Do zinc and probiotic double supplementation on cigarette smoke induced inflammation in rat alter Malondealdehyde serum level and INOS (*Inducible Nitric Oxide Synthase*) expressionon lung tissue?

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Abstract

Introduction: Air pollution causes significant morbidity and mortality. One of the most common forms of air pollution is cigarette smoke (CS). Inhalation of CS causes oxidative stress, inflammatory response, generate nitrogen oxides and toxic free radicals.Zinc and probiotic are thought to be antioxidants to fight against free radicals.
Aim: Investigate mechanism of action of zinc and probiotic supplementations on the components ofmalondialdehyde(MDA) serum level and Inducible Nitric Oxide Synthase (iNOS) expression that underlie the effects of CS induced inflammation of Wistar rats.
Material and Method: Experimental research posttest only-control group. 30 Wistar rats which met inclusion criteria, randomized into five groups: Group KS: food only, K(-): Smoke, P1: Smoke+Zinc, P2: Smoke+Probiotics and P3: Smoke+zinc+probiotic.Two smoking cigarettes were given twice while supplementation once a day for 7 days, then rats were sacrificed. Blood sampleand lung tissuewere taken for serum MDAtest and immunohistochemistryINOS expression of lung tissue.
Result: Highest mean of MDA was P1 (191.20 ± 98.57), lowest mean was K(-)(101.27 ± 42.07). No difference of MDA level between K(-) and treatment groups (p> 0.05). INOS expression showed highest mean in K(-)(37.50 ± 11.40), lowest mean in P3 (17.50 ± 5.24). K(-) against P1, P2 and P3 had significant difference (p<0,05), while no significant difference shownbetween the group treatments.
Discussion: Supplementation only zinc or probiotics alone as well as double supplementation had no effect on changes in MDA levels in rats exposed to CS. It may due to brief duration period of treatment, inhibited zinc absorption because of cadmium. Zink and probiotic double supplementation had the lowest INOS expression compared to the other solo treatment groups, indicating zinc and probiotic may have the effect of reducing INOS production especially in lung tissue and airway. Study reported inhibition of iNOS could prevent or improve the manifestations of chronic obstructive pulmonary disease (COPD) in rats. This suggests that concurrent administration of zinc and probiotics can be a therapeutic option for COPD patients.

Keywords: Cigarette Smoke, Zinc, Probiotic, MDA serum level, INOS expression
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1. Introduction

Air pollution causes significant morbidity and mortality in patients with inflammatory airway disease, increases atopic allergic sensitization, and increases susceptibility to infection.[1] One of the most common forms of air pollution is cigarette smoke. Inhalation of cigarette smoke causes oxidative stress and consequence of the inflammatory response induced by smoke, further oxidation of NO occurs in CS (cigarette smoke) to generate nitrogen oxides and toxic free radicals.[2]

Cigarette smoke is associated in variety of diseases and poses a serious challenge to health systems worldwide. Cigarette smoke affects both innate and adaptive immunity.[3] In developed countries, smoking is the root of 24% of all mortality in men and 7% of all mortality in women. These figures rise to more than 40% in men in central and eastern Europe and to 17% in women in the United States.[4] Basic Health Research of Indonesia stated that in 2018, there is an increase prevalence of smoking in adolescents aged 10 -18 years from 2013 (7.20%) to 2018 (9.10%). Indonesia has the highest smoking prevalence rate in the world and it still has the potential to increase. Every year, more than 240,000 deaths happen due to smoking in Indonesia or in other word, there are 660 deaths every day.[5]

Zinc and probiotic are taught to be antioxidants by various mechanisms such as fight against free oxygen radicicals.[6-8] Both play a role in determining immune responses.

No studies have assessed the effect of zinc and probiotics as double supplementation on exposure to cigarette smoke as a tool for air pollution. The difference from the assessment of the measured parameters are Malondialdehyde(MDA) level andInducible Nitric Oxide Synthase(iNOS) expression in the lung tissue of rats exposed to cigarette smoke.

The aim was to investigate mechanism of action of zinc and probiotic supplementations on the components of MDA serum level and iNOS expression that underlie the effects of cigarette smoke induced inflammation of Wistar rats.

