Divine Cigarette Smoke Inhibit AgNOR Expression of Injured Nasopharyngeal Mucosa

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ABSTRACT

*Background:*Divine cigarette smoke contains nano sized particles that may conduct electrons up to millivolts, giving energy and electrons in injured cells to promote their reversibly autorepair. The aim of this study was provedthe inhibition effect of divine cigarette on AgNOR expression of nasopharyngeal mucosa that exposed by gaseous formaldehyde and cigarette smoke.

*Methods:*The post test only controlled group design using 32 C_3H mice divided into 4 groups; Control group (C1)exposed by 10% solution of gaseous formaldehydeand cigarette smoke for 18 weeks and control group 2 (C2) for 9 weeks. Treatment group 1 (T1) exposed by10% solution of gaseous formaldehydeand divine cigarette smoke for 18 weeks and treatment group 2 (T2) for 9 weeks.Nasopharyngeal tissue was processed for AgNOR expression.

*Results:*Kruskal-Wallis test for AgNOR count was significantly different (P=0.021) between groups. Mann-Whitney test for AgNOR count between C1 vs T1 (P=0.018),T1 vs T2 (P=0.017) were also significantly different, but between C2 vs T2 was not different (P=0.065).

*Conclusion:*divine cigarette smoke given together with gaseous formaldehyde had significant effect on inhibiting AgNOR expression that can be explored in the further study.

Keywords: Divine cigarette;formaldehyde; cigarette smoke;AgNOR

INTRODUCTION

The NPC'setiology is multifactorial.Various risk factors such as carcinogenic substances have been believed to cause the occurrence of NPC.One of such carcinogenic material is formaldehyde.Research conducted by Conolly in 2003 proved that formaldehyde can cause squamous cell carcinoma in nasopharyngeal mucosa of rat F344.The study was conducted by giving chronic formaldehyde steam for 6 weeks in F344 rat until squamous cell carcinoma appeared. The effect of formaldehyde carcinogenesis derived from its corrosive to the mucosa so that when inhaled continuously can cause severe necrosis in the nasopharynx and nasal cavity.*Formaldehyde* also triggers the occurrence of DNA mutations.The CO group in the formaldehyde reacts with the amine group in the protein to produce methane or hexamethylenetramin which then reacts with the DNA in the body.[1]Nanobiology is a new discipline that can revolutionize the world of health. This method has considerable potential for cancer treatment that can be used by doctors.Nanobiology incorporates biological nanoscience tools, ideas and materials to deal with biological issues that can be solved with nanotechnology. The way nanobiology works is by building molecular devices and working to build molecular machines that utilize the concept of nature.[2,3]

The molecules in nanobiology are 1 - 100 nm for example ribosomes, enzymes, DNA, etc.These units are generally macromolecules. They are complex arrangements composed of several components of the monomer.They work very specific and even considered to have intelligence because they know when, how, where and with whom they must work.[3]Kretek divine is one of the products that use the principle of nanobiology. Kretek divine is the result of technology conversion from cigarettes containing free radicals to healthy cigarettes containing complex nanostructures that can deliver electrons up to milivolt levels.[4]Cigarette smoke contains toxic components such as Hg, NH3, CO, CO2, NO, NO2, and others. In the kretek divine, these toxic components are converted into components of a very small size (nano-sized) that have a potential energy that can create more environmentally and human-friendly complex structures.[5]In the nanoparticles in the kretek divine is also contained a scavenger that is a free radical carrier in the body. The presence of this scavenger causes the nanoparticles to bind and remove free radicals inside and outside the cell so the body becomes cleaner.Then the nanoparticles can energize and electron the diseased cells to promote self-improvement of healthy cells, to optimize themselves.[6]Because of these various advantages, the kretek divine should be tested as a new treatment method in NPC therapy.

