Dysplastic Model of Oral Squamous Cell Carcinoma in Male Wistar Rat: Chemically Induction with Dimethyl Benz(A) anthrance (DMBA)

Ahyar Riza[1], Denny Satria[2], Alfath Nugroho[3], Jeremias Lumban Toruan[3]

1. Department of Oral and Maxillofacial Surgery, Faculty of Dentistry, University of Sumatera Utara, Medan, Indonesia
2. Department of Pharmaceutical Biology, Faculty of Pharmacy, University of Sumatera Utara, Medan, Indonesia
3. Faculty of Dentistry, University of Sumatera Utara, Medan, Indonesia

Abstract

Oral cavity squamous cell carcinoma (OSCC) is the sixth most common cancer in the world with a high incidence and poor prognosis. 7,12 Dimethylbenz(a)anthracene is a prototype of polycyclic aromatic hydrocarbons which has been shown to cause cancer in mice.

This study was conducted to determine the optimization of the OSCC model in rats with respect to frequency, dose, duration of administration, type and length of wounds to the carcinogen DMBA (7,12 dimethylbenz(a)anthracene). In this in vivo study, 42 male wistar rats (Rattus novergicus) were used and divided into 6 treatment groups. Mice were then sacrificed on day 29 and day 43 for histopathological analysis using hematoxylin-eosin staining. The 2017 WHO classification was used to assess the degree of dysplasia. Data were analyzed using the Mann-Whitney test.

The results showed were that almost all rats had dysplasia with a different percentage of degrees in each group, only 1 rat in group 2 did not have dysplasia. In general, dysplasia occurs most in the moderate and severe categories. The degree of severe dysplasia entirely occurred (100%) in groups 3, 4 and 5.. It can be concluded that optimizing the OSCC rat model can be done by using a higher dose, a longer administration time and the length of the wound given.

Experimental article (J Int Dent Med Res 2022; 15(2): 606-610)

Keywords: Oral squamous cell carcinoma (OSCC), Dimethylbenz(a)anthracene, optimization of OCSCC model.

Received date: 01 December 2021 **Accept date:** 10 January 2022

Introduction

Oral cavity squamous cell carcinoma (OSCC) is the sixth most common cancer in the world. OSCC can occur in the buccal mucosa, floor of the mouth, tongue and other tissues in the oral cavity.[1,2] This type of cancer occurs in more than 500,000 cases every year and has the highest mortality rate globally.[1] The World Health Organization (WHO) reports that the prevalence and the highest mortality rate of OSCC is divided into several regions around the world, such as the Southeast Asia region and the European region.[1] Based on the International Agency for Cancer Research in 2014, the incidence of all types of oral cancer in Indonesia was 319 cases diagnosed during the last fifteen years in Dharmais Indonesia National Cancer Hospital (DNCH) and nearly 70% are diagnosed at an advanced stage. The prognosis of OSCC is poor with an estimated survival rate of only 2-3 years in DNCH.[3] Some of the effects felt by patients with OSCC are impaired speech, swallowing, nausea, vomiting, and weakness which have an effect on decreased body functions that make the patient unable to function optimally. [4]

The high incidence and poor prognosis of OSCC have prompted more and more researchers to conduct in-depth investigations into the pathogenesis and potential therapeutic targets of SCC.[3] Despite improvements in cancer treatment in the form of surgery, radiotherapy and chemotherapy, or a combination of the three, there was no significant improvement. significant in survival. Therefore new treatments, such as gene therapy are being developed.[4]

Research on OSCC can be done both in vitro and in vivo. In vitro experiments have advantages such as being relatively simple, safe,

Corresponding author:
Ahyar Riza, drg, Sp. BM (K)
Departement of Oral and Maxillofacial Surgery,
Faculty Of Dentistry, University of Sumatera Utara,
Jl. Alumni. No. 2, Medan North Sumatera, Indonesia.
Zip code: 20155
E-mail: ahyar.riza@usu.ac.id

and using specific species, but in vitro results have the disadvantage of differences between cell culture and physiology which can affect the process and give inconsistent results.[5,6] On the other hand, in vivo experiments using animal models is representative of the whole organism and avoids safety, ethical, and research-related issues that arise in human experimentation.[6] Animal models can accurately reflect the biologically significant behaviors of tumors, such as invasion and metastasis. For these reasons a suitable animal model is a prerequisite for clarifying the initiation and development of SRM. Previous research has revealed that several types of animals can be used to model OSCC, including hamsters, rats, mice, dogs, and cats. Rats and mice are the most commonly used animals in the modeling of OSCC.[6,7,8,9]