2. Material and methods

This study was laboratory experimental research with the posttest only-control group. The study protocol was approved by the Local Ethical Committee of Laboratory Animals at Diponegoro University. The place for procurement, maintenance, sampling, intervention on exposure to cigarette smoke were carried out at the Experimental Animal Laboratory, Faculty of Medicine, Diponegoro University for 14 days. Examination and analysis of rats' MDA serum levels was carried out at the GAKI Laboratory, Faculty of Medicine, Diponegoro University, Semarang and the expression of iNOS in rat lung tissue was carried out at the Anatomical Pathology Laboratory of Sebelas Maret University, Solo.

Animal and treatment

A total of 30 male Wistar rats aged 15 weeks old, weighing around 150g – 200g, healthy and active during procedure were used in our study. The environmental setting was dim light in our laboratory animals. Food was standard food for animal laboratory. The rats were acclimatization for 7 days, and were randomized into five groups as follows: Group KS: standard control, given food only (n:6), Group K(-): Smoke, Group P1: Smoke+Zinc, Group P2: Smoke+Probiotics and Group P3: Smoke+zinc+probiotic.

A 26,5 x 40 x 23 cm cabin with a thickness of 0.5 mm, ventilation above was prepared for the rats and given standard feeding. Two smoking cigarettes were given each in the morning at 07.00 am and 02.00 pm local time every day for 7 days. Using long oval pipe which connect the cigarette and the syringe of 50 cc, the smoke was inspired to the syringe and then puffed into the cabin through the round hole, until the cigarette was fully burned. The duration of smoking of a cigarette was recorded as a mean of eight minutes. The feeding procedure was given two times a day at 08.00 and 15.00 local time. This was to ensure the optimal absorption of zinc.

Administration of zinc and probiotic via probe was given 30 minutes after the first cigarette smoke exposure with a dose of zinc 3.5 mg/kgBW followed by probiotic 2.5×10^9 CFU/g for each of treatment group. Zinc and probiotics will be diluted into 4ml of water and given through a probe. The second cigarette smoke ignited at the 02.00 pm local time.

After 7 days, all rats were sacrificed thereafter. Termination of euthanasia used was an overdose anesthetic technique using ether. After animal dies, all buried in place provided by the laboratory. Lung tissue was carefully dissected and sectioned, fixed on 10% formaldehyde solution until further use. Blood samples were taken through the medial canthus sinus orbitalis using a 5cc syringe and put into a red cap vacutainer tube. Allow the sample to clot for 2 hours at room temperature then frozen to -26^0C for one week.

Biochemical MDA serum level evaluation

The biochemical study assessed was Malondialdehyde (MDA) level analyzed using the ELISA method. Sample used was blood serum and calculated using spectrophotometer with wavelength of 450 nm.

Histopathological of INOS Expression evaluation

Lungs were collected and sectioned, fixed on 10% formalin, dehydrated and embedded in paraffin. Immunohistochemistry of paraffin-embedded rat lung using iNOS Rabbit pAb (A0312) at dilution of 1:100 and evaluated by semi-quantitative analysis of the lung sections by double pathologists using the blind protocol. Histopathological changes evaluated in percentage of INOS expression shown in microscope using 10x and 40x magnifications.

Statistical analysis

The data of MDA serum level and percentage of INOS expression obtained were edited, coding, and entered in a computer file. After clearing, the data were analyzed statistically using the SPSS program.

Descriptive analysis of MDA serum levels and INOS expressions were shown the mean value and standard deviation as well as Box-Plot. Normality test of both data using Shapiro Wilks test. MDA serum levels shown not normally distributed, then the unpaired difference test was carried out using the non-parametric Kruskal Wallis test.

INOS expressions data were normally distributed, the unpaired difference test was carried out using the one-way ANOVA parametric test. From the results of the INOS difference test (%) using the one-way ANOVA test, it was found that $p < 0.05$ and levene > 0.05, based on the treatment group there were differences with homogeneous data variants. To find out the differences between groups, it was followed by using the Post Hoc LSD test.

3. Results

Evaluation of the MDA serum level

Multiple analyses were performed between each group. The MDA serum level in all groups was presented in Box-Plot graphic in Figure 1. It showed the highest mean of MDA level was group P1 which was191.20 ± 98.57. The lowest mean is group K (-) namely 101.27 ± 42.07.

