Anticarcinogenesis effect of Gynura procumbens (Lour) Merr on tongue carcinogenesis in 4NQO-induced rat

D. Agustina*, Wasito**, S.M. Haryana***, and A. Supartinah ****

* Oral Medicine Department, Faculty of Dentistry, Gadjah Mada University
** Pathology Department, Faculty of Veterinary Medicine, Gadjah Mada University
*** Histology and Molecular Biology Department, Faculty of Medicine, Gadjah Mada University
**** Pedodontics Department, Faculty of Dentistry, Gadjah Mada University

Jogjakarta - Indonesia

ABSTRACT

In Indonesia Gynura procumbens (Lour) Merr leaves have been long used as various cancers medication. Many in vitro and in vivo studies have demonstrated anticarcinogenesis of ethanol extract of Gynura procumbens leaves. The aim of this study was to investigate the anticarcinogenesis of the ethanol extract of Gynura procumbens leaves on 4 nitroquinoline 1-oxide (4NQO)-induced rat tongue carcinogenesis. Fifty six 4 week old male Sprague Dawley rats were used in this study and divided into 7 groups. Gro up 1, 2 and 3 were lingually induced by 4NQO for 8 weeks. In groups 2 and 3 the extract was given simultaneously with or after 4NQ O induction finished, each for 10 weeks and 26 weeks, respectively. Groups 4, 5 and 6 were induced by 4NQO for 16 weeks. However, in groups 5 and 6 the extract was given as well simultaneously with or after the 4NQO induction, each for 18 weeks, respectively. Group 7 served as the as untreated control group. The results from microscopical assessment showed that tongue squamous cell carcinomas (SCC) developed in 100% (3/3) of group 1. However, only 33.3% (2/6) and 25% (2/8) of rats in groups 2 and 3, respectively demonstrated tongue SCC. Among groups 4, 5 and 6, no significant difference of tongue SCC incidence was observed. From these results it is apparent that the ethanol extract of Gynura procumbens leaves could inhibit the progression of 4NQO-induced rat tongue carcinogenesis in the initiation phase.

Key words: Gynura procumbens, 4NQO, rat, tongue carcinogenesis


INTRODUCTION

Oral cancer is a serious public health problem although the incidence of this disease is much lower in comparison with, other malignancies such as prostate, colon, breast and uterine cancers. Overall mortality rate of oral cancer remains high, at approximately 50%, despite of modern medical services, which is probably due to the advanced stage of the disease at presentation.1 Around 86.8% of patients seeking medication usually have developed advanced condition or even metastatic.2 Those patients tend to have poor prognosis and low survival rate, resulting a short life expectancy. Conventional cancer treatment such as radiotherapy and chemotherapy often generate unfavorable effects to the patients, such as lowering the patient's immune system due to the destruction of normal cells. The extent of involved areas causing surgical treatment to create disfigurement and dysfunction of oral and facial tissues, which further leads to quality of life decrease in oral cancer patient. Based on these facts, currently more Indonesians return to the use of traditional herbal remedies as an empirical alternative therapy which is potential to cure cancers by loweing the side effects with a lower cost.

According to interview with local residents in Magelang, Jawa Tengah, Gynura procumbens (Lour) Merr has been long used to treat several cancers such as leukaemia, uterine and breast cancers.3 This plant is easily found in Indonesia and in various parts of South East Asia. Three fresh leaves are consumed daily for certain period of time, depending on the disease severity. Previous study reported that consumption of the ethanol extract of Gynura procumbens leaves might inhibit the occurrence of lung cancer up to 23% in newborn mice after being induced by benzopyrene on the first, eighth and fifteenth days.4 In vitro study also showed that the ethanol extract of Gynura procumbens leaves had a cytoxic effect on myeloma cell culture with LC50 of 72 μg/ml.5 Recent data demonstrated that the extract was also effective in inhibiting the pre-initiation and initiation phases of stomach carcinogenesis in Swiss mice after induction of benzopyrene, which is showed by, the percentage of animals bearing tumour and the number of tumour nodules.6 A Similar result also occurred which is in mammary carcinoma of Sprague Dawley rat induced by DMBA for
10 times with a dosage of 20 mg/kg body weight during the initiation phase. The incidence of mammary carcinoma was reduced up to 40% until the end of the experiment. The most recent study showed an anti angiogenic effect of the ethanol extract of Gynura procumbens leaves on the chorioallantois membrane of chicken after bFGF induction.