Carcinogenic agents can cause tissue structure to become abnormal so that changes occur in the process of tissue homeostasis and continue to experience abnormalities that induce cancer. One example of a carcinogenic substance is DMBA (*7,12 dimethyl benz(a)anthrance*). *7,12 Dimethylbenz(a)anthracene* is a prototype of polycyclic aromatic hydrocarbons, and various studies using this material have been shown to cause cancer in mice.[9] This compound is metabolized as electrophilic diolepoxide which then binds to adenine and guanine in DNA to form harmful compounds. . Induction of these carcinogens can cause oxidative stress and cause lesions on DNA bases to oxidize to form DNA adducts. In 1991, Lin and Chen revealed that after 8 weeks of cancer induction with application of 0.5% DMBA 3 times a week and arecaidine 6 times a week for 4 weeks, the period of cancer initiation became faster.[10,11]

The dose and frequency of exposure to DMBA (*7,12 dimethyl benz(a)anthrance*) is thought to have an effect on the rapid growth of squamous cell carcinoma in mice. In a previous study regarding the effect of the frequency of DMBA administration by Muchsin D in 2016, three mice with mild dysplasia were obtained, two mice were given DMBA twice per week for two weeks, and one mouse was given DMBA twice per week for four weeks. The state of moderate dysplasia in one mouse with DMBA exposure twice for four weeks and two mice with DMBA exposure four times for four weeks.[20] In another study regarding curcumin extract as a chemopreventive agent in a mouse animal study model by Maulina T et.al in 2019, with exposure to 100 g 0.5% DMBA 3 times a week for 4 weeks on the buccal mucosa of Sprague-Dawley rats effectively induces dysplasia. The use of DMBA in this study can be considered as a strong tumor inducer.[13]

Currently a lot of research is being done in the development of therapies to significantly improve survival for cancer patients. Thus, early diagnosis of OSCC will enable practitioners to monitor, diagnose and treat the disorder at an early stage of dysplasia or even carcinoma in situ.[1] This study was conducted to determine the optimization of the OSCC model in mice with respect to frequency, dose, duration of administration, type and length of wounds to the carcinogen DMBA (*7,12 dimethylbenz(a)anthracene*). It was developed to obtain an optimal OSCC model in mice in order to maximize research on OSCC.

Materials and methods

Ethical Clearance

The research method used has been approved by the Animal Research Ethics Committee, Faculty of Mathematics and Natural Sciences, University of North Sumatra (No.0125/KEPH-FMIPA/2021).

Types of Research and Research Groups

This research is an in vivo laboratory experiment with a posttest only control group design. The animals used in this study were 42 male wistar rats (Rattus novergicus). The mice were then divided into 6 treatment groups:

- Group 1 (control) was given a streak of 1 cm 100 µg DMBA 0.5% three times a week for 4 weeks;
- Group 2 was given 1 cm 100 µg DMBA 0.5% streak twice a week for 4 weeks;
- Group 3 was scratched 1 cm 200 µg DMBA 0.5% three times a week for 4 weeks;
- Group 4 was scratched 1 cm 100 µg DMBA 0.5% three times a week for 6 weeks;
- Group 5 was scratched 2 cm 100 µg DMBA 0.5% three times a week for 4 weeks;
- Group 6 was given an incision of 1 cm 100 µg DMBA 0.5% three times a week for 4 weeks.

Mice were then sacrificed on day 29 and day 43 (group 4) for histopathological analysis.

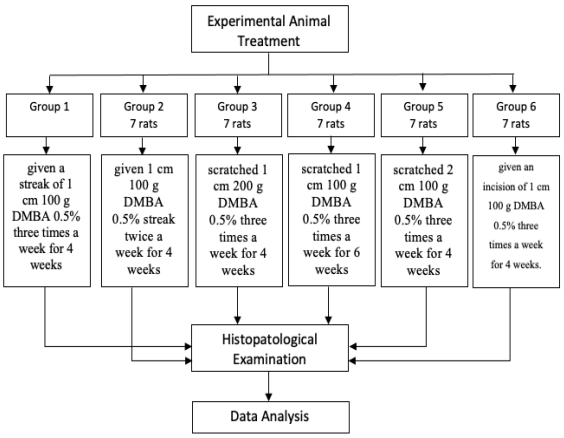

Figure 1. Research Flow.