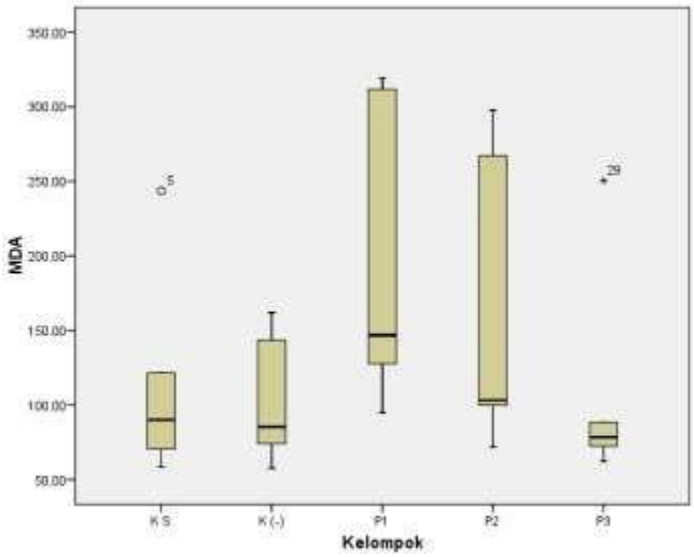

Figure 1. Box-Plot Graph of Serum MDA Level Production

No difference in MDA based on the KS group with K (–) (p value > 0.05). Data was not normally distributed. No difference in MDA between K(-) and the treatment groups. (p> 0.05)

Histopathological Result

Figure 2 shows the lowest mean of all INOS expression was in the KS group, which was 15.00 ± 4.74 and the lowest mean in the treatment group was in the P3 group of 17.50 ± 5.24. Meanwhile, group K (-) had the highest mean INOS expression, which was 37.50 ± 11.40.

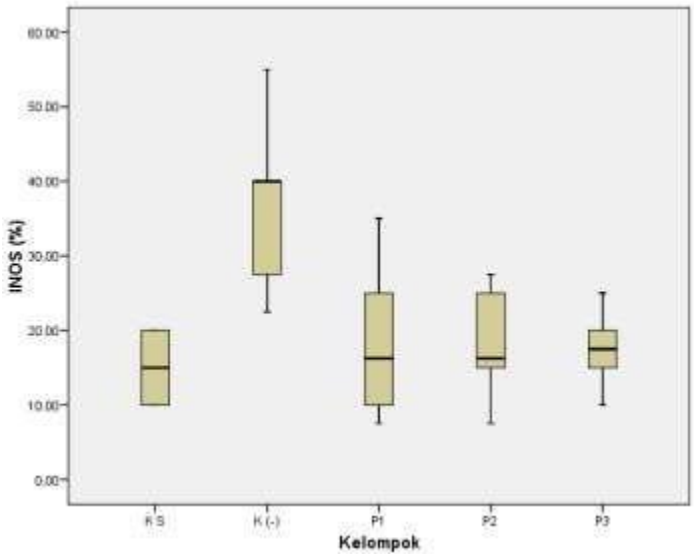

Figure 2. Box-Plot Graph of Lung tissue INOS Production

Statistical analysis revealedthere was a difference in INOSexpression (%) between KS (Fig. 3A, B) group with K (–) (p < 0.05)(Fig. 4A, B). There were differences INOS expression (%) between the treatment group, with homogeneous data variants (p<0,05; levene>0,05).

It was found that K (-) against P1, P2 and P3 had a significant difference (p<0,05), while no significant difference shownbetween the group treatments. (Fig. 5A, B)

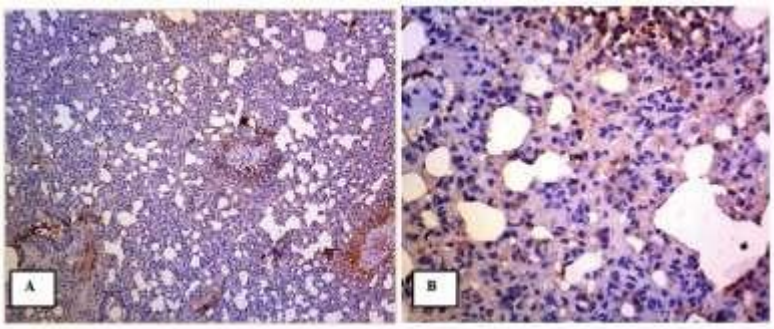

Figure 3. A) INOS expression in the KS group at 10x and B) 40x magnification with 15% expression of INOS.

