesion in the microscopic examination was hyperplasia of the squamous epithelium. Dysplasia and papillomas were rarely seen, while carcinomas were not observed. Chemoprevention activity of protocatechiuc acid in applied experimental model was confirmed.

Keywords: protocatechuic acid, N-nitrosomethylbenzylamine, experimental carcinogenesis, tongue, rat

STRESZCZENIE

W pracy oceniono wpływ naturalnego roślinnego związku fenolowego – kwasu protokatechowego, na rozwój zmian rozrostowych nabłonka wielowarstwowego płaskiego rogowaciejącego języka indukowanych przez karcynogen – N-nitrozometylobenzyloaminę u szczurów. Karcynogen podawano podskórnie w dawce 0,5 mg/kg m.c. trzy razy w tygodniu od 3 do 8 tygodnia doświadczenia, natomiast kwas protokatechowy w paszy w dawkach 1000 lub 2000 ppm przez pierwsze 7 lub 24 tygodnie. Częstość i liczba zmian makroskopowych na powierzchni grzbietowej języka była najwyższa u zwierząt otrzymujących wyłącznie karcynogen. Liczba zmian była istotnie niższa w grupie otrzymującej kwas protokatechowy w większej dawce przez całe doświadczenia. W badaniu mikroskopowym najczęściej występującymi zmianami były rozrosty nabłonka płaskiego; dysplazja i brodawczaki płaskonabłonkowe występowały rzadziej. Nie stwierdzono obecności raków. Badania potwierdziły działanie chemoprewencyjne kwasu protokatechowego w zastosowanym modelu doświadczalnym.

Słowa kluczowe: kwas protokatechowy, N-nitrozometylobenzyloamina, doświadczalna karcynogeneza, język, szczur