The results of this study will be seen on the preventive or curative effects of kretek divine through apoptotic index and histopathological changes in nasopharyngeal

epithelium of C3H mice.The preventive effect of kretek divine is played by a scavenger that can capture free radicals in the body so that this scavenger can prevent the process of carcinogenesis in the body.[7]The curative effect of kretek divine is due to the nanoparticles in the kretek divine that can energize and electron the sick cells to optimize themselves into healthy cells.[6]In accordance with previous studies that tried to test a potentially anti-cancer substance, the results of this study with kretek divine is expected to increase apoptotic index and make histopathologic changes of nasopharynx epithelium to be better.[8,9]Previous research attempting to test nanobiology for NPC therapy has never existed. Other studies only mention that kretek divine with the method of balur can cure liver cancer.[4]Therefore, research kretek divine as NPC therapy is the first study that tested nanobiologi as NPC therapy.

This study proves the difference of kretek divine effect on nasopharyngeal carcinogenesis of C3H mice exposed to formaldehyde compared with regular clove cigarettes by assessing apoptotic index and histopathologic changes through painted HE.Cell proliferation activity is known by AgNOR painting.This is due to the expression AgNOR can describe the velocity of the cell cycle and related to the time of doubling the tumor.The proliferation activity of cancer cells determines the growth rate of the tumor period, so AgNOR becomes a diagnostic and prognostic tool against tumor behavior.The improvement and repairment of AgNOR painting techniques has made AgNOR more standardized, reliable, simple and fast.[10]

Nucleolar Organizer Region (NOR) is a DNA arch that occupies a special area in chromosomes 13, 14, 15, 21, and 22 that are known as the place of ribosomal DNA genes (rDNA). NOR plays an important role in protein synthesis, helps maintain the extension of the DNA configuration, and regulates its transcription.[11]Visualization of NOR by using silver staining was introduced by Ploton et al in 1986 which was later modified by Ofner et al in 1994 by adding formalin to fixation.[12]To do the scoring amount of silver deposit in this technique used different methods.The most commonly used method is to manually count the number of nuclear intra nuclear points per cell on at least 100 cells using a light microscope. Another more recent method is to calculate the total area of silver deposits with the help of computers.[13]

Material and Methods

This research is a laboratory experimental research with post test only control group design.The experimental animals in this study were C3H strain rat. The sample of the study was the C3H strain obtained from the Faculty of Veterinary Medicine of the Bogor Agricultural University which fulfilled the inclusion criteria; Male rat, age ± 3 months, body weight ± 20 grams and no visible anatomical abnormalities. The excluded mice are visible pain (inactive motion) and die during treatment. The determination of the

sample size is done according to the WHO rule which is at least five individuals per group. C3Hrats in this research are divided into 4 groupsnamely; K1 group:Positive Control group 1is a group that exposed to regular cigarette smoke and induced with 20% steam formalin andgiven standard diet contained 54mg/kgWBof 10%formalindaily for 18 weeks; K2group: Positive Control Group 2 is a group that induced by 20% steam formalin and given standard diet contained 54 mg/kgWB of 10%formalinfor9weeks,then for the next another 9weeks regular cigarette smoke is given; P1 group: Treatment Group 1 is a group that given divine kretek exposure and induced by 20% steam formalin and given standard diet contained54mg/kgWB of 10%formalindaily for18 weeks; P2 Group:Treatment Group 2 is a group that induced by 20%steam formalinand given standard diet contained 54mg/kgWB of 10%formalinfor 9weeks,then for another next 9weeks given *divine* kretek smoke exposure. During the research, the experimental animal is placed on a cage and being feed in*ad libitum*way. The independent variables in this study were exposure to divine kretek and exposure to regular clove cigarettes. Dependent variable is apoptotic index.

***Divinekretek*exposure**

Divine kretek exposure is smoke exposure of the burning result of kretek cigarette that was have been added RDE liquid that contained metionin amino acid and fenilanin in form of nano particle. One divine kretek cigarette burned for one group of mice was followed by spraying smoke as much as 2 x 20 cc of the rectum of rat.Spraying is done by adding a cigarette on a pipe connected to 60 cc spuit, then burned and after that the smoke cigarette sucked by spuit to reach 20cc. The purpose of spraying smoke on the anus of this rat is to increase the dose and also to prevent the effect of formalin in the gastrointestinal tract.(Sarjadi 2012, personal communication, Jan 10th).

