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Original Article

The effects of cigarette smoke nanoparticles in the colorectal carcinogenesis of wistar rats

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Abstract

Smoking cigarette is one of risk factors for developing colorectal carcinoma. Various researches have been made to suppress the toxicity of cigarette smoke. One of such effort is to reduce the size of cigarette smoke particles using nano water solution containing aromatic groups and EDTA. This study aims to assess the effects of this cigarette smoke nanoparticle regarding colorectal carcinogenesis. The experimental study was carried out with *sequential post test only control group design*. Thirty Wistar rats were divided into 3 groups: exposure to cigarettes smoke, exposure to smoke of cigarettes containing nano water solution and control for 14 weeks and 28 weeks. Colorectal epithelial morphology was assessed on the histopathology examination, whereas the expression of APC, KRAS, MSH2, MLH1, and p53 was assessed on immunohistochemistry procedure. Our results showed that cigarette smoke nanoparticles had better effects regarding colorectal epithelial morphology, especially through the increased expression of APC.

Keywords: Cigarette smoke, cigarette smoke nanoparticle, colorectal cancer, APC, KRAS, MSH2, MLH1, p53.

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Introduction

Colorectal cancer (CRC) is the third most common types of cancer and the fourth highest cancer as a cause of deaths in the world. In 2012, WHO recorded 1,361,00 new cases of colorectal cancer and 694,000 of them died (Ferlay et al., 2013). Data on epidemiological studies show a strong and consistent association between smoking and the incidence of colorectal adenoma (Giovannuci, 2001). Smoking is also significantly associated with colorectal cancer incidence and mortality, especially against rectal cancer (Botteri *et al*., 2008).

The efforts to suppress the toxicity of cigarettes has a lot to do including the discovery of kretek Divine as a local wisdom of Indonesia developed from the perspective of medical science in the field of nanomaterials. Applications of Divine cigarettes utilizes nanoparticle structure which is expected to be able to neutralize radicals contained in cigarettes especially metal mercury radical (Hg*) (Zahar and Sumitro, 2011b). In the concept of Divine nanoparticles, the toxicity of cigarettes hypothesized comes from Hg radicals as *sensitizer* which have a high-energy (in an excited state) that are bound to a complex of smoke nanoparticles and oscillates with Aurum atom (79Au) in the complex of phenanthrene together with nicotine of tobacco (Sumitro and Zahar, 2010). Transformation of toxic tobacco is performed using DIVINE solution, a solution of water containing nano-compounds with aromatic groups such as aromatic amino acid and EDTA, which serves as a Hg scavenger (Zahar and Sumitro, 2011a).

Research on the application of Divine cigarettes smoke nanoparticles in biological systems, especially in the prevention of colorectal cancer has not been done yet. This study is necessary because the nanoparticles have a different character from the original material came from and will have a different impact on the biological system. The interaction between nanomaterials with biological systems need to be supported by a study to verify the benefits, safety and the risk assessment of these materials. This study is assessing the comparative effects of cigarettes smoke with Divine cigarettes smoke in the colorectal carcinogenesis by observing the epithelial morphology and expression of APC, K-RAS, MSH2, MLH1, and p53.

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Method

This study was a sequential experimental research laboratory with post test only control group design using 30 male Wistar rats inbreed strain aged 1-2 months and weight between 150-200 g. The research subjects were randomly allocated into three treatment groups ie negative exposure group as a control group (K), cigarettes smoke exposure group (AK) and Divine cigarettes smoke exposure group (AKD). Exposure to smoke cigarettes using commercial filter cigarettes with the same brand throughout the study. Exposure to Divine cigarettes smokes use filter cigarettes with the same brand that has been smeared by the Divine solution. Exposure to cigarette smoke and Divine cigarette smoke done passively in the closed fumigation (*sidestream smoke*).

