

Artikel Penelitian

Rosuvastatin Attenuated Elastic Fiber Degradation in Chronic Obstructive Pulmonary Disease Sprague-Dawley Rats

Rosuvastatin Menghambat Destruksi Serabut Elastik pada Tikus *Sprague-Dawley* Model Penyakit Paru Obstruktif Kronik

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ABSTRACT

Chronic obstructive pulmonary disease (COPD) is an incurable disease which causes disability and death. The main pathogenesis of COPD is oxidative stress due to cigarette smoke which initiates various reactions and lead to lung elastic fibers destruction. Statins are known to have antioxidant effects and reduce mortality in COPD. We studied the effects of cigarette smoke exposure cessation and rosuvastatin on oxidative stress and the level of elastic fiber destruction in COPD model rats. Thirty 10-week old male Sprague-Dawley rats were divided into 2 groups: Control (n=6, did not received fumigation nor treatment) and Smoking (n=24, received fumigation for 70 days) groups. Afterwards, the smoking group was divided into 4 groups: Sham, R2, R5, R10, and received 0.9% NaCl, 2.5, 5 and 10 mg/kg/day of rosuvastatin, respectively. Examination of malondialdehyde (MDA) and desmosine serum were conducted to measure oxidative stress and elastic fiber degradation level, respectively. After smoke exposure, MDA and desmosine levels of COPD rats were found to be significantly higher (p=0.000 and 0.000) than controls. The MDA level in Sham, R2, R5 and R10 groups decreased significantly after therapy (p=0.000; 0.033; 0.015; 0.002). However, the post-treatment desmosine level was increase significantly in Sham and R2 groups (p=0.006 dan 0.012) and insignificantly (p=0.117 dan 0.278) in the R5 and R10 groups. It can be concluded that the cessation of exposure to cigarette smoke can reduce oxidative stress, but not elastic degradation process. The administration of rosuvastatin of 5 or 10 mg/kg/day attenuated elastic degradation process.

Keywords: COPD, rosuvastatin, oxidative stress, malondialdehyde, elastic fibers degradation

ABSTRAK

Penyakit paru obstruktif kronik (PPOK) merupakan salah satu penyebab utama kecacatan dan kematian di dunia. Patogenesis utama PPOK adalah stres oksidatif akibat asap rokok yang memicu penghancuran serabut elastik paru. Obat golongan statin terbukti memiliki efek antioksidan dan menurunkan mortalitas pada PPOK. Pada penelitian ini dikaji efek penghentian paparan asap rokok dan pemberian rosuvastatin terhadap stres oksidatif dan destruksi serabut elastik pada tikus model PPOK. Tiga puluh ekor tikus *Sprague-Dawley* jantan usia 10 minggu dibagi menjadi 2 kelompok, yaitu Kontrol (n=6) yang tidak mendapat perlakuan khusus, dan *Smoking* (n=24) yang mendapat paparan asap rokok selama 70 hari. Selanjutnya kelompok *Smoking* dibagi menjadi 4: *Sham*, R2, R5, R10 (n=6), yang secara berturut-turut mendapatkan terapi NaCl 0,9%, rosuvastatin 2,5; 5; dan 10mg/kgBB/hari. Tingkat stres oksidatif serta degradasi serabut elastik pre dan pascaterapi diperiksa dengan mengukur kadar *malondialdehyde* (MDA) serum menggunakan metode *thiobarbituric acid-reactive substance* dan desmosin serum dengan ELISA. Kadar MDA dan desmosin pre-terapi tikus PPOK lebih tinggi bermakna (p=0,000 dan 0,000) dibanding kelompok kontrol. Uji t berpasangan digunakan untuk membandingkan kadar MDA dan desmosin pre dan pascaterapi intragroup. Kadar MDA pasca terapi kelompok sham, R2, R5 dan R10 menurun bermakna (p =0,000; 0,033; 0,015; 0,002). Kadar desmosin kelompok sham dan R2 meningkat bermakna (p=0,006 dan 0,012), dan tidak bermakna pada kelompok R5 dan R10 (p=0,117 dan 0,278). Dapat disimpulkan bahwa penghentian paparan asap rokok dapat menurunkan stres oksidatif, namun demikian, destruksi elastin masih terus terjadi. Pemberian rosuvastatin 5 atau 10mg/kg/hari mampu menekan destruksi serabut elastik.