Regular cigarette smoke exposure

Regular cigarette smoke exposure is smoke exposure of the burning result of kretek cigarette with no adding of RDE liquid.One regular kretek cigarette burned for one group of rat was followed by spraying smoke as much as2x20cc of the rectum of rat.Spraying is done by adding a cigarette on a pipe connected to 60 cc spuit, then burned and after that the smoke cigarette sucked by spuit to reach 20 cc.(Sarjadi 2012, personal communication, Jan 10th).

AgNORMethod

The activity of nasopharyngeal epithelial cell proliferation was assessed using the AgNOR method. The counting method of AgNOR patch number was performed according to Ploton method by counting the number of AgNOR interphase per cell on 100 tumor cells with 1000x magnification using emersi oil, then taken average. AgNOR

Painting appears as a brown or black dot in the epithelial cell nucleus.[8]In this study examination of patches of AgNOR done by anatomical Pathologist blindly.

Formaldehyde Induce

The induction of nasopharyngeal carcinogenesis with formalin was performed by a modified Conolly method using steams from a 20% formalin solution which flowed into a 50x30x20cm3 semi-closed chamber and additionally supplemented with an oral standard diet containing 54 mg / kgBB of formalin 10% per day for 9 weeks.[8]Male mice of the C3H strain were acclimated in the laboratory by each treatment group and given standard feed for one week ad libitum. The terminology was performed at week 9 for the calculation of apoptotic index according to Aihara M et.al method by calculating apoptotic body per 100 tumor cells from significant area with 400x enlargement at 10 field of view from each preparation, in one paraffin block then taken the mean value.

StatisticAnalysis

Data are grouped by their variables in nominal, ratio, and ordinal scales. For the calculation of the apoptotic index, a normality test was performed using Shapiro Wilk. Hypothesis test is performed by using one way anova test as parametric test if it qualifies.If the parametric test requirements are not met then Kruskal-Wallis test is used as non parametric test followed by posthoc Mann Whitney test while for histopathology change the score, the data is analyzed using Kruskal Wallis test. The data obtained is processed using a computer.

EthicalClearance

Ethical Clearance of this study was obtained from the Komisi Etik Penelitian Kesehatan/Commission of Health Research Ethics (CHRE/KEPK) Faculty of Medicine Diponegoro University / RS. Kariadi Semarang.The experimental animals in this study will be fed in ad libitum, well maintained, kept in cage cleanliness, well treated during transportation and maintained according to the behavior and biological needs of experimental animals.

Research Result

This study used 32 C_3H mice divided into four groups, they are those that received exposure to regular cigarette kretek smoke during formaldehyde induction (K1),a group that received exposure to regular cigarette kretek smokeafterformaldehyde induction(K2), a group that received exposure to *divine*kretek smoke during formaldehyde induction (P1),and a group that received exposure to divine kretek smoke after formaldehyde induction(P2). Two rats die during the adaptation process before the treatment begins, and there are two female rats to be excluded.In the first 9 weeks of the study, one rat from each group was terminated, to be assessed for its nasopharyngeal carcinogenesis. Then four other rats were excluded because of death. At

the end of the study, the remaining rats were 20 individuals, with a minimum sample size of 5 individuals per group still fulfilled.Furthermore, all remaining rats are terminated for their nasopharynx. The nasopharyngeal preparation is then stained with HE painting. Later then a histopathological scores performed on each preparation blindly.

Results of histopathology score of nasopharyngeal epithelium of C3H mice.

In this study changes in histopathological scores only reach the stage of mild dysplasia so that the apoptotic body.can not be seen.