Induction of oral epithelial dysplasia by DMBA

Induction was carried out at the Focus Medical Laboratory, Medan, Indonesia. Rats were anesthetized using ketamine hydrochloride intraperitoneally at a dose of 10 mg/kg BW. The buccal mucosa of each group 1, 3, 4, 5 rats was scraped and group 6 was incised three times a week (every Monday, Wednesday, and Friday) and group 2 twice a week (every Monday and Friday) with a syringe and blade no. 15 (Onemed, Indonesia) containing 100 µg and 200 µg DMBA 0.5% (Sigma-Aldrich corporation D3254, USA) and corn oil (Tropicana slim, Indonesia) as solvents, with a length of 1 cm and 2cm measured using a UNC-15 (Osung, Korea) for four and six weeks. On day 29 and day 43, rats were sacrificed by cervical dislocation. The frequency of DMBA used in this study was based on a previous study on curcumin extract as a chemo-preventive agent in a mouse research model by Maulina T et al. in 2019, who found that application of 100 g DMBA 0.5% to the buccal mucosa of Sprague-Dawley rats three times weekly for four weeks was effective in inducing dysplasia.[14] A 2016 study by Muchsin D on the effect of DMBA frequency found that DMBA exposure twice a week can cause dysplasia.[15]

Research Animals

The animals used in this study were 42 male wistar rats (*Rattus novergicus*) with inclusion criteria of eight weeks of age with an average body weight of 200-300 grams. Rats must be in a healthy condition characterized by active movement, clean fur, clear eyes and have never received any treatment before. Mice were obtained from and placed in CV. Focus Medical Indonesia veterinary house. Mice were acclimatized for 1 week before any treatment to ensure good adaptation.

Histopathological Examination

After rats were sacrificed on days 29 and 43, dysplasia was assessed by hematoxylin eosin staining at Anatomical Pathology, Faculty of Medicine, University of North Sumatra, Medan, Indonesia. The buccal mucosa of rats was cut and fixed with 10% formalin. The tissue is cut into small pieces for the dehydration process. Dehydration is carried out by 80% to 95% for 1 hour 30 minutes. After that, each tissue was put in toluene for 30 minutes. The tissue was then infiltrated with liquid paraffin at 58-60℃ for 30 minutes to 6 hours in an incubator to remove toluene from the tissue and replace it with paraffin. Paraffin blocks were cut using a rotary microtome with a thickness of 4-5 m. The cut tissue was placed in a water bath at 46℃ and collected on a clean slide. Slides are labeled using non-removable ink. The slides were then stained with hematoxylin-eosin staining.[16,17,18] The stained tissue slides were then observed under a light microscope with magnifications of 40x, 100x, and 400x. The degree of dysplasia in this study was scored using the WHO 2017 classification system, with a score of 0 for no dysplasia, 1 for mild dysplasia, 2 for moderate dysplasia, and 3 for severe dysplasia.[19]

Statistic analysis

The data were compared using the Independent t-test if the data were normally distributed and the Mann-Whitney test if the data were not normally distributed to determine the significant difference in scores in the degree of dysplasia between the control group and each treatment group. The results are considered significant if the p-value is below 0.05. Statistical analysis was performed using IBM SPSS version 21.

Results

This research is an in vivo laboratory experiment with a posttest only control group design. The animals used in this study were 42 male wistar rats (*Rattus novergicus*). The mice were then divided into 6 treatment groups:

- Group 1 (control) was given a streak of 1 cm 100 µg DMBA 0.5% three times a week for 4 weeks;
- Group 2 was given 1 cm 100 µg DMBA 0.5%

streak twice a week for 4 weeks;
- Group 3 was scratched 1 cm 200 µg DMBA 0.5% three times a week for 4 weeks;
- Group 4 was scratched 1 cm 100 µg DMBA 0.5% three times a week for 6 weeks;
- Group 5 was scratched 2 cm 100 µg DMBA 0.5% three times a week for 4 weeks;
- Group 6 was given an incision of 1 cm 100 µg DMBA 0.5% three times a week for 4 weeks.

Mice were then sacrificed on day 29 and day 43 (group 4) for histopathological analysis.