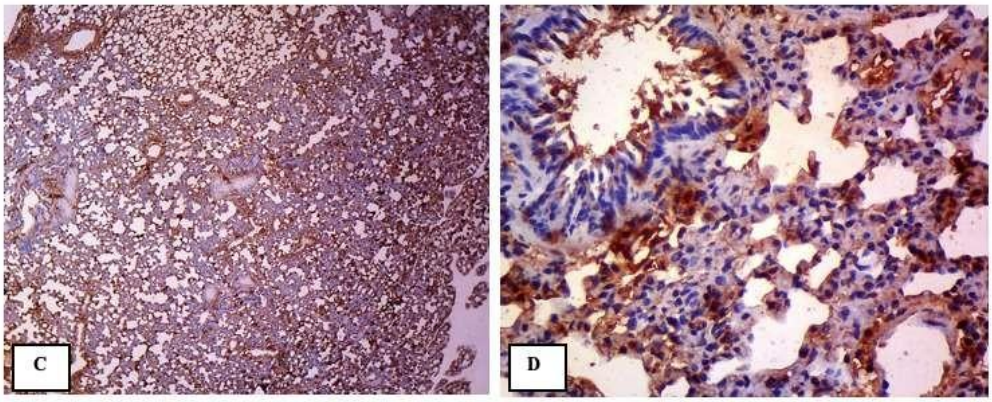

Figure 4. C) INOS expression in group K(-) at 10x magnification and B) 40x magnification with 55% expression of INOS.

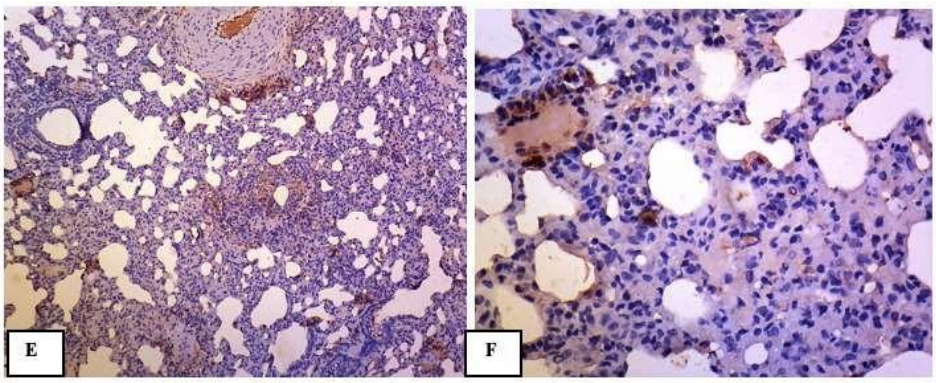

Figure 5.E) INOS expression in the P3 group with 10x magnification and F) 40x magnification with 10% of INOS expression.

4. Discussion

This study shows that, when compared with each other, no significant difference was observed between MDA serum levels and administration of Zinc-Probiotic oral in any of the groups. However, the INOS expression shows statistically significant distinguish in group exposed to cigarette smoke and group exposed to cigarette smoke with treatment with zinc only, probiotics only and shows a significance advantage when given both.

Oxidative damage to lipid compounds that occurs when free radicals react with PUFA (Poly Unsaturated Fatty Acid). Oxidative stress occurs because ROS in cells cannot be stabilized, causing damage to biomolecular DNA, RNA, proteins and lipids. In lipids, there will be lipid peroxidation, where the hydroxyl radicals react with unsaturated fatty acids.

One of the products of lipid peroxidation is malondialdehyde[9]

Free radicals produced in the body will be neutralized by antioxidants in the body. If the level of free radicals is too high, the ability of endogenous antioxidants is inadequate to neutralize free radicals, resulting in an imbalance between free radicals and antioxidants.[10]

Zinc functions as an antioxidant by various mechanisms. Zinc is an inhibitor of NADPH oxidase that produces ROS. Zinc is also a co-factor of the enzyme superoxide dismutase (SOD), which catalyzes the dismutation of $O^{-}2$ to H2O2.[11] Zinc as an essential micronutrient acts as an anti-oxidant, anti-apoptotic, and anti-inflammatory.[12]

Probiotics are live microorganisms that have health benefits when consumed or applied to the body.[13] Probiotics play a role in determining defense mechanisms including innate and adaptive immune responses. One of the main mechanisms of action of probiotics is through regulation of the immune response. Genomic and proteomic studies of probiotics identified several specific genes and compounds derived from probiotics, mediating immunoregulatory effects.[7, 14]

The study showed the supplementation of zinc alone, probiotics alone as well as zinc and probiotics together had no effect on changes in serum MDA levels in rats exposed to cigarette smoke.