Table1. Nasopharyngeal mucosal's Histopathology Score

Group	Test	*p*
Regular kretek cigarette compare to*divine*kretek duringinductionof *formaldehyde* (K1-P1)	Mann-Whitney	0,014*
Divine kretek during inductioncompare to *divine* kretek after inductionof *formaldehyde* (P1-P2)	Mann-Whitney	1
Regular kretek cigarette during induction compare to regular kretek after inductionof *formaldehyde* (K1-K2)	Mann-Whitney	0,05
Regular kretek cigarette after inductioncompare to *divine* kretek after induction of*formaldehyde* (K2-P2)	Mann-Whitney	0,513

* significant($p<0,05$)

Mann-Whitneytest between group that exposed to regular kretek cigarette smoke during induction of *formaldehyde*(K1)and group that exposed to divine kretek smoke during inductionof *formaldehyde*(P1) is ($p=0.014$) or occured significant differences, meanwhile other Mann Whitney test result showed no differences ($p>0,05$).

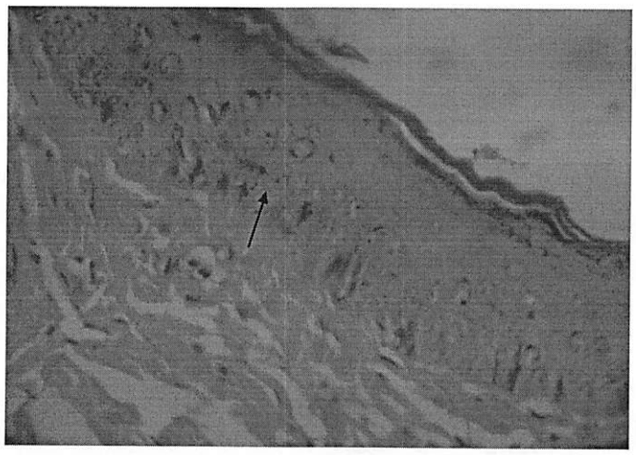

Figure1. Normal nasopharyngeal mucosa (HE 400X, score1)

Figure1.Normal Nasopharyng Ephitelium.It is showed cellproliferation in normal boundaries. There is no hyperplasia or dysplasia.

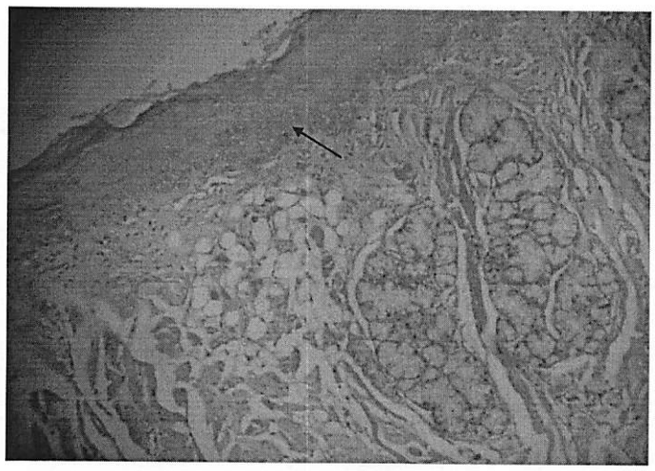

Figure2. Mild dysplasia(HE 400X, score 4)

Figure2.Nasopharyngeal dysplasia; the figure showed the thickening on mucosal epithelium, disappearing of silia and of proliferation or hyperplacia on lamina basalis and parabasal which are not more than one third of the epithelium. (see arrows).

Table2. Kruskal-WallisTest on the ammount of AgNORspot

Variable	Test	*p*
Cell Proliferation Activity	Kruskal-Wallis	0.021

Table2showed the result of Kruskal-Wallistest on cell proliferation activity using method of AgNOR spot counting and the value is p=0.021 or there is a significant differences ($p<0.05$).Next will done the *post hoc* Mann-Whitney test to know the differences between the treatment group.