Kata Kunci: Degradasi serabut elastik, *malondialdehyde*, PPOK, rosuvastatin, stres oksidatif

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INTRODUCTION

Smoker prevalence in many countries in the world, including Indonesia is increasing every year (1). Cigarette smoking is the most important risk factor for cardiovascular disease and chronic obstructive pulmonary disease (COPD), which cause progressive airflow limitation (2). The pathogenesis of COPD starts from free radicals in cigarette smoke that triggers the activation of alveolar macrophage cells (MA) as the first-line defense cells. The activated alveolar macrophages release a variety of inflammatory mediators that attract neutrophils and monocytes, which subsequently increase the production of Reactive Oxygen Species (ROS) such as superoxide, hydrogen peroxide and hydroxyl radicals ($\cdot\text{OH}$) (3).

In a physiological state, human body has its own mechanism to balance the level of oxidants by the activity of endogenous antioxidants. However, ROS from cigarette smoke could reduce this activity. Oxidant burden from cigarette smoke and inflammatory cell's product can lead to oxidant-antioxidant imbalance or oxidative stress. The level of oxidative stress can be measured indirectly by measuring the levels of *malondialdehyde* (MDA) as the product of lipid peroxidation(3).

In addition to ROS, inflammatory cells also produce a variety of protease, which is a proteolytic enzyme that can degrade proteins, including lung elastin. Under normal circumstances, proteases are essential for the defense mechanism and antiproteases are present to protect the surrounding tissue from the destructive effects of proteases. However, the ROS burden could lead to α 1-AT deficiency in the body (4). Increased production of proteases accompanied by the inactivation of antiproteases promotes elastin degradation which results in the COPD condition(3).

When elastin is degraded by proteases, desmosine, which is a component of cross-links in mature elastin is detached. Since the released desmosine cannot be absorbed, reused or catabolized by the body, it will be disposed of in its unchanged form. Desmosine is a unique amino acid which is only found in mature elastin, the degree of elastin damage can be detected by measuring the desmosine level in the blood(5).

The progress of COPD can be reversed by smoking cessation, but there is no available drug to cure the disease (6). Giving an antioxidant to prevent tobacco smoke-induced oxidative stress and COPD has shown promising results but its remedial effect on COPD patients is very limited (7-12). Statins, such as rosuvastatin are known to have antioxidant effects. A recent study and meta-analysis on many studies of statin concluded that there is a mortality reduction in COPD patients receiving statin therapy (13-15). However, Criner G *et al* found no significant difference of mortality rate in COPD patient with statin compare to without statin (16). Compared to other statins, rosuvastatin has the longest half-life (19 hours) as well as rarely causes myopathy, liver function test abnormalities and adverse interactions with other drugs (17). This study aimed to determine the effect of smoke cessation and administration of rosuvastatin in reducing the oxidative stress and elastic degradation in COPD rats by measuring serum MDA and desmosine levels.

METHODS

Animals

Ten weeks old male *Sprague-Dawley* rats (n=30) were purchased from Integrated Research and Testing Laboratory, Universitas Gadjah Mada, Yogyakarta, Indonesia. Rats were maintained in cages with light-dark cycle every 12 hours. Food and water were given ad libitum. Ethical clearance was obtained from The Medical and Health Research Ethics Committee, Faculty of Medicine, Public Health, and Nursing Universitas Gadjah Mada, Yogyakarta, Indonesia (KE/FK/452/EC).