Termination of research subjects carried out on two step by the end of the 14th week (phase I), 5 rats of each

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group, and the rest was terminated at the end of the 28th week (phase II). Tissue processing was done to performed histopathological examination of the colorectal epithelium. At the end of the study, all samples performed an immunohistochemical examination. Avidin biotin complex method was used with primary antibodies : APC polyclonal antibody (biorbyt orb10109; dilution 1:100, UK); KRAS polyclonal antibody (biorbyt orb49092; dilution 1:100, UK); MSH2 monoclonal antibody (Biocare Medical CM219; dilution 1:100, USA); MLH1 monoclonal antibody (Biocare Medical CM220; dilution 1:100, USA); and p53 polyclonal antibody (biorbyt orb129712; dilution 1:100, UK). Histopathology of colorectal was assessed by scoring the following conditions: 1. the normal colon, 2.inflammation without dysplasia, 3. dysplasia and 4. carcinoma. Expression of APC, KRAS, MSH2, MLH1, and p53 assessed using the Allred score (Allred *et al*., 1998) in 10 fields of view at 400x magnification.

Independent Samples Kruskal-Wallis test followed by post hoc analysis using the Mann-Whitney U test were performed on all variables except KRAS expression of termination phase II that using One-way ANOVA. Multivariate analysis was used to test the minor hypothesis. Discriminant function analysis was used to explain how the role and contribution of the expression of APC, KRAS, MSH2, MLH1, and p53 on the function as the discriminator between the three groups. The result was Fisher's number that represents the dependent variable that has a major role in the function of distinguishing between the three study groups. Statistical analysis was performed with a 95% confidence interval with a limit of significance if $p < 0.05$

Results

Overview

This study was approved by Health Research Ethics Committee (KEPK) of Diponegoro University School of Medicine and Dr. Kariadi Hospital Semarang. The Research was conducted at Animal Facility of The Institute for Integrated Research and Testing Gadjah Mada University Yogyakarta and Anatomical Pathology Laboratory of Medical Faculty, Diponegoro University/ Dr. Kariadi Hospital Semarang. The Histopathological and immunohistochemical assessment conducted by two pathologists blindly. Kappa test showed very good agreement between the two examiners on all variables, colorectal epithelial morphology, expression of APC, KRAS, MSH2, MLH1 and p53 (Kappa value > 0.81; p <0.05).

Descriptive Data Analysis

The initial weight of the samples in this study was 176.2 ± 12.687 g with a minimum weight 152 g and 195 g maximum. The average weight of samples that terminated at phase I were 310.04 ± 41.845 g in K, 313.14 ± 41.369 g in AK group and 286.76 ± 40.367 g in AKD. The average weight of samples that terminated at phase II were 403.12 ± 53.857 g in K, 407.58 ± 44.198 in AK and 370.82 ± 38.190 in AKD.

Morphology of the colorectal epithelium from samples that terminated in phase I as follows: in K was 100% normal morphology, in AK was 20% normal morphology, 20% inflammation, 20% dysplasia and 40% carcinomas, whereas in the AKD was 60% normal morphology, 20% inflammation and 20% carcinoma. Morphology of the colorectal epithelium from samples that terminated in the last phase showed as follows: in K was 80% morphologically normal and 20% carcinoma, in AK was 100% carcinoma, whereas in AKD was 80% normal and 20% carcinoma.