Those findings promote the anti carcinogenic potency of Gynura procumbens leaves for cancer therapy which should be elucidated in more detail. Current literatures, report no previous study or research on anti carcinogenic effect of the ethanol extract of Gynura procumbens leaves in oral carcinogenesis. In the light of different etiology that leads to different pathways in oral carcinogenesis, it is interesting to investigate the role of the ethanol extract of Gynura procumbens in inhibiting the oral carcinogenic process. The goal of the present study is to elucidate the inhibitory effect of the ethanol extract of Gynura procumbens leaves in oral carcinogenesis by comparing clinical and histopathological appearances in rat tongues between rats given 4 nitroquinoline 1-oxide (4NQO) and the extract with those given by 4NQO alone.

4 nitroquinoline 1-oxide (4NQO) is a full carcinogenic agent. It can function either as an initiator or a promoter of cancer process. It can also be classified as an indirect carcinogen since it must be metabolically activated in vitro to produce “ultimate carcinogens”, which are involved in the induction of tumours. Previous in vitro studies have been used 4NQO to induce malignancy in oral cavity, either by water drinking or direct application on oral mucosal tissue for a certain period of time. Squamous cell carcinoma (SCC) produced by 4NQO behaved in a similar way to those of humans. According to Nauta et al., creating an oral carcinogenic model in rat by inducing 4NQO is appropriate to mimic a human oral carcinogenesis since they have similarities either in histopathologic or immunologic characteristics. The significance of this study may serve as scientific evidence for the industrial development of Gynura procumbens leaves as anti oral carcinogenic agent.

MATERIALS AND METHODS

Animals used in this study were 56 male Sprague Dawley rats (F54), aged 4 weeks with average body weight of 63 g (PPOM, Food and Drug Research Center, Jakarta, Indonesia).

Carcinogen agent used was 4-nitroquinoline 1-oxide (4NQO) (Sigma Chemical Company, Australia), dissolved in propane-1,2-diol (PD) (Sigma Aldrich Chemic Gmbh, Germany) to a final concentration of 0.5% (m/v).

Ethanol extract of Gynura procumbens (Lour) Merr leaves was obtained by Soxhletation method, with 80% ethanol and suspended in 0.5 ml aquadest.

4 nitroquinoline 1-oxide (4NQO) solution was applied using a no. 2 artists’ brush which delivered a relatively constant volume (0.15 mg). A single brush stroke was applied from posterior to anterior of the dorsal surface of rat tongue three times weekly. The extract was given through oral intubation twice weekly with a dosage per administration equivalent to 3.5 g dry leaves (simplicia)/kg body weight.

![Figure 1. Design of the study.](image-url)
Design of the study (Figure 1): Group 1 (n = 6) consisted of rats given 4NQO alone for 8 weeks (4NQO-8 wk); Group 2 (n = 9) consisted of rats given 4NQO for 8 weeks and the extract simultaneously. The ethanol extract of Gynura procumbens was administered for 10 weeks commencing one week before the 4NQO exposure and terminated one week after 4NQO induction finished (4NQO-8 wk + extract); Group 3 (n = 11) consisted of rats given 4NQO for 8 weeks and the extract afterwards. The extract was administered for 26 weeks, starting 1 week after the cessation of 4NQO treatment until the end of the experiment (4NQO-8 wk → extract); Group 4 (n = 9) consisted of rats given 4NQO alone for 16 weeks (4NQO-16 wk); Group 5 (n = 9) consisted of rats given 4NQO for 16 weeks and the extract simultaneously. The ethanol extract of Gynura procumbens was administered for 18 weeks commencing one week before 4NQO exposure and terminated one week after 4NQO induction finished (4NQO-16 wk + extract); Group 6 (n = 6): rats were given 4 NQO for 16 weeks and the extract afterwards. The extract was administered for 18 weeks, starting 1 week after the cessation of 4NQO treatment until the end of the experiment (4NQO-16 wk → extract); Group 7 (n = 6) served as an untreated control group.