Induction of COPD

The rats were divided into 2 groups: Control (n=6) and Smoking groups (n=24). The Control group did not receive fumigation, while Smoking group received cigarette smoke exposure at 8 cigarettes/day. Each cigarette used in this study contained 38 mg of tar and 2.4 mg of nicotine. Cigarette smoke exposure was given using the whole body exposure method. Every day, the rats from Smoking group were moved into the smoking apparatus. The smoking apparatus consist of three major parts. Cigarette burn chamber in the bottom, smoke exposure chamber in the middle and ventilation in the top of the apparatus. The smoke exposure chamber was a 50 cm x 50 cm x 50 cm of glass chamber. Burn chamber and smoke exposure chamber were separated by a metal nets. This nets allowed the smoke from the cigarette entering the smoke exposure chamber. For smoke exposure, cigarettes were burned in the burn chamber, and the smoke from the cigarette entered the box (Figure 1). Since all the smoke was released from the tip of the cigarette, all the smoke produced was side stream smoke. The rats were exposed to cigarette smoke for 70 consecutive days, as described by Zhang *et al*, with a slight modification (smoking exposure was given everyday instead of 6 days/ week)(18).

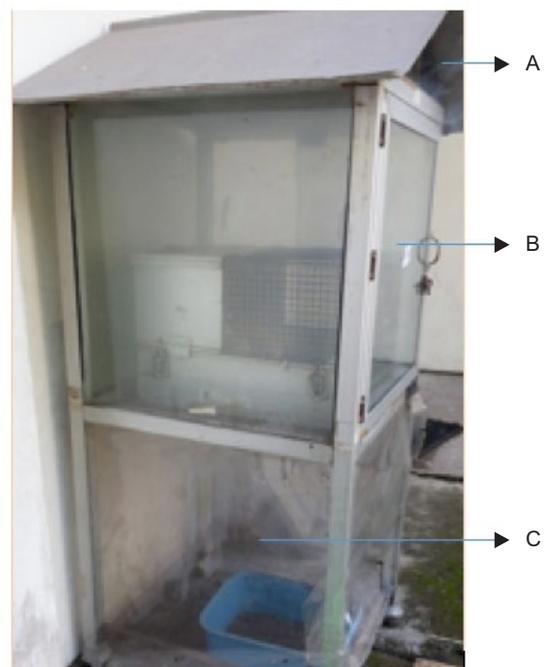


Figure 1. Smoking apparatus

Note: Smoking apparatus consist of (A) ventilation in the top of apparatus, (B) smoke exposure chamber to place the rats, and (C) bottom part to plasce and burn the cigarette.

Treatment

On Day 71, exposure to cigarette smoke was discontinued. The rats from Smoking group were divided further into 4 groups: Sham, R2, R5 and R10 groups (n = 6 for each group). Subsequently, each rat of Sham, R2, R5 and R10 groups received treatment for 27 days via oral administration. Sham group received 2 ml of NaCl 0.9%; R2, R5, R10 groups received 2.5 mg/kgBW, 5 mg/kgBW and 10 mg/kgBW of rosuvastatin, respectively. The rosuvastatin tablet was crushed into powdered and diluted in NaCl 0.9% solution, and given to the rats through a feeding tube.

Sample Collection

Blood samples were taken one day after the last cigarette smoke exposure (for pre-treatment measurement) and one day after the last treatment (for post-treatment measurement). The blood was taken from the retro-orbital vein using a micro-hematocrit capillary tube. Within one hour after collection, the pre and post-treatment blood were centrifuged at 4000 rpm for 20 minutes to collect the serum.

Oxidative Stress Level Measurement

The level of oxidative stress was determined by measuring serum MDA levels using the thiobarbituric acid-reactive substance (TBARS) method according to the previous study (19,20). Briefly, 1 ml serum was mixed with 4 ml 'TBA reagent', incubated in water bath at 90°C for 80 minutes and cooled in ice. After being mixed with butanol extraction solution and centrifuged, the pink color complex in the supernatant was measured spectrophotometrically at 510, 532 and 560 nm. The actual absorbance was determined by the following equation:

$$\text{Actual } A_{532} = 1.22 [(A_{532}) - (0.56) \times (A_{510}) + (0.44) \times (A_{560})]$$

TBARS concentration ($\mu\text{mol/l}$ solution) was read using 1,1,3-3-tetramethoxypropane as a standard.

