

Squamous Cell Carcinoma of the Tongue in Sprague-Dawley Rats using 4-Nitroquinoline 1 Oxide Model

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ABSTRACT

Background. 4-nitroquinoline 1-oxide (4NQO) is a water soluble carcinogen which is often used to induce cancer in oral cavity. **Objective.** The purpose of this study was to know the incidence of histopathological lesions in the tongue of rats after administration of 4-nitroquinoline 1-oxide (4NQO) for a model of oral carcinogenesis in the animal experimental laboratory. **Materials and Methods.** This study was conducted in the Department of Pathological Anatomy, Faculty of Medicine, University of Indonesia (FMUI). 4NQO (30 ppm) was administered by drinking water *ad libitum* to the Sprague Dawley (SD) rats during different time inductions (4-9 and 36-46 weeks). The present of tumor in oral cavity of the 4NQO-treated rats were observed macroscopically and microscopically. Tumor volume were measured based on the formula established by G. Carlsson. The degree of histological changes was determined according to observation towards their Hematoxylin and Eosin (HE) staining results. **Results.** Dysplasia and invasive squamous cell carcinoma (SCC) were respectively found in the tongue of the group given 4NQO for 4-9 and 36-46 weeks. The tongue from rats (4NQO for 36-46 weeks) exhibited whitish protuberant nodular lesions with the longest diameter was 17 mm. The histopathological lesion incidences for dysplasia and invasive SCC respectively were 25% (5/20) and 75% (15/20). The rats had tumor in their tongues with variation of degree alteration which proportional to the induction time. The extension of 4NQO induction time increasing the degree of change in the tumor. **Conclusion.** 4NQO represents a good model of carcinoma of tongue and gives consistence results with histological changes degree being related to duration of exposure.

Key words: Tongue carcinoma, Sprague-Dawley, 4NQO, incidence.

ABSTRAK

Latar belakang. 4-nitroquinolin 1 oksida (4NQO) merupakan karsinogen yang dapat larut dalam air yang sering digunakan untuk menginduksi kanker dalam rongga mulut. **Tujuan.** Maksud dari studi ini adalah untuk mengetahui insidensi lesi histopatologi pada lidah tikus setelah pemberian 4-nitroquinolin 1 oksida (4NQO) sebagai sebuah model karsinogenesis dalam suatu laboratorium hewan coba. **Bahan dan Metode.** Studi ini dilakukan di Departemen Patologi Anatomi, Fakultas Kedokteran Universitas Indonesia (FKUI). 4NQO (30 ppm) diberikan melalui air minum *ad libitum* pada tikus Sprague Dawley (SD) dalam waktu induksi yang berbeda-beda (4-9 dan 36-46 minggu). Keberadaan tumor dalam rongga mulut pada tikus yang diberi 4NQO diamati secara makroskopik dan mikroskopik. Volum tumor diukur berdasarkan formula yang ditetapkan oleh G. Carlsson. Derajat perubahan histologic ditentukan menurut hasil observasi pewarnaan Hematoksilin dan Eosin (HE). **Hasil.** Displasia dan karsinoma sel skuamosa masing-masing ditemukan pada lidah kelompok yang diberi 4NQO selama 4-9 dan 36-46 minggu. Lidah dari tikus (yang diberi 4NQO selama 36-46 minggu) menunjukkan lesi nodular menonjol berwarna keputihan dengan diameter terpanjang 17 mm. Insidensi lesi histopatologik dysplasia dan SCC invasive masing-masing yakni 25% (5/20) dan 75% (15/20). Tikus-tikus itu memiliki tumor pada lidahnya dengan derajat perubahan bervariasi yang proporsional dengan waktu induksi. Perpanjangan waktu induksi 4NQO meningkatkan derajat perubahan tumor tersebut. **Kesimpulan.** 4NQO merupakan suatu model karsinoma lidah yang bagus dan memberikan hasil yang konsisten dengan derajat perubahan histologik yang berkaitan dengan lamanya pemaparan.

Kata kunci: Karsinoma lidah, Sprague Dawley, 4NQO, insidensi.