The study used 42 Wistar rats (*Rattus novergicus*) but during the study it was found that one rat from group 1 died. Thus, 41 rats survived until the day of sampling according to the day. Each rat was treated according to its respective group, then the degree of dysplasia was assessed based on the 2017 WHO classification. The results obtained were that almost all rats had dysplasia with a different percentage of degrees in each group, only 1 rat in group 2 did not have dysplasia. In general, dysplasia occurs most in the moderate and severe categories. The degree of severe dysplasia entirely occurred (100%) in groups 3, 4 and 5, thin can be seen in table 1. It can be seen in the histological picture (in Figure 1) the characteristics of dysplastic cells by category.

Group	N	No Dysplasia	Category Cell Dysplasia		
			Mild	Moderate	Severe
Group 1	6	0 (0,0%)	1 (16,7%)	3 (50,0%)	2 (33,3%)
Group 2	7	1 (14,3%)	3 (42,9%)	3 (42,9%)	0 (0,0%)
Group 3	7	0 (0,0%)	0 (0,0%)	0 (0,0%)	7 (100%)
Group 4	7	0 (0,0%)	0 (0,0%)	0 (0,0%)	7 (100%)
Group 5	7	0 (0,0%)	0 (0,0%)	0 (0,0%)	7 (100%)
Group 6	7	0 (0,0%)	0 (0,0%)	1 (14,3%)	6 (85,7%)

Table1. Distribution of Dysplastic Cells with DMBA Administration with Differences in Frequency, Dose, Duration of Administration, Type and Length of Wounds Between Control and Treatment.

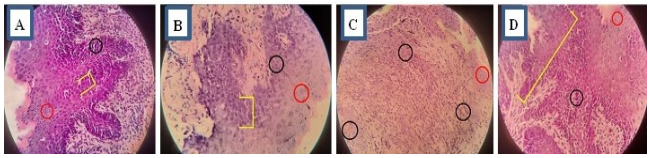

Figure 1. Histology of the mouse model slide.

Figure 1. Histology of the mouse model slide in the image marked with red (normal cells), yellow (total thickness of the epithelium involved), and black (dysplastic cells); A. Mild dysplasia, characterized by abnormal changes in the shape, number, size and color of the nucleus, and changes in the basal to parabasal layers; B. Moderate dysplasia, more and more abnormal nucleus shape and presence of mitoses in 2/3 of the basal layer but still within normal limits; C. Severe dysplasia, marked abnormal nucleus and changes in more than 2/3 of the basal layer and there is a focus of invasive cells; D. Severe dysplasia, marked abnormal nucleus and changes in more than 2/3 of the basal layer.

Group	N	Avarage ± SD	P-value
Group 1	6	2.167± 0.753	0.067
Group 2	7	1.286 ± 0.756	
Group 1	6	2.167± 0.753	0.01*
Group 3	7	3.000 ± 0	
Group 1	6	2.167± 0.753	0.021*
Group 4	7	3.000 ± 0	
Group 1	6	2.167± 0.753	0.014*
Group 5	7	3.000 ± 0	
Group 1	6	2.167± 0.753	0.056
Group 6	7	2.860 ± 0.378	

Table 2. Differences in Average Dysplasia Score with DMBA Administration with Differences in Frequency, Dose, Duration of Administration, Type and Length of Wounds Between Control and Treatment. **Mann-Whitney* test; $P < 0.05$; significant.

The results obtained and can be seen from table 2. There was no significant difference in the degree of dysplasia although group 1 showed a higher degree of dysplasia than group 2 (P=0.067). The same is also shown by the variable type of wound. The table shows that there is no significant difference in the degree of dysplasia between group 1 and group 6 (P=0.056). The table shows the results of significant differences in the degree of dysplasia between group 1 and group 3 (P=0.01); group 1 and group 4 (P=0.021). and between group 1 and group 5 (P=0.014).

In the average value of the degree of dysplasia in all groups, there are 3 groups that have the highest average degree of dysplasia, namely 3,000 in group 3, namely the group that was given a scratch of 1 cm 200 µg DMBA 0.5% three times a week for 4 weeks. 4 is the group that is given a 1 cm 100 µg DMBA 0.5% streak three times a week for 6 weeks, and group 5 is the group that is given a 2 cm 100 µg DMBA

0.5% streak three times a week for 4 weeks. For group 6, the group that was given an incision of 1 cm 100 µg DMBA 0.5% three times a week for 4 weeks had a lower average degree of dysplasia of 2,860, then followed by group 1 which was the control group, namely the group that was given a 1 cm scratch. 100 µg DMBA 0.5% three times a week for 4 weeks with a mean degree of dysplasia of 2,167. The group with the lowest average degree of dysplasia was group 2, namely the group that was given a scratch of 1 cm 100 µg DMBA 0.5% twice a week for 4 weeks with an average degree of dysplasia of 1,286.