Elastin Degradation Level Measurement

The determination of elastin destruction level was performed by measuring the level of desmosine serum using ELISA. The procedure was based on the manufacturer's protocol (MyBiosource, catalog number: MBS 748709).

Statistical Analysis

The statistical analyze was performed using SPSS (Statistical Product and Service Solutions). After fumigation, the MDA and desmosine level between Control and Smoking groups were analyzed using unpaired t-test. After treatment, the post-treatment serum MDA levels and desmosine levels between Control, Sham, R2, R5, and R10 groups were compared using One Way ANOVA. Serum MDA and desmosine levels between

pre and post-treatment in each group were analyzed using paired t-test. All the results were shown in mean \pm SD. The statistical significance was set at p value of <0.05.

RESULTS

Serum MDA Level

After fumigation, serum MDA levels in the Smoking group were significantly higher than Control group (Table 1). This result indicated an increase of oxidative stress in rats exposed to cigarette smoke.

Table 1. Serum MDA and desmosine levels in control and smoking groups after 70 days of cigarette smoke exposure

	Control Group	Smoking Group	p value
MDA ($\mu\text{mol/L}$)	1.46 \pm 0.30	2.59 \pm 0.42*	0.000
Desmosine (ng/mL)	1.76 \pm 1.07	8.47 \pm 3.91*	0.000

Note: Control group (did not receive cigarette smoke exposure). Smoking group (received cigarette smoke exposure for 70 days) * p<0.001 (unpaired t-test analysis compare to control group).

One day after smoke cessation, the Smoking group was further divided into 4 groups (Sham, R2, R5, and R10). At the end of the study, 29 rats were survived, but one rat from R2 group died in the middle of the study. After 27 days of rosuvastatin treatment, serum MDA level was measured again. Paired t-test analysis showed that post-treatment serum MDA levels in Sham, R2, R5, and R10 groups were significantly lower than the pre-treatment level (Table 2). The analysis of the post-treatment MDA level between groups using one way ANOVA showed no significant difference. This suggested that smoking cessation with or without rosuvastatin reduced MDA level.

Serum Desmosine Level

Following cigarette smoke exposure, serum desmosine levels in the Smoking groups were significantly higher than that of the Control group (Table 1). This suggested that smoke exposure induced elastic fiber degradation in rats.

Twenty-seven days after the administration of NaCl 0.9% or rosuvastatin, the level of serum desmosine was measured again. We found that the post-treatment desmosine level increase in all treatment groups compared to pre-treatment levels. Paired t-test analysis between pre and post-treatment within groups showed significantly higher post-treatment desmosine level than the pre-treatment level in Sham and R2 groups. However there was no significant difference between pre and post-treatment desmosine level in R5 and R10 groups.

Table 2. Pre- and post-treatment serum MDA and desmosine levels in Sham, R2, R5 and R10 groups

	Sham (n=6)	R2 (n=5)	R5 (n=6)	R10 (n=6)
MDA pre-treatment ($\mu\text{mol/L}$)	2.49 \pm 0.28	2.73 \pm 0.67	2.64 \pm 0.49	2.53 \pm 0.29
MDA post-treatment ($\mu\text{mol/L}$)	1.31 \pm 0.36*	1.76 \pm 0.32*	1.77 \pm 0.32*	1.53 \pm 0.52*
Desmosine pre-treatment (ng/mL)	6.41 \pm 3.25	7.03 \pm 2.52	8.30 \pm 2.76	11.9 \pm 4.76
Desmosine post-treatment (ng/mL)	14.56 \pm 7.19*	17.76 \pm 3.90*	14.76 \pm 7.16	18.0 \pm 8.30

Note: Sham group (cigarette smoke cessation + 2ml NaCl 0.9%); R2 group (cigarette smoke cessation + rosuvastatin 2.5mg/kg/day); R5 group (cigarette smoke cessation + rosuvastatin 5mg/kg/day); R10 group (cigarette smoke cessation + rosuvastatin 10mg/kg/day); * p <0.05 paired t-test between pre-treatment and post-treatment level within group).