INTRODUCTION

Anatomically, the tongue is divided into the oral tongue and the base of the tongue which are a subsite of the oral cavity and the oropharynx respectively. Squamous cell carcinomas (SCC) are malignant neoplasia that originate in the squamous epithelium.¹ There are the decreasing of incidence of oral cavity squamous cell carcinoma (OCSCC) and the reverse for oral tongue squamous cell carcinoma (OTSCC)¹ and oropharyngeal SCC (OPSCC).²

OTSCC is the most common type of oral cancer, and contributes to about 40-60% of all oral cancer deaths. Incidence of OTSCC in the United States was 12,770 in 2012.¹ Comprehension about histopathology and molecular mechanisms of tongue carcinogenesis may support the development of novel strategies for the prevention and therapy of tongue cancer. The animal models often required in the researches that associated with the comprehension. The most used carcinogen to induce OTSCC specifically and several organs generally that applied in low concentration via water *ad libitum* is 4-nitroquinoline 1-oxide (4NQO). The carcinogen mediated experimental

carcinogenesis is similar to human oral carcinogenesis.^{3,4} This study is the first research of oral cancer animal model in animal facilities of Department of Pathological Anatomy, FMUI. Our study is intended to know the incidence of histopathological lesions in the tongue of Sprague-Dawley (SD) rats after administration of 4NQO for a model of oral carcinogenesis. Histological determination of a tumor is the accepted end point in carcinogenicity testing.⁵

MATERIALS AND METHODS

This study was conducted in the Department of Pathological Anatomy, Faculty of Medicine, University of Indonesia (FMUI). 4NQO (30 ppm) was administered by drinking water *ad libitum* to the Sprague Dawley (SD) rats during different time inductions (4-9 and 36-46 weeks). The present of tumor in oral cavity of the 4NQO-treated rats were observed macroscopically and microscopically. Tumor volume were measured based on the formula established by Carlsson. The degree of histological changes was determined according to observation towards their Hematoxylin and Eosin (HE) staining results.

Animals.

All procedure and care given to the SD rats were conducted according to and after was obtained the approval from respectively Institutional Animal Care Guidelines and the Institutional Research Ethics Committee of Faculty of Medicine, University of Indonesia (FMUI). SD rats bred by Badan Pengawas Obat dan Makanan RI (BPOM-RI) on May in 2014. The research conducted in the animal experimental laboratory, Department of Pathological Anatomy, FMUI. Twenty five male SD rats,³ weeks old were used for this study. They were housed two to a plastic cage with the top wire. The rats were fed with standard rat diet. At 6 weeks of age the rats were transferred to the holding room and randomized into experimental and control groups. The holding room was maintained at 27±2°C and 50±10% humidity, with 12 hrs light/dark cycle.

Chemicals.

4NQO was purchased from Sigma-Aldrich (≥ 98 % pure, lot no. 5LBG4397V, Pcode: 1001565723). Drinking water containing 50 ppm of 4NQO was prepared twice a week and was given in dark bottles.

Development of animal model.

After 2 weeks of acclimatization. At 6 weeks of age, each rat was randomly divided into a treatment group (n=20) and a control group (n=4) which the drinking water contained 4 NQO (30 ppm) and no 4NQO respectively. The mean weight of the rats at the time was 58.88 gram (SD±6.16 gram). Animals were weighed twice weekly. The rats were analyzed macroscopically for lesion on their tongue surface. The rats which shown the precancerous lesion and weight loss after 4-9 weeks 4NQO-induced carcinogenesis⁶ (as group I) euthanized under deep ether anesthesia. Macroscopic observations were performed and then the tongue were processed for histopathological confirmation after being fixed in 10% buffered formalin, embedded in paraffin blocks, and processed by methods using hematoxylin and eosin stain.^{6,7} Determination of the length of induction is based on some information about oral cancer carcinogenesis induced 4NQO in some strains of rat.⁸ The design of this longitudinal study was designed to monitor and the development of oral carcinogenesis. Choosing of the rat that euthanized

in group I based on the present reduction of body mass significantly and the morphological changes on the tongue surface macroscopically. Other rats in the group I continued to be induced by 4NQO up to 36-46 weeks (as group II).⁹ The number of rats that had been euthanized at 4-9 weeks to be replaced included in group II. All the rats of group II were killed when they became moribund.