Biala et al[15] in their 2016 study who treated acute nicotine to induce deep oxidative stress in brain structures, showed a significant increase in MDA concentrations and suppression of antioxidant enzyme activity (SOD and GPx) after nicotine administration, in all brain structures examined. Interestingly, a recent 2020 study by Khalifeh et al[16] with male rats exposed to water pipe tobacco smoking (WTS) or intraperitoneal nicotine injections showed that these therapies mediate stress resistance via BDNF and TRKB signaling but not anxiety. This may lead no increase of MDA production in rat serum.

Furthermore, fairly short duration of exposure to cigarette smoke and administration of treatment which was only given for seven days may also be the reason. Research conducted by Leeuwenburgh et al[17] proved that physical exercise in rats increased antioxidants and antioxidant enzymes in skeletal muscle and heart muscle after physical exercise for 10 weeks. After 10weeks, glutathione levels in active muscles increased by 33%, glutathione peroxidase activity increased 62% and superoxide dismutase levels increased by 27%. The antioxidant activity will be able to reduce malondialdehyde levels in the body. Research conducted by Debin et al[18] concluded that *Dendrophthoe pentandra'*swater extract therapy could reduce MDA levels and improve liver damage in hypercholesterolemia rats after 14 days of administration with doses of 400mg/kg BW and 800mg/kgBW. Ismiyati[19] which examined the antioxidant vitamin C dose of 8.57 mg/kgBW/day in rats exposed to cigarette smoke (one cigarette per fifteen minutes for sixty minutes) for thirty days was significantly different from untreated rats which resulted in an increase of MDA levels. Further research is needed on the effect of zinc and probiotics as antioxidants with longer duration of cigarette exposure and administration of zinc and probiotic.

Inhibited zinc absorption can also affect the results of Serum MDA levels. Research conducted by Ajose et al[20] found that cigarettes are the highest source of Cadmium. This study compared the levels of cadmium and zinc in smokers and non-smokers and their correlation to the prevalence of prostate cancer. Cadmium levels in smokers are higher than in the non-smoker group, while zinc levels in smokers are lower than levels in non-smokers, this indicates that smoking is a major factor in inhibiting zinc absorption and can be used as a biomarker in the incidence of prostate cancer. Reinforced by a follow-up study by Richter et al[21], cadmium levels in urine, serum, plasma, and blood were significantly correlated with smoking status in which cadmium inhibits the biochemical function of zinc and zinc supplementation.

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Hotchkiss et al[22] stated that iNOS protein will be produced by macrophages when activated by cytokines and toxins. iNOS has never been found physiologically in normal cells, iNOS remains active for 24-36 hours and can synthesize 100-1000 times more NO than nNOS and eNOS.

The group of rats exposed to cigarette smoke given any supplementation showed a significant difference to negative control group, where all the groups which were given the supplementation experienced a decrease in INOS expression. Administration of zinc and probiotic double supplementation showed significant differences in INOS expression compared to group supplementation solo. The results of the group with double supplementation had the lowest INOS expression compared to the other solo treatment groups, indicating zinc and probiotics may have the effect of reducing INOS production especially in lung tissue and airway.

Seimetz et al[23] reported that inhibition of iNOS could prevent or improve the manifestations of chronic obstructive pulmonary disease (COPD) in rats. This suggests that concurrent administration of zinc and probiotics can be a therapeutic option for COPD patients.

5. Conclusion

No statistical difference in the production of serum MDA levels in rats exposed to cigarette smoke with and without zinc, probiotics, as well as administration of both.

There was a statistically significant difference in the expression of INOS between rats' lung tissues in rats exposed to cigarette smoke and group given treatment. The group which was given the treatment showed a lower expression of INOS, and the lowest was in the double supplementation group.

6. Limitation

The limitation of this study namely brief duration period of treatment and no positive controls as standard control drugs, such as vitamin C.

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