DISCUSSION

Cigarette smoke exposure is known to be the main risk factor for COPD. Cigarette smoke consists of many toxic particles, including ROS (3). Lipid content in the cell membrane is the principal target molecule of oxidation by ROS (21). The increase of MDA level after smoke exposure observed in the present study was consistent with the findings in other studies on animals and humans (22) This finding suggests that cigarette smoke exposure increases oxidative stress. Some studies revealed that the increase of oxidative stress in smoking subjects were caused by the higher concentration of ROS derived from cigarette smoke as well as activated neutrophils and macrophages. Moreover, cigarette smoke also leads to the depletion of endogenous antioxidants level such as vitamin E, vitamin C, superoxide dismutase (SOD), Catalase (CAT) and glutathione peroxidase (GPX). This in turn worsens the oxidative stress condition (22,23).

Serum MDA Levels

To date, pharmacology therapies that are available for COPD are for reducing the symptoms, but not for curing the disease. Therapy that is known to inhibit the progressivity of COPD is smoking cessation. In this study, smoke cessation has been shown to reduce MDA levels. Other studies also demonstrated that smoking cessation reduced the levels of MDA (24). However, the present study found that rosuvastatin did not reduce the levels of serum MDA greater than smoke cessation only. Statistical analysis using one-way ANOVA did not show any significant difference between groups in post-therapy MDA levels.

Some studies reported that rosuvastatin reduced the level of MDA and increased antioxidant levels in subjects with stroke, atherosclerosis, dyslipidemia, and obesity (25-27). On the other hand, one previous study showed that although antioxidant administration was able to reduce the level of serum MDA on rats which were exposed to cigarette smoke, its administration on normal rats did not show any effect on MDA levels (28). Therefore, there is a possibility that upon the optimum redox balance of the oxidant-antioxidant system, the body will maintain this balance. Overall, our study shows that cigarette smoke cessation is capable of reducing the serum MDA level in COPD model rats. However, the ability of rosuvastatin to reduce the level of oxidative stress in COPD model rats was not evidenced in this study.

Serum Desmosine Level

This study showed that the Smoking groups which were given cigarette exposure had higher levels of desmosine than the Control group. This result was in agreement with other studies on human (29). ROS contents in cigarette

smoke are potent to inactivate α 1-AT, which is a major antiprotease, by oxidating the active side of this enzyme (30). This mechanism induces a protease-antiprotease imbalance that leads to elastin degradation in cigarette exposure.

Smoke cessation was expected to reduce the serum desmosine. The antioxidant effects of rosuvastatin as an adjuvant therapy was expected to restore the function of α 1-AT which in turn suppress the elastin degradation rate. However, this was not the case of our study. Our study found a significant increase of desmosine levels in the Sham and R2 groups following treatment, but not significant in R5 and R10 groups. This result may suggest that the elastin degradation process continued after smoking cessation. Furthermore, there is a possibility that elastin degradation was affected by other factors besides oxidative stress.

One of the factors that could possibly increase elastin degradation after smoking cessation is inflammation. Studies in humans showed that the inflammatory process in COPD subjects was persistent and even increased after smoking cessation (31). Since inflammatory cells such as neutrophils and macrophages are capable to produce a variety of proteases (3), possibly further destruction of elastin continue to occur. However, the administration of 5 mg/kgBW or 10 mg/kgBW of rosuvastatin was shown to attenuate the destruction process, since there was no significant difference in desmosine levels between pre and post-treatment. However, this possibility needs to be confirmed. In the future, it is important to measure the inflammation level of the subjects. Furthermore, to ensure that the increase of desmosine level comes from the lungs, the measurement of the desmosine protein levels and mRNA expression in the lungs are needed.

In conclusion, this study demonstrated that cigarette smoke cessation decreased oxidative stress as characterized by the reduction of serum MDA levels. However, the antioxidant effect of rosuvastatin could not be observed. This study found an increase of desmosine level following smoke cessation, suggests that elastic degradation continued after smoke cessation. The administration of 5 or 10 mg/kgBW/day of rosuvastatin has been shown to attenuate elastic degradation process.

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