APC expression from samples that terminated in the first phase showed the average score in K was 6.80 ± 1.304, in AK was 3.40 ± 3.847 and in AKD was 5.40 ± 3.130. APC expression from samples that terminated in the last phase showed the average score in K was 6.20 ± 3.493, in AK was 0 (constant) and in AKD was 5 ± 3.082. KRAS expression from samples that terminated in the first phase showed the average score in K was 7.00 ± 1.000, in AK was 3.60 ± 3.782 and in AKD was 4.60 ± 2.793. KRAS expression from samples that terminated in the last phase showed the average score in K was 4.00 ± 3.742, in AK was 3.00 ± 3.317 and in AKD was 3.00 ± 2,449. MSH2 expression from samples that terminated in the first phase showed the average score in K was 3.80 ± 1.095, in AK was 2.20 ± 2,280 and in AKD was of 3.60 ± 2.074. MSH2 expression from samples that terminated in the last phase showed the average score in K was 3 ± 2, in AK was 0 (constant) and in AKD was 3 ± 2. MLH1 expression from samples that terminated in the first phase showed the average score in K was of 3.60 ± 0.894, in AK was 1.80 ± 2.490 and in AKD was 3.60 ± 2.074. MLH1 expression from samples that terminated in the last phase showed the average score in K was 2.80 ± 1.789, in AK was 0 (constant) and in AKD was 3 ± 2. p53 expression from samples that terminated in the first phase showed the average score in K was 0 (constant), in AK was 1 ± 1.414 and in the AKD was 0.60 ± 1.342. p53 expression from samples that terminated in the last phase showed the average score in K was 0.80 ± 1.789, in AK was 0 (constant) and in AKD was 0.60 ± 1.342.

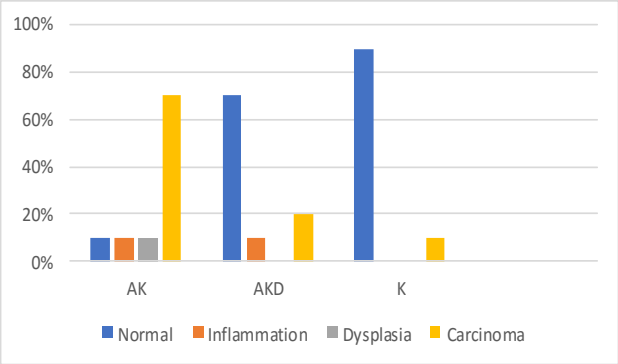

Figure 1. The proportion of colorectal histological changes in all three groups (N=30)

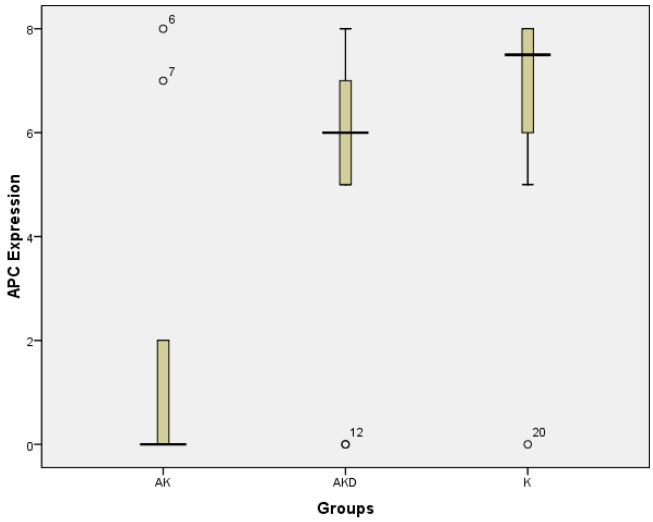

Figure 2. The expression of APC, data are presented as the ratio of three groups (N=30)

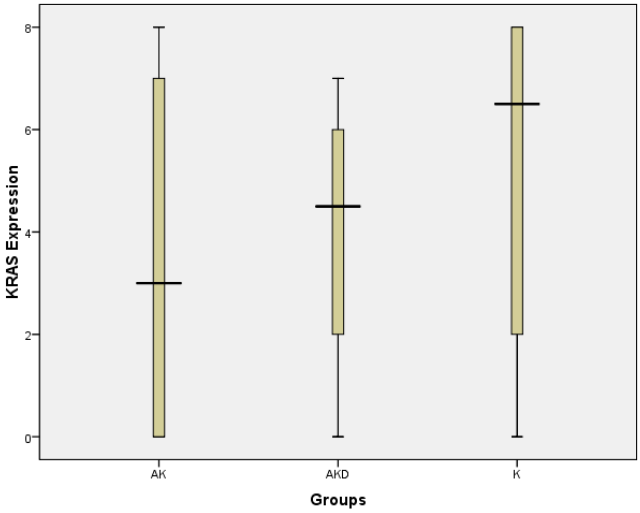

Figure 3. The expression of KRAS, data are presented as ratio of three group (N=30)