Table3.*PostHoc*Mann-Whitneyteston the ammount of AgNOR spot

Group	Mann-Whitney (p-value)
Regular kretek cigarette compare to divine kretek during induction (K1-P1)	0,018*
Regular kretek cigarette compare to divine kretek after induction (K2–P2)	0.0065
*Divine*kretekduring induction compare to *divine*kretekafter induction (P1-P2)	0.01

* significant(p<0,05)

Table3showed the significant differences Mann-Whitneytest result (p<0,05)between the group that had been given regular kretek cigarette during the*formaldehyde*induction compare to the group that had been given d*ivine*kretek during the *formaldehyde*(K1-P1) with the value*p*=0.018,and it is also showed the significant differences between group that had been given Divine kretek during induction compare to divine kretek after induction of formaldehyde (P1 -P2)with value *p*=0.017.

Figure3showed the nasopharyng muchose epythellium on the AgNOR painting.TheAgNORspot appeared as the brown or black dot inside the epythellium core cell.

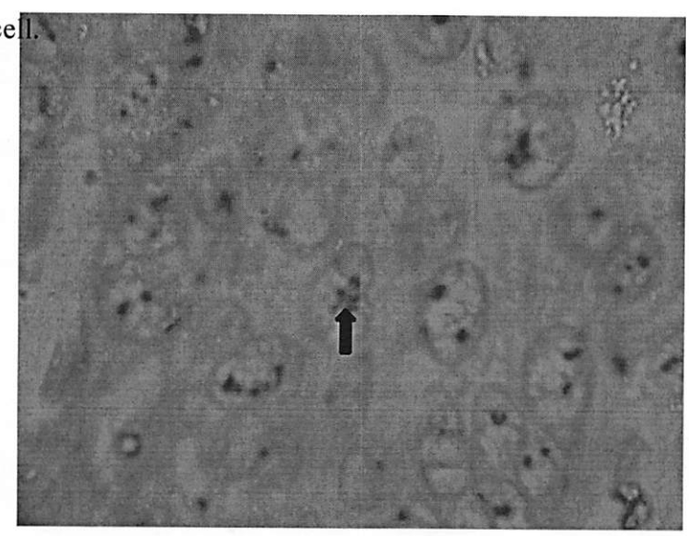

Figure3. Nasopharyngeal mucosa on the AgNORpainting (1000x).The arrow showed the AgNOR spot.

DISCUSSION

*Divine*kretekis one of the innovation on the science and technology. Divine kretek contained big molecule block (nano structure) that having high density electron which is able to catch the free radical and donate electro to the body.[14]This nano structure is having stable structure that able to trigger the Hg ion catching process on the nicotine. The nature of this nano structure making the *divine* kretekcould eliminate Hg toxicity on the cigarette smoke.[15,16]

In the making of *divine*kretek,some chemical substance are added as the*scavenger*(free radical carrier inside the body)which is in a form of aromatic substance with a complex structure. This complex structure is rich on high density electron therefore it is having high enough potential energy to transfer electron.[17]Because of this potential energy contain and its *scavenger*nya then the *divine* kretek hoped to give energy and electron to the sick nasopharyng cell that later could push and self optimize to become healthy cell and could neutralize, bind and dump the free radical from the body.[18]

This research is conducted by induced the *formaldehyde* on C3H rat through20% formalin steamand dan10%formalin diet so the NPC hoped to be appear on thatC3H rat. Then for the intervention *divine*kretekand regular kretek are given in form of smoke exposure.The control groups (K1 And K2) that are given regular cigarette smoke in this research are having purpose to analyze whether regular cigarette or divine kretek that are having preventive or curative effect. Exposing regular cigarette smoke on K1 group and divine kretek on P1 group for 18 weeks are having purposes to prove the preventive effect on one or both of the groups, meanwhile exposing regular cigarette smoke on K2 and divine kretek smoke on P2 for 9 weeks are having purposes to prove the curative effect on one or both of the group.The preventive and curative effect could be seen on the apoptotic index and the changing of nasopharyng muchose ephytellium hispathology in which the apoptotic index would become higher and the changing of hispathology would look better than before.The Apoptotic is a programmized cell death.[19]In the malignancy transformation, the transformation cell is unable to be eliminate using apoptotic mechanism therefore it would appeared apoptotic resistancy.[11,20]This is one of the preventive mechanism which is hoped from the *divine*kreteknamely apoptotic increasing.Apoptoticthat happened on tumor cell could be evaluate using apoptoticindex.[19,20]Based on the previous theory and study,the apoptoticbody can only be seen on the cancer cell.FitrianiLumongga's research stated that apoptoticappeared because of P53gen mutation, which isa tumor suppressor gene.The function of this P53gen is to prevent cell replication on the genetically