Pathological examination.

The grades of oral epithelial dysplasia are hyperplasia, dysplasia (the term was introduced by Reagon in 1958 in relation to the cells exfoliated from lesions of the uterine cervix)⁹ and carcinoma *in situ*.^{10,11} Histopathological features of oral squamous dysplasia comprise morphological or architectural (Bulbous, teardrop-shaped rete ridges, loss of polarization and orientation, exaggerated intercellular spacing) and cytological features (increased nuclear/cytoplasmic (N/C) ratio, nuclear hyperchromasia, nuclear polymorphisms with prominent nucleoli and excessive or abnormal mitotic figures).^{10,11} When a quarter of the lowest layer epithelium shown dysplastic characteristics, a diagnosis of mild dysplasia is appropriate term. When half and three-fourths of the epithelium exhibit at least two of the features we use respectively the term moderate and severe epithelial dysplasia.^{9,11} Carcinoma *in situ* will occur after dysplasia, it represents an intraepithelial malignancy. There are not invasion and keratin pearl formation.

SCC is characterized by squamous differentiation (often seen as keratinization, sometimes with keratin pearl formation) and invasive growth with disruption of the basement membrane. There are three types of SCC differentiation types. They are well, less well or moderate and poor-differentiated (anaplastic) types.¹¹ In the well-differentiated type, the cancer cells are obviously squamous in type and consist of masses of prickle cells (often found keratin pearls in the mass) with a limiting layer of basal cells. Each pearl consisting of a central area of keratin surrounded by whorls of prickle cells. The keratin pearls are rarely or absent in moderate differentiated of SCC, and the prickle cells and their nuclei are more pleomorphic. There are mitotic figures abundant usually and many may atypical.^{11,12} In anaplastic type of tumor, the cancer

cells are even more irregular and may hardly be recognizable as being of epithelial origin.¹¹

Tumor dimensions (a=longest diameter and b=shortest diameter) were measured by Anyview software digital microscope mobile. The volume of tumor (V) were calculated by G. Carlsson formula, $V = a \times (b)^2 / 2$.^{13,14} The length of the diameters (longest and shortest) which showed of Anyview software must be divided with the magnification.

Statistical analysis.

Statistical analysis was conducted with SPSS standard version 20.0 software. The correlation between duration of treatment with the occurred type of histopathological lesion was analyzed by predictive ordinal logistic regression (CI 95%). Correlation between the duration of 4NQO application with body mass of rat and volume

tumor in the group II was analyzed by Pearson correlation test.

RESULTS

General observation.

A total of 24 rats survived at the end of the experiment. Five rats in group II died from unidentified cause. No macroscopic metastases were observed in any of the rats. The mean of their body mass are shown in the table 2. *Occurrence of tongue lesions.* On the time course study, an early induction of grossly visible tongue lesions including dysplasia and tumor was found but not obviously. At group II, the incidence and number of tongue tumors were greater than that of the group I (Table 1). The mean of their body mass and measurement of tumor in 2 dimensional are shown in the table 2.

Table 1. Incidence of histopathological lesion in tongue of rats in the 4-nitroquinoline 1-oxide (4NQO)* model for Oral Tongue Squamous Carcinoma Cell.

Group	Weeks	uber of animal	Lesions			
			Normal	Hyperplasia	Dysplasia	Carcinoma
Control		4	4	0	0	0
I		20				
	4		0	0	Mild (n=2)	0
	7		0	0	Mild (n=2)	0
	9		0	0	Moderate (n=1)	0
II		20				
	36 - 46		0	0	0	invasive (n=15)**

Note:*4 NQO - 30 ppm by drinking water, ** with papillary tumor as SCC variant (n=5),* OTSCC grade I (n=8) and OTSCC grade II (n=7).

Tabel 2. Clinicopathological characteristic for the rats.