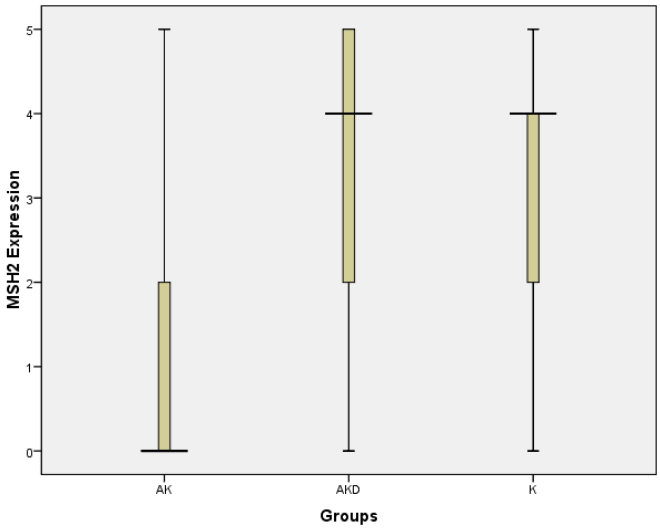

Figure 4. The expression of MSH2, data are presented as ratio of three groups (N=30)

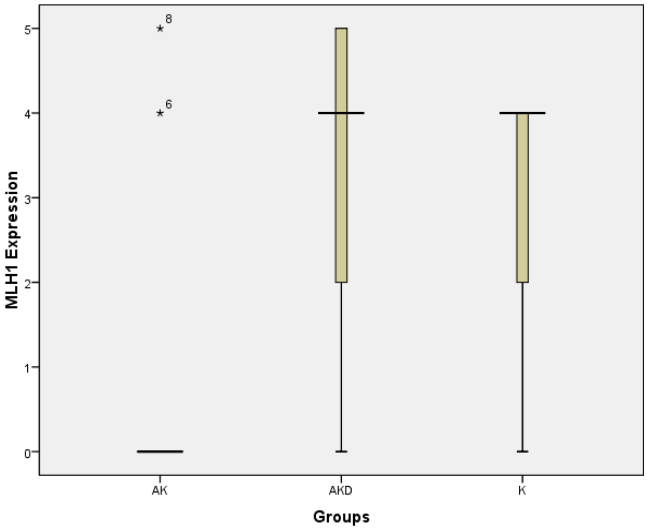

Figure 5. The expression of MLH1, data are presented as the ratio of three groups (N=30)

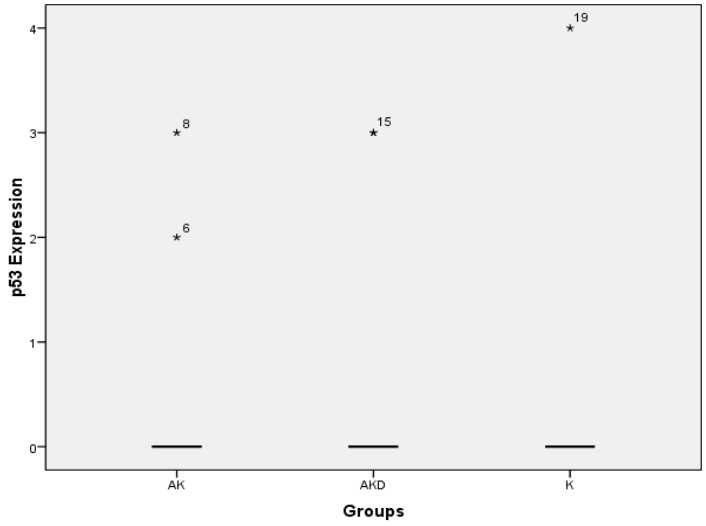

Figure 6. The expression of p53, data are presented as the ratio of three groups (N=30)

Overall of the three groups samples obtained colorectal epithelial morphology as follow : in AK group, 10% normal, 10% inflammation, 10% dysplasia, and 70% carcinoma; in AKD group, 70% normal, 10% inflammation, and 20% carcinoma; in K group, 90% normal and 10% carcinoma (Figure 1).