Animal experimentation: Every 2-4 rats were housed in stainless steel and plastic cages at constant humidity (50–55%) and temperature (± 25 °C) with 12 hours light in the Animal House of Pharmacology and Toxicology Department, Faculty of Pharmacy, Gadjah Mada University, Jogjakarta, Indonesia. They were fed AD-II pellets (PT Japfa, Sidoarjo, Indonesia) and had tap water available ad libitum.

During the course of study, the animals were weighed biweekly and general inspection of the general health was carried out daily to record any macroscopic changes. Should the general condition deteriorate significantly, the rat was terminated by overdosed ether. To observe the progression of tongue carcinogenesis, three rats from groups 1, 2, 3, 4, 5, and 7 were terminated in the middle of the experiment (the 19th week) to allow histological examination. The survivors (17 of 19 rats, 89%) Determination of systemic problems presence was based on histopathological examination, tissues and gross lesions were fixed in 10% buffered formalin, embedded in paraffin blocks and stained with hematoxylin-eosin (H-E). Tongue lesions were diagnosed according to the criteria of WHO: “Histological Typing of Cancer and Precancer of the Oral Mucosa”.16

RESULTS

At the beginning of the experiment, both the experimental and control groups appeared to be in good health assessed by subjective assessment of their physical mobility and fur luster. There was no significant difference of the body weight among the groups of the experiment at the end of the study. In general, the health of the experimental rats throughout the experimental period appeared poorer than that of the untreated rats.

Three rats from groups 1, 2, 3, 4, 5, and 7 (total: 18 rats) were terminated in the middle of the experiment for observation of carcinogenesis progression. Histological assessment to those tongues demonstrated no dysplastic changes occurred in groups 1 (4NQO-8 wk), 2 (4NQO-8 wk + extract) and 3 (4NQO-8 wk → extract). However, each one of three rats from groups 4 (4NQO-16 wk) and 5 (4NQO-16 wk + extract) had tongue SCC. The tongue of three rats from group 7 (untreated control) remained normal.

Nineteen (50%) rats survived until the end of the experiment (the 36th week), however the other nineteen (50%) had to be necropsied before the end of the experiment. Three of group 2, five of group 3, two of group 4, five of group 5 and four of group 6 failed to survive until the 36th week. Possible causes of deaths were the presence of tongue SCC (10 of 19 rats, 53%) and systemic problems (9 of 19 rats, 47%) such as chronic respiratory disease (CRD), splenomegaly and liver infection of Taenia crassicollis. Those systemic problems were also noted in the survivors (17 of 19 rats, 89%). Determination of systemic problems presence was based on histopathological

Table 1. Histopathological examination of 38 rats’ tongues from rats necropsied either before or at the end of the experiment