	Group I		Group II		Control	
	Value	P value	Value	P value	Value	P value
Mean of						
Body mass (g) (final)*	99.55-65.84		209.40 ± 11.93	0.18	363±47.34	
d1 (mm)*	-	0.00	14.75 ± 1.14	0.98	-	0.907
d2 (mm)*	-		9.58 ± 1.08	0.08	-	
tumr volume (mm ³)*	-		708 ± 224.10	0.19	-	

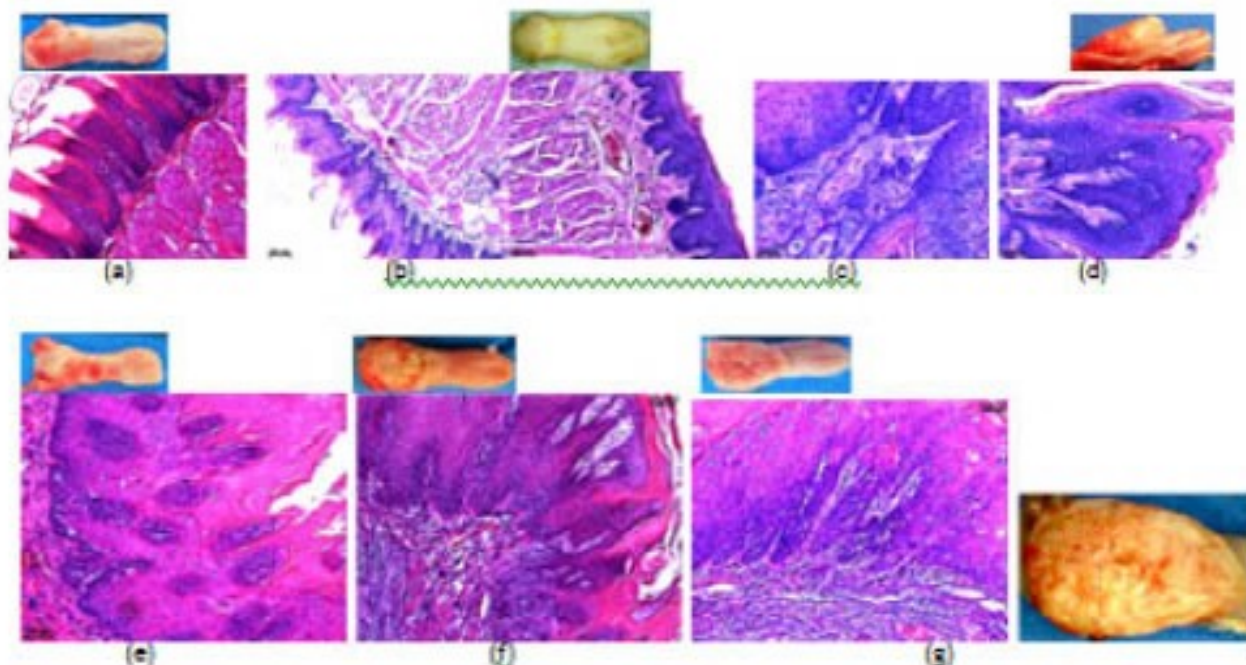


Figure 1. Macroscopically (upper) and microscopically (lower) examinations of tongue tissue of the rat. Normal tongue (1.a); dysplasia, after 7 and 9 weeks 4 NQO-treated respectively (1.b., the tongue was soaked in 10% buffer formalin) and (1.c); OTSCC after 36 and 46 weeks of 4 NQO administration respectively (1.d) and (1.e); the ocell carcinoma spread into submucosa and underlying muscle layers (not continuous view of the preparat) (1.d. left) and the epithelium layer (1.d. right); OTSCC with papillary tumor variant on the side region of the tongue, the papillary tumor showing koilocytes cells and broad-based bulbous to exophytic growth with rounded projections resembling a cauliflower-like growth pattern (1.f); The measurement of OTSCC volume with *Anyview* software in magnification 30x (1.g. right). (Hematoxylin and Eosin staining; x100 magnification, bar = 50

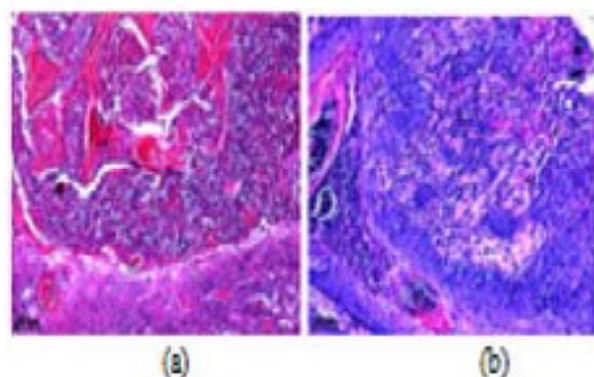


Figure 2. Differentiation types of invasive OTSCC. Well-differentiated (grade I) (2.a.) and moderate-differentiated (grade II) (2.b.) invasive OTSCC respectively after 36 weeks and 42 weeks 4 NQO administration. (Hematoxylin and Eosin staining; x100 magnification, bar = 50 μ m).