Immunohistochemical analysis of all samples in three groups showed: APC score in AK was 1.70 ± 3.129, in AKD was 5.20 ± 2.936, in K was 6.50 ± 2.506; KRAS score in AK was 3.30 ± 3.368, in AKD was 3.80 ± 2.616, in K was 5.50 ± 3028; MSH2 score in AK was 1.10 ± 1.912, in AKD was 3.30 ± 1.947, in K was 3.40 ± 1.578; MLH1 score in AK was 0.90 ± 1.912, in AKD was 3.30 ± 1.947, in K was 3.20 ± 1.398; p53 score in AK was 0.50 ± 1.080, in AKD was 0.60 ± 1.265, in K was 0.40 ± 1.265 (Figure 2-6).

Statistic Test

In the first termination phase, there were no significant differences between groups in all dependent variables ($p \geq 0.05$). In the last termination phase, there were significant differences between groups on the morphology of colorectal epithelium variable ($p = 0.018$), the expression of APC ($p = 0.025$), the expression of MSH2 ($p = 0.033$), and the expression of MLH1 ($p = 0.032$). There were no significant differences for the expression of KRAS and p53 ($p > 0.05$). Ordinal regression analysis performed on colorectal epithelial morphology variable showed significant differences between AK with AKD ($p = 0.012$) with coefficient (B) = 2.512 and OR = 6.305. While K with AKD was not significant ($p = 0.285$). Multivariate analysis using Wilks' Lambda for the expression of APC, MSH2 and MLH1 variables showed significant result with p value = 0.018 and Box's M ($p = 0.249$). Between-subjects effects tests found significant differences for the three dependent variables, APC expression ($p = 0.003$), MSH2 expression ($p = 0.013$) and MLH1 expression ($p = 0.008$). Post hoc analysis using Bonferroni test found significant differences as follows: in APC expression between K with AK ($p = 0.003$; MD = 4.80) and between AKD with AK ($p = 0.033$; MD = 3, 50). In MSH2 expression between K with AK ($p = 0.026$; MD = 2.30) and between AKD with AK ($p = 0.035$; MD = 2.20). In MLH1 expression between K with AK ($p = 0.022$; MD = 2.30) and between AKD with AK ($p = 0.016$; MD = 2.40).

The interaction and contribution of the dependent variable to the discriminant function of the three groups were analyzed using discriminant function analysis. APC expression was the variable that still included in the last analysis. Classification functions were analyzed using Fisher's linear discriminant function. Increased expression of APC score had a positive role of had interaction in the three groups. Expression of MSH2 and MLH1 have little interaction with the groups. The results of classification analysis showed the same distinguishing power of the original grouped and cross-validated grouped that is 60%.

Discussions

This study proved that cigarette smoke is one of the colorectal epithelial morphological changes toward malignancy and inflammation risk factor. It takes a long time for cigarettes smoke to induce neoplastic transformation in the colorectal epithelium. It needs between 14 to 28 weeks in rats or when converted to the man between 20-40 years. This result is similar to previous research in population and laboratory (Giovannucci et al., 1994, Novrial et al., 2017). Morphological changes induced by cigarette smoke indicates similar to *colitis-associated colon cancer (CAC)* or *'inflammation-dysplasia-carcinoma pathway'* (Figure 7), one of colorectal cancer subtype related inflammatory bowel disease (Terzic et al., 2010).