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of rats</th>
<th>N</th>
<th>MoD</th>
<th>SeD</th>
<th>CIS</th>
<th>MicSCC</th>
<th>WellSCC</th>
<th>SCC Incidence (%)</th>
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<tr>
<td>1</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
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Group 1: 4NQO-8 wk; Group 2: 4NQO-8 wk + extract; Group 3: 4NQO-8 wk → extract; Group 4: 4NQO-16 wk; Group 5: 4NQO-16 wk + extract; Group 6: 4NQO-16 wk → extract; Group 7: untreated control; N: Normal; MoD: Mild Dysplasia; SeD: Severe Dysplasia; CIS: Carcinoma in situ; MicSCC: Micro-invasive Squamous Cell Carcinoma; WellSCC: Well Differentiated Squamous Cell Carcinoma; For SCC Incidence, CIS, MicSCC and WellSCC were categorized as SCC.
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Macrosopic or clinical appearances of 38 rat tongues showed hyperkeratosis, white, granular/nodular, thickening (Figure 3), ulcerative (Figure 4) lesions or the presence of tumor mass (Figure 5) on the posterior dorsal surface of the tongue, which could be clearly differentiated from the normal condition (Figure 2). Lesion diagnosed as well differentiated SCC might have macroscopic appearance as hyperkeratosis, granular/nodular, thickening, ulcerative lesions or tumor mass.

Results histopathological examination of HE sections on 38 rats’ tongues from rats necropsied either before or after the experiment are shown in Table 1 (note: 18 rats necropsied in the middle of the experiment were not included). Percentage according to histopathological diagnosis in groups of study is demonstrated in Table 2 and on Figure 6 in which mild, moderate and severe dysplasia were categorized as dysplasia and carcinoma in situ (CIS), micro-invasive SCC and well differentiated SCC were grouped together in SCC.

Normal histological appearance of the dorsal surface of the rat tongue is characterized by regular epithelial stratification. There is no epithelial down growth and polarity of the basal cells is significantly present (Figure 7). Moderate hyperplasia was especially demonstrated by epithelial down growth and basal and spinous cells proliferation. On the other hand, bulbous rete ridges was relatively prominent as well (Figure 8). Severe dysplasia showed that more severe atypic cells involving the whole thickness of epithelial layer, irregular epithelial stratification and loss of intercellular adherence (Figure 9). Well differentiated SCC was characterized by the prominent appearance of keratinization on tumor cells more than 75% and many tumor nests infiltrated into lamina propria even deeper to musculatory area (Figure 10).

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Group of Study</th>
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<tr>
<td></td>
<td>1</td>
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<tr>
<td>Normal</td>
<td>0</td>
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<tr>
<td>Hyperplasia</td>
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</tr>
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<td>0</td>
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<tr>
<td>SCC</td>
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SCC: Squamous cell carcinoma

Table 2. Percentage according to histopathological diagnosis in groups of study

Figure 2. Macroscopical appearances of dorsal surface of rat tongue indicated as normal appearance from untreated control group.

Figure 3. A white thickening lesion (an arrow) taken from a rat from group 2 (4NQO-8 wk + extract) that has been histologically diagnosed as hyperplasia.

Figure 4. An ulcerative lesion (an arrow) taken from a rat from group 3 (4NQO-8 wk → extract) that has been histologically diagnosed as well differentiated SCC.

Figure 5. A tumor mass (an arrow), a pedunculated lesion taken from a rat from group 4 (4NQO-16 wk) that has been histologically diagnosed as well differentiated SCC.
Figure 6. Percentage based on the histopathological assessment of lingual mucosa of 7 groups of rats used in this study.

Figure 7. Microscopical appearances of dorsal surface of rat tongue indicated as normal appearance from untreated control group, H&E, 100X.

Figure 8. Moderate hyperplasia, H&E, 100X taken from a rat from group 3 (4NQO-8 wk → extract).

Figure 9. Severe dysplasia, H&E, 200X taken from a rat from group 3 (4NQO-8 wk → extract).