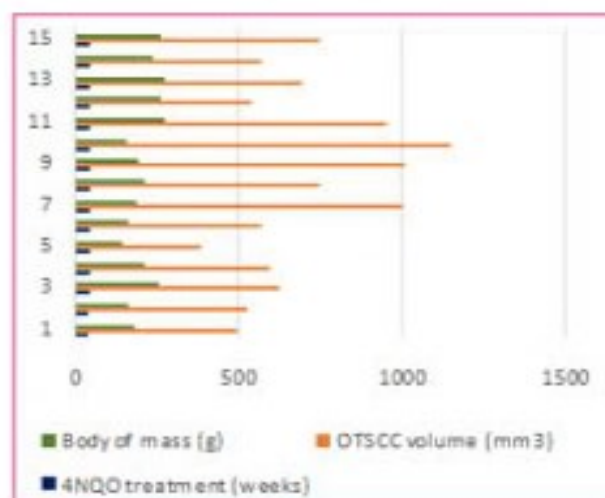


Figure 3. Clinicopathological characteristic for rats OTSCC+ in the group II

Statistical results.

Model fitting information in ordinal regression analysis proved that complementary log-log model with duration of 4NQO administration ($p=0.000$) as independent variable which histopathological lesion types as dependent variable, as a good model. Goodness of fit test shown the value of

Chi-square for Pearson was 10.573 with $p=0.999$ (> 0.05). It means that the data were appropriate with the ordinal logistic regression model prediction. The signification of duration of 4NQO administration value in parameter estimates was 0.002 (< 0.005). Nagelkerke value from Pseudo R-square was 95.6%. Shapiro-Wilk test results

informed that the distribution data of body mass and volume tumor of rats was normal. Coefficient correlations between the duration of 4NQO application with body mass of rat and volume tumor in the group II were 0.515 ($p = 0.049$) and 0.441 ($p = 0.066$) respectively.

DISCUSSION

The objective of chemical carcinogenesis models is to provide further understanding about the multistep process of carcinogenesis include biomolecular process in a certain cancer in order to develop strategies for early diagnostic, cancer prevention and cancer therapy. There are available many commercial pure carcinogens. 4NQO is the appropriate carcinogen for induction oral cancer in animal model.(17) 4NQO is a potent mutagen and carcinogen even can stimulate apoptosis in various types of cell caused its capability UV-mimetic agent leading to DNA damage.(18) Carcinogenesis requires time and appropriate environment. Our study presents the incidence of histological lesion in our animal model for OTSCC in different of duration of carcinoma induction with 4NQO by drinking water ad libitum. There were some differences observed and measured. They are discussed as follows.

In our study there were no differences of histopathological found in tongue of the rat after 4 weeks 4NQO administration with those given for 7 weeks. The differences of the features exhibited between the tongues from the rat which 7 and 9 weeks 4NQO-treated. The alteration occurred in the quarter of the lowest layer epithelium (fig. 1. b) whereas in the tongue of the rat which administrated 4NQO 2 weeks longer the histological changes got in the half of epithelium. The histological changes were architectural changes (teardrop-shaped rete ridges, loss of polarization and orientation, exaggerated intercellular spacing) and cytological changes (nuclear/cytoplasmic (N/C) ratio, nuclear hyperchromasia, nuclear polymorphisms with prominent nucleoli and excessive or abnormal mitotic figures). So there were epithelial dysplasia in mild and moderate forms as shown in figures 1. b. and 1.c. respectively.