In this study, decreased expression of APC in the cigarette smoke group induced dysplasia and carcinoma. This phenomenon indicates that the APC mutation occurs at a late stage of carcinogenesis, as one of the characteristics of the CAC (Ullman and Itzkowitz, 2011, Barbaro et al., 2014). Decreased expression of MSH2 and MLH1 indicate alteration of DNA repair system (Wimmer et al., 2014). Negative expression of both MMR genes protein indicates that colorectal carcinoma induced by cigarette smoke is unstable or classified as MSI-H (Carethers, 2014). Loss of MSH2 and MLH1 expression can be caused by mutations or by DNA hypermethylation (Raskov et al., 2014). Decrease in expression of KRAS as a result of cigarette smoke was not proven in this study that indicates KRAS mutations do not occur, so that the tumor formed by cigarette smoke was a KRAS wild-type tumors, consistent with the previous study that proved correlation between smoking with increasing incidence of KRAS wild type adenoma (Wark et al., 2006), and wild type KRAS gene tumor (Weijenberg *et al*., 2008). Increased expression of p53 as a sign of cigarette smoke induced mutation was also not evident in this study. This may indicate that p53 gene transcription was altered. The alteration may be caused by epigenetic changes which occur in the p53 gene promoter (Lo and Sukumar, 2008). Immunohistochemical expressions are shown in figure 8.

Multivariate analysis showed expression of APC has a positive role or have interaction in all three groups, while the expression of MSH2 and MLH1 have no interaction with the group statistically. The results of the classification analysis obtained the same distinguishing power of the original grouped and cross-validated grouped that is 60%. This means that statistically, the variables which excluded from the analysis (expression of MSH2 and MLH1) have a small interaction to the expression of APC, although the univariate analysis showed significant in all three groups. This indicates variable expression of MSH2 and MLH1 have a distinguishing function individually, but only slightly interacts with the expression of APC. Expression of MSH2 and MLH1 in discriminant analysis seemed to have a small contribution even though when viewed on the mechanisms of carcinogenesis both are a pretty big role.