Figure 10. Well differentiated SCC, H&E, 100X taken from a rat from group 1 (4NQO-8 wk).
**DISCUSSION**

Decreased general health in treated rats was especially the result of the 4NQO treatment. 4NQO treatment led to the presence of a big tumor mass on the dorsal surface of the tongue caused rats to have difficulties in eating, causing limitation of food consumption. On the other hand, the long duration of the experiment caused rats more susceptible to many infection from their environment. This assumption was based on the finding that almost 50% of unsurviving rats and 89% of surviving rats had CRD, splenomegaly or liver infection of worm cyst such as Taenia crassicollis. This incidence suggests that the longer the experimental duration the more susceptible the animals are to infectious agents. The severity of infection was indicated by the number of animals suffering splenomegalies. Other factors that contribute to CRD and liver infection by Taenia crassicollis were sanitation, maintenance and humidity of the bedding. In this study, replacement of the cage, drinking bottle and bedding were carried out once a week. Infection might be prevented by more frequent sanitation maintenance. 

Histopathological examination on rat tongue in the middle of the experiment (the 19th week) to observe the progression of tongue carcinogenesis indicated that the longer the 4NQO induction the earlier tongue SCC will develop. This statement is supported by the finding that no SCC developed until the 19th week in rats given 4NQO for 8 weeks. However, two out of 6 rats developed tongue SCC by the 19th week in groups induced by 4NQO for 16 weeks. This means that longer 4NQO induction might cause more genetical changes, leading to a faster progression of the carcinogenic process.

According to Tanaka et al., the initiation phase in 4NQO-induced tongue carcinogenesis in rat was achieved by 4NQO induction for ≤ 10 weeks. The data on the incidence of tongue neoplasms among the groups 1, 2 and 3 (Table 1) indicated that administration of ethanol extract of Gynura procumbens leaves on the initiation phase (8 wk 4NQO induction) could suppress the progression of tongue carcinogenesis in the models used. However, the inhibition was more effective if the extract was given in a longer period than 4NQO induction after the animals have been initiated as conducted in group 3 in which the reduction of tongue SCC incidence was up to 75%. If the administration of the extract was calculated, rats in group 2 were only given the extract for 10 weeks, but rats in group 3 were given the extract for 26 weeks. It suggests that the longer the administration of the extract against the induction of carcinogen, the more the suppression on oral carcinogenesis leading to a lower incidence of tongue SCC. The above assumption was proven by the results of groups 4, 5 and 6 were compared. It was clear that in the groups 5 and 6, the duration of 4NQO induction compared to that of the extract administration was not so different (the 4NQO: 6 wk, the extract: 18 wk), so the inhibition of tongue carcinogenesis could not be achieved. Apparently, more frequency of extract administration was needed to be able to inhibit the progression of tongue carcinogenesis.

Our study suggests that Gynura procumbens leaves are possible new dietary preventive agents against tongue carcinogenesis. Anticarcinogenic component contained in the leaves is especially flavonoids. Flavonoids are found include in polyphenolic compounds that might be able to dysregulate cancer cell development. These include antioxidant activities, scavenging effect on activated carcinogens and mutagens, the action on proteins that control cell cycle progression, and altered gene expression. The core structure of the flavonoids, 2-phenyl-4H-1-benzo[4-one (flavone) affects proliferation, differentiation, and apoptosis in human colon carcinoma cell line. Even its apoptotic inducer is stronger than clinically established anti tumour agent, camptothecin. The results of the study corroborated the results of the previous investigations that administration of ethanol extract of Gynura procumbens leaves could block the pre-initiation and initiation phases of stomach carcinogenesis in Swiss mice induced with benzopyrene and that of mammary carcinoma in Sprague Dawley rat induced by DMBA and the extract could reduce the occurrence of lung tumor up to 23% in new born mice after being of after induction with benzopyrene.

In conclusion, the results of our study demonstrate the inhibitory effect of ethanol extract of Gynura procumbens (Lour) Merr leaves in the initiation phase of 4NQO-induced tongue carcinogenesis in rats especially viewed from clinical and histopathological aspects. Further experiments to elucidate the inhibitory mechanism of Gynura procumbens leaves in molecular level is strongly recommended for the development of chemopreventive agent of Gynura procumbens in oral carcinogenesis.

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