damage cellby stopping the G1 phase cycle or interphase.This gen also function to state apoptotic if the cell damage is quite vast and repair state in failure.[21]Hidayat Sulistyoin his study stated that apoptoticonly happen on heavy dysplasia state.[8] However,this result of the study showed that hispathology changing that occured only reach light dysplasia therefore the apoptoticindexcounting can not be done.One of the cause why carcinogenesis on this study is not on heavy dysplasia is the technical fault on the uncertain dose of*formaldehyde* in ppm value. This happened because the insufficient tool and the value of *formaldehyde*in the air should be 10ppm.[22]In order to see the differential meaning and the preventive and curative effect of the*divine*kretek is by the histopathology changing score. Hispathology score result showed significant differences on group that given *divine* kreteksmoke during *formaldehyde*induction(P1) and K1 group.

The result showed that there was significant difference of AgNOR spots (p = 0,017) in groups exposed to kretek divine during induction of formaldehyde (P1) and groups exposed to kretek divine after formaldehyde induction (P2). The number of AgNOR spots group exposed to divine kretek during induction of formaldehyde (P1) was lower than the group exposed to divine kretek after formaldehyde induction (P2). A significant difference between the two groups showed that the preventive effect of divine kretek was better than its curative effect.

The significant differences in the amount of AgNOR spots (p = 0.018) were also evident in the group given regular kretek cigarette smoke during formaldehyde induction (K1) with groups exposed to kretek divine during formaldehyde induction (P1) showing the inhibitory effects of nasopharyng carcinogenesis.However, in the group that was given regular kretek cigarette after inductionof *formaldehyde*(K2)and the group that was given divine kretek exposure after the induction of *formaldehyde*did not show a difference (p = 0.065), and the mean number of AgNOR spots of the group that was given divine kretek exposure after induction of*formaldehyde*(P2)is higher than the group that was given regular kretek after the induction (K2).This suggests that divine kretek only has a preventive effect, while its curative effect is not proven.

The preventive effect of clove divine is derived from nanoparticles having spin interaction abilities derived from conjugated HO-Phenanthrenedienyl.These highly electron-density particles are capable of transferring electrons into cells, thus optimizing the healthy cells.[2343]In addition, various free radicals (which can break DNA) in the body such as nitrogen, oxygen, and mercury that can enter the body due to exposure to formaldehyde will be captured by scavanger(Amino acid methionine and phenylalanine)inside the *divine*kretek.[15,18,24]

The increased activity of cell proliferation in an initiated tissue is a very important change in the early stages of tumor promotion which is a typical sign of precancerous lesions.[8]At the time of proliferation, the increase in protein synthesis for cell growth is accomplished by altering the biosynthesis of the ribosome.The biosynthesis of the ribosome itself can be demonstrated by the quantity of the nuclear organization region (NOR) which is precisely in the nuclear structure and enclosed by the thick fibrillar component.[25]The values of AgNOR spotts is obtained differenton normal tissue, reactive proliferation tissue, benign proliferation, and malignancy.[26]

Increasing the number of AgNOR reflect the progression of neoplastic cells, where the nature and temperament transformed into malignant cells, or cancer cells that have already appeared also undergone a process of excessive cell proliferation activity.[8]The study of carcinogenesis in wistar colon rats performed by Aswiyanti showed that there were differences in the number of AgNOR spots on the control wistar rat colon with treatment. Wistar rat colon that got celery diet has lower count results than wistar mouse colon that did not get celery diet although at the malignancy have not arise in the colon.[11,27]Other studies have suggested that malignant cells in infiltrative breast duct carcinomas have more AgNOR points per nucleus.[27]