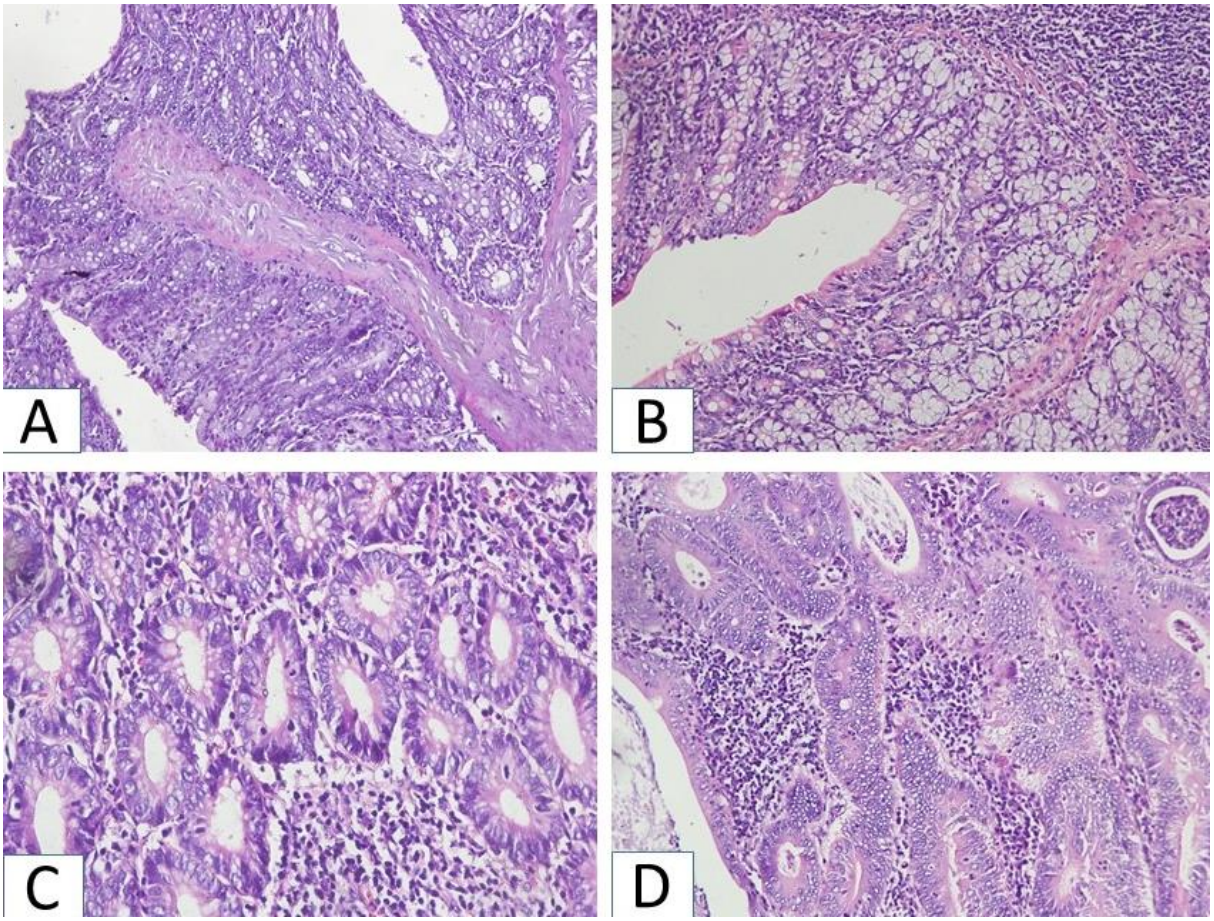

Figure 7. The histologic features found in this study indicated a colitis associated colorectal cancer related to cigarette smoke. A. Normal colon; B. Colitis without dysplasia; C. Colitis with dysplasia; D. Adenocarcinoma. (HE. 200x, 200x, 400x, 200x).