Discussion

This study aims to determine the optimization of the OSCC model in rats with respect to the frequency, dose, duration of administration as well as the type and length of the wound using the carcinogen *dimethylbenz(a)anthracene*. In this study, the degree of dysplasia was assessed based on the WHO category score 2017. The susceptibility of mouse strains, genetic mutations, and carcinogenic doses can affect carcinogenesis in mice.[15] It is known that cancer is the result of a complex interaction between genetic and environmental factors that convert normal cells into cancer cells. [20] The modeling of dysplasia in this study used the compound *Dimethylbenz [a] anthracene* (DMBA). The compound *dimethylbenz [a] anthracene* (DMBA) was chosen because it is a class of Polycyclic Aromatic Hydrocarbons (PAHs) which are the most potent carcinogens in making cancer models and have been widely used in cancer studies. This is also based on research conducted by Maulina et al in 2019 using DMBA in the manufacture of a dysplasia model with a scratch method for 4 weeks resulting in moderate to severe dysplasia.[15] Likewise, research conducted by Pourshaidi et al in 2019 used the DMBA smear method in model making. dysplasia within 10 weeks resulted in mild to moderate dysplasia.[14] Based on this DMBA has the ability to trigger carcinogenesis.

In this study, the carcinogenic agent DMBA (*7,12 dimethylbenz(a)anthracene*) was used to induce dysplasia. The mechanism of cancer caused by DMBA occurs because these compounds are metabolized into active metabolites, such as epoxide diols and free radicals. The two active metabolites can bind to DNA to form DNA adducts in the carcinogenesis process. The result of DMBA induction causes oxidative stress so that DNA lesions form and form DNA adducts.[21,22] Carcinogenesis is the stage of changing normal cells into cancer cells, namely the initiation, promotion, and progression phases. At the initiation stage, there is a change in the DNA of the cell nucleus that allows normal cells to gradually turn into cancer cells. Cancer initiators can be obtained from repeated exposure to the environment or chemicals that can cause genetic changes in DNA so that it can trigger DNA lesions. Failure of DNA lesion repair mechanisms can lead to mutations in certain genes such as oncogenes and tumor suppressor cells, which are cell cycle regulators used in cell growth, cell division, cell differentiation, and apoptosis. DMBA not only causes oxidative stress but can induce the expression of genes that act as cell cycle regulators or suppressors. At the initiation stage, DMBA acts as an initiator that will cause mutations in DNA. Mutations that occur in this gene encourage the transformation of cells to become abnormal, but in this process it is still reversible.

Based on the results of this study, it was shown that there was an increase in the degree of dysplasia in the method of administration that was triggered deeper, longer, and longer into the mucosa. Administration of DMBA that was triggered deeper, longer, and longer into the mucosa resulted in a degree of severe dysplasia, whereas administration of a shorter, shallower, and lesser amount of DMBA resulted in a degree of mild to severe dysplasia. Administration of DMBA with a deeper incision resulted in the degree of severe dysplasia because in the incision method, DMBA was cut at a submucosal depth. The submucosa contains components in the form of nerves and blood vessels, so that carcinogens can spread more quickly to surrounding tissues and the incidence of dysplasia becomes more severe.[15] The degree of dysplasia produced in this study is directly proportional to the 2018 study of Maulina et al. who succeeded in forming moderate to severe dysplasia using the scratch method and using the compound *dimethylbenz [a] anthracene* (DMBA) as an inducer of dysplasia. The compound *dimethylbenz [a] anthracene* (DMBA) is reported to have the ability to bind to DNA and cause gene mutations that play a role in malignant

transformation.[15] Another study conducted by Pourshaidi et al in 2019 which also used DMBA as an inducer of dysplasia in the oral cavity with the smear method resulted in mild to moderate dysplasia.[14] Based on this, scratch length, scratch depth, frequency, dose, and duration of DMBA administration are factors that determine the potential for DMBA penetration to the mucosal tissue in triggering the incidence of dysplasia.

Conclusions

There was a significant difference in the average degree of dysplasia with different doses, duration of administration and wound length and there was no significant difference in the average degree of dysplasia with differences in the frequency of administration and the type of wound. It can be concluded that optimizing the OSCC rat model can be done by using a higher dose, a longer administration time and the length of the wound given.

Declaration of Interest

The authors report no conflict of interest.

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