The number of AgNOR spots has a correlation with various other anatomical pathology examinations. AgNOR staining results have a high correlation with flow cytometric and immunohistochemical staining. Hartini proved that there is a positive correlation between the value of AgNOR spotting number with histology grading infiltrative breast ductal carcinoma.[27]While Khaisuntaha proves that the number of AgNOR spots has a negative correlation of -0.008 with apoptotic index in cervical cancer.[28]Based on the results of this study, it is proved that kretek divine has the potential to prevent the process of nasopharyngeal carcinogenesis characterized by a significant decrease in the amount of AgNOR.

Limitations of this study are; 1) the measurement of induction doses of carcinogenesis exposing the nasopharyngeal mucosa is uncertain, due to the limitations of the tool, according to the theory should the exposure given to cause malignancy in the nasopharynx of at least 10 ppm,2)Nasopharyngeal malignancy in Rosenmuller's fossa in rat, can not be localized accurately,3)This study does not show the mechanism of free radical capture by divine kretek.

CONCLUSION

The administration of exposure to divine kretek smoke during formaldehyde induction (to prove the preventive effect) can significantly decrease the activity of cell proliferation.The administration of exposure to clove divine smoke after formaldehyde induction (to prove curative effect) can not decrease cell proliferation activity.The administration of exposure to divine kretek smoke during formaldehyde induction may decrease cell proliferation activity better than exposure to clove divine smoke after induction of formaldehyde (the divine kretek preventive effect is stronger than its curative effect).

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P3 MANAGEMENT OF TONGUE CANCER AT PKU MUHAMMADIYAH YOGYAKARTA HOSPITAL

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Introduction: Squamous cell carcinoma (SCC) of the tongue is the most frequent intra oral head and neck cancer. It occurs in the elderly men, a decrease has been seen in males, contrary to the increase in female subjects. We describe the management of a female 55 years old with SCC of the tongue T3N1M0 stage. **Case reports:** A 55-year-old women with recurrent stomatitis and intense pain with a tongue lesion present for last one months. On examination, an ulcerative lesion of size 2.5 x 1 cm was seen on the left inferior lateral of tongue. FNA histopathologic result was invasif squamous cell carcinoma. The staging of the tumor was evaluated and it was found to be stage III (T3N1M0). Patient was treatment consisted of hemiglossectomy and radical neck dissection. The margin of the excised tissue was found to be free of tumor but there was histological evidence of metastasis into the level 3 lymph nodes. After surgery, treatment was completed with radiation and chemotherapy. **Comments:** For most oral cavity cancers, surgery is the treatment of choice, radiation or chemoradiation is added postoperatively. Selective neck dissection is indicated if the risk of nodal disease exceeds 15 to 20%.

Keywords: Squamous cell carcinoma, radical neck dissection, metastasis

P4 DIVINE CIGARETTE SMOKE INHIBIT AGNOR EXPRESSION OF INJURED NASOPHARYNGEAL MUCOSA

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Background: Divine cigarette smoke contains nano sized particles that may conduct electrons up to millivolts, giving energy and electrons in injured cells to promote their reversibly autorepair.
Aim: To prove the inhibition effect of divine cigarette on AgNOR expression of nasopharyngeal mucosa that exposed by gaseous *formaldehyde and cigarette smoke*.
Method: The post test only controlled group design using 32 C_3H mice divided into 4 groups; Control group (C1) exposed by 10% so*lution* of gaseous *formaldehyde* and cigarette smoke for 18 weeks and control group 2 (C2) for 9 weeks. Treatment group 1 (T1) exposed by 10% so*lution* of gaseous *formaldehyde* and divine cigarette smoke for 18 weeks and treatment group 2 (T2) for 9 weeks. Nasopharyngeal tissue was processed for AgNOR expression.
Results: Kruskal-Wallis test for AgNOR count was significantly different (P=0.021) between groups. Mann-Whitney test for AgNOR count between C1 vs T1 (P=0.018), T1 vs T2 (P=0.017) were also significantly different, but between C2 vs T2 was not different (P=0.065).
Conclusion: Divine cigarette smoke given together with gaseous *formaldehyde* had significant effect on inhibiting AgNOR expression that can be explored in the further study.
Keywords: Divine cigarette, gaseous *formaldehyde, cigarette smoke,* AgNOR