Histopathological examination found the tumor spread into the submucosa and underlying muscle layer, forming small nests with typical

keratin pearl formation (fig. 1.d-g). Figure 1.d. (left) indicating the basaloid-appearing cells showing marked increased mitotic activity, pleomorphism, hyperchromatic nuclei, and scanty cytoplasm. Note the existence of peripheral nuclear palisading.

The present of koilocytes cells and exophytic growth with a cauliflower-like growth pattern are characteristic for papillary tumor as variant in squamous carcinoma.(4) Koilocytes is as thought a microscopic indicator of HPV infections.(19) In 1956, Koss and Durfee, et. al., named the squamous cells with perinuclear clearing surrounded by a thin coil of cytoplasm, as "koilocytes" (Greek word, *koilos* = hollow cell),(19) Despite true prevalence of HPV in OSCC is not clear, there was 30% (5/15) of the rats with OTSCC in group II had papillary tumor(s) on their surface tongue.

We found three types of the pattern of tumor invasion (POI) on architectural feature of the tongue tissues of group II. POI refers to the manner in which cancer infiltrates tissue at the tumor/host interface. POI type 3 where the island at tumor greater larger than 15 cells/island which the type 4 where the island consists ≤ 15 cells/island. POI type 5 (20-22) The types were POI type 3, 4 and 5 which showing in figure 1.d. and 1.e., 1.f., and 1.g. respectively. Clinically, the prognosis of the OTSCC patient shows POI type I which a pushing-border invasion pattern is better than for those with other POI types.(23)

Figure 1 (d-g) exhibits eosinophils in filtrate was associated with stromal invasion, it was prominent within the tissue surrounding the OSC tumor mass.(24) This is appropriate with suggestion of Falconieri et al. and Oliveira et al, but is contrary to Dorta and colleagues study that prove high rates of tumor-associated tissue eosinophilia represent a favorable prognostic factor in clinical TNM stage II and III OSCC from floor of the mouth, oral tongue, retromolar area and inferior gingiva.(25, 26) The initial recruitment and activation of eosinophils toward the tumor microenvironment is principally related with Th2 response.(26) Advancing lesions of the OTSCC- induced 4NQO have down regulated and up regulated cytokine production of Th1 and Th2 respectively. The enhancing of Th2 cytokine also presented in peripheral blood of tobacco smoking.(27) This is one of the reasons of using 4NQO as carcinogen in oral cancer researches.

As shown in figure 2., there were lack of keratin pearls in the mass of OTSCC grade II. We found OTSCC grade I and OTSCC grade II in tongue of 8 and 7 rats in group II respectively. Statistical results of both treatment groups proved that the equations for our ordinal regression model obtained were 0.209 - 0.123 duration of 4NQO administration; 1.925 - 0.123 duration of 4NQO administration and 5.012 - 0.123 duration of 4NQO administration. The Nagelkerke value indicated that variable of duration of 4NQO administration capable to explained 95.6% of variation of the histopathological lesion degree.

The using Carlsson formula to measured volume tumor was caused it is the best formula to measure an unremoved tumor like OTSCC. (14) 'Anyview' software helped us to measure a volume tumor in mm unit accurately. Clinicopathological data (fig. 3) exhibited probability the effects of duration of the carcinogen toward body mass of rat and its volume tumor. Although the biggest tumor was not present in the smallest rat, the 3 smallest tumor possessed by the 3 rats after 36 - ≤ 42 weeks 4NQO treated.

Pearson correlation test evidenced that the strength of correlation between duration of 4NQO administration was quite significantly with tumor volume of the rats but not correlation significantly with the rat body mass. All rats in group II suffered OTSCC and survived more 180 days. Our animal model produced a consistent OTSCC 75% (15/20) in incidence with cancerous lesions produced which similar pathology with tongue cancer in human. The points are the key elements for the ideal animal model. (28)

CONCLUSION

4NQO represents a good model of carcinoma of tongue and gives consistence results with histological changes degree being related to duration of exposure. Authors' Disclosures of Potential Conflicts of Interest The author(s) indicated no potential conflicts of interest.

ACKNOWLEDGEMENTS

This study was supported by a Grant-in-Aid from the Ministry of Research, Technology and Higher Education of the Republic of Indonesia

(contract no. 100/K3/KM/2015).

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