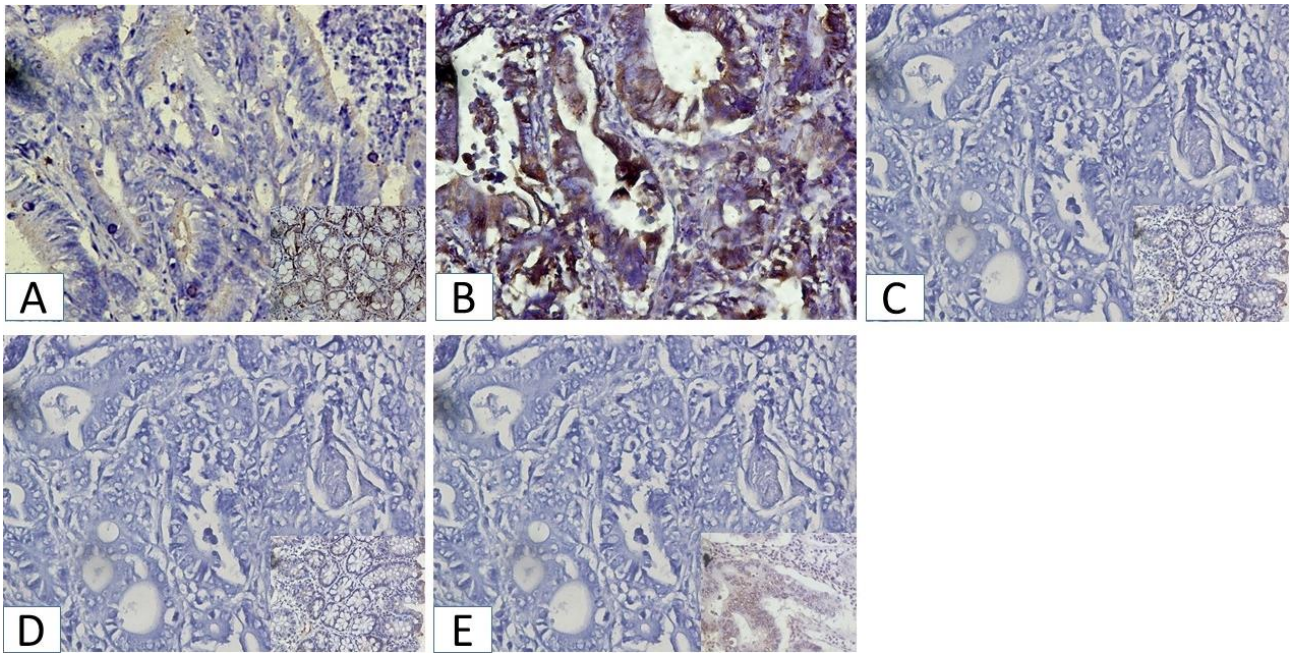

Figure 8. Immunohistochemical expression of colorectal carcinoma. A. Loss expression of APC (insert positive expression in normal mucosa, IHC 400x); B. Positive expression of KRAS (IHC 400x); C and D. Negative expression of MSH2 and MLH1 (insert positive expression in normal mucosa, IHC 200x); E. Negative expression of p53 (insert positive control, IHC 200x).

In the CAC, MMR gene mutations such as MSH2 and MLH1 plays a role in the initiation phase along with p53 gene mutation, whereas mutations in the APC gene plays a role in the promotion and progression phase (Ullman and Itzkowitz, 2011, Barbaro et al., 2014). Different functions at different stages are estimated to cause a less interaction between expression of MSH2 and MLH1 with the expression of APC. Discriminant function of APC by 60% can still be considered good but not too high. This indicates that there are other variables besides the involvement of APC, MSH2, and MLH1 in this process. The role of p53 although not significant may contribute through epigenetic changes that can not be identified in this study. Based on the analysis, the expression of APC, MSH2, and MLH1 can be used as a marker to see trends in colorectal carcinogenesis, especially in smokers.

This study proved the potential effect of the Divine solution as an inhibitor of genotoxic effect of carcinogens in cigarette smoke. The Divine solution contained compounds of aromatic groups such as aromatic amino acid and EDTA. EDTA serves as a metal chelator and has ability to form a low molecular weight complex with the metal atom as a central coordinator (Flora and Pachauri, 2010; Kannan *et al*., 2010), whereas biradical compounds such as aromatic amino acid, acetosal, manitol and methionine acid have capacity as scavenger and produce energy for metabolic system (Sumitro, 2011; Roy and Das, 2015). This low molecular weight complex has a high electron density and is very likely to act as a radical scavenger with Hg radical atom as central coordinator. Previous research using Electron Spin Resonance (ESR) proved that this low molecular weight complex had similarity with other biological complex structures such as chlorophyl, cytochrome, and hemoglobin which have significant role in electron transfer (Zahar and Sumitro, 2011b). Mg becomes metal element as central coordinator in chlorophyl complex structure, whereas Cu in cytochrome and Fe in hemoglobin (Bertini *et al*., 2009).

At the time cigarette is burning, radicals produced then bound and controlled by this complex structure. Furthermore, with the excess capacity of its electrons, Divine smoke inhaled into the circulation could potentially be a scavenger for radicals in the body and then create order in the biological systems (Zahar and Sumitro, 2011b). Regularity means in this study are the optimal functions of genes that regulate cell proliferation and DNA repair. This genomic stability obtained from the balance of free radicals in the body through the role of low molecular weight complexes compounds formed by the Divine solution. This situation produces a favorable micro environment for growth and repair at the cellular level marked by better colorectal epithelial morphology at AKD group compared to the AK group. In conclusion, we reported that cigarette smoke nanoparticles produced by Divine solution are capable of acting as low molecular weight complex that has a high electron density and this complex structure has a capacity as radical scavengers to reduce the toxicity of cigarettes smoke.

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