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CERTIFICATE OF APPRECIATION

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Awal Prasetyo

In recognition of your valuable support & contribution as

SPEAKER

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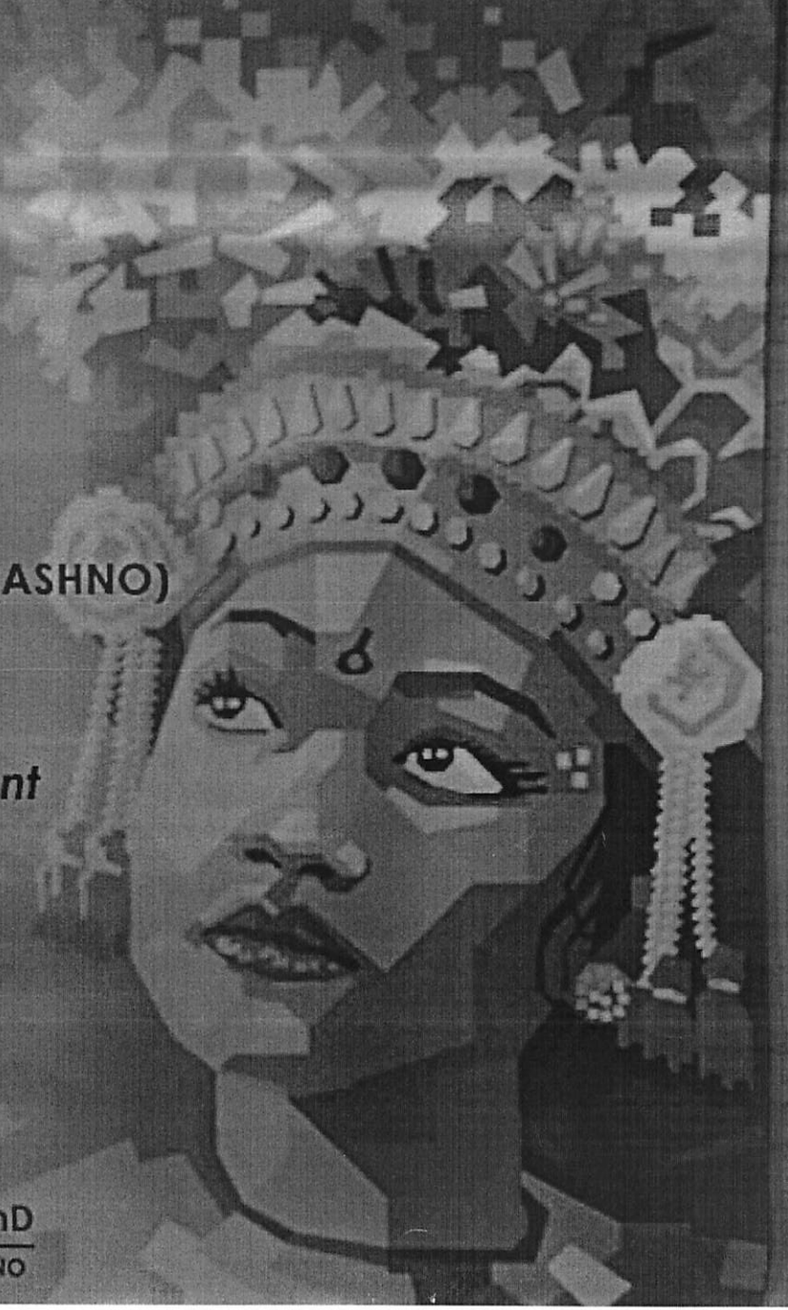

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