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***Original Article***

# The automatic transmission motorcycle ultrafine particles $PM_{0.1}$ effects in the alveolar enlargement, depleted of septum alveolus, and lung inflammation

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**Abstract**

Motorcycles smoke have been responsible inducing lungs related to diseases for a long time. This study was aimed to reveal the effect of the repeated exposure automatic transmission motorcycle smoke in the development of lungs damage in the wistar mice by observing the alveolar, the septum alveolar, and the tissues inflammation. Threes groups of the wistar mice had been labelled as control, C1, and C2 and keep in 30 x 20 x 20 $cm^3$ chamber. The C1 group was introduced by the motorcycle smoke for 60 second and C2 for 100 seconds. The smoke was flowed into a chamber by using an air pump with the rate of 2 m/s. The smoke was exposed repeatedly to the mice as long as 10 sequence days, and the lung was observed for every 2 day. The alveolar size was observed by measuring the line core length. The septum alveolar was indicated by the depleted septum alveolus, and the inflammation was identified by the morphological way. All of the result were statistically analysed. The alveolar line core grew longer for the C1 and C2 group of 11±6 µm and 20±2 µm compared to the control. The Depleted Septum Alveolar increased up to 77% for the C1 group and 79% for the C2 group. The tissue inflammations were found in every sample. The $R^2$ from statistical approach showed the strong correlation between the lung damage and the repeated exposures. The lung damage in terms of alveolar size enlargement, the increase of damage septum alveolus, and tissues inflammation were related to repeated exposure of motorcycle smoke.

**Keywords:** Alveolar enlargement, inflammation, mice's lung, motorcycle smoke, repeated exposures, septum alveolus.

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## Introduction

A number of motorcycles has increased in Asia, e.g., more than 224 million in China (Li et al., 2015), 12 million in Taiwan (Lin et al., 2008), 121 million in India (DayalSharma et al., 2011), and they reached 84 million in Indonesia in 2013. Consequently, the particulate matter concentration has increased in the air. The concentration becomes significantly high at the closer area of the highway rather than any other areas (Tri et al., 2014) that proves the effect of the number vehicles contributes to the air pollution. The emissions are in terms of gasses, e.g. hydrocarbon (Lin et al., 2008) $NO_2/NO_x$ (Keuken et al., 2012), and particulate matters in various size distribution such as: $PM_{10}$ (particles with the diameters < 10µm), $PM_{2.5}$ (particles with the diameters < 2.5µm) (Srimuruganandam and Nagendra, 2012), and $PM_{0.1}$ ( particles with the diameters < 0.1µm) (Ibald-mulli et al, 2002; Wardoyo et al., 2006; Tang et al., 2012) growing in the high concentration. The usage of carburetor fuel supply systems, air-fuel ratio in the engine, engine aging, fuel without a catalyst, and compressed engine ratio have affected the concentration of the particulate matter emissions (Hassani & Hosseini, 2016).

The vehicle emission has been significantly found to affect the human health (Kampa & Castanas, 2008) particularly associated to the development of lung diseases (Schaumann et al., 2014), influenza-like illness (Liang et al., 2014; Feng et al., 2016), asthma (Buonanno et al., 2015), respiratory diseases (Huang et al., 2016), chronic obstructive pulmonary diseases (*COPD*) (Karakatsani et al., 2012) and allergy response (Baldacci et al., 2015). The effect of the cigarette smoke particulate matters on lung has been identified in the previous study in terms of the depleted of septum alveolus (DSA) that lead into the tissues inflammation (Yamamoto et al., 2006; Bourdon et al., 2012; Xu et al., 2013); inflammation that was identified by the hemorrhage (Pinkerton et al., 2000; Nørgaard et al., 2010); and the development of the emphysema (Stinn et al., 2013). In the long exposure case by cigarette smoke (Hoek et al., 2013) was found the similar damage as the short terms (Gualano et al., 2008; Braakhuis et al., 2014; Jiang et al., 2014). However, the specific influence the motorcycle emission on lung has not been investigated yet. Especially the impacts of ultrafine particle motorcycle emission on the lung alteration in terms of alveolus geometrical alteration, depleted of septum alveolus, and inflammation have been unkown as well. By the fact that is the human exposure to motorcycle emission in their daily activity especially in ASIA country is very high. This research has become a pretty urgent to be conducted. This study was aimed to find the evidence about the effect of the repeated exposures of the motorcycle emission on the lung alteration.

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## Methods

### Experiment Method

The particulate matter concentration was measured in terms of the ultrafine particle concentration contained in the automatic transmission motorcycle smokes by using a TSI P-Trak Ultrafine Particle Counter Model 8525. The measurements were sampled for every 10 seconds. The

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total concentration is calculated by using Eq. 1 (Wardoyo et al., 2006; 2007).

$$C_T = Q \sum_0^n Cs \quad \text{.....(1)}$$

Where; $Q$ is the total debit, $C_T$ is the total concentration of $PM_{0.1}$, Cs is a measured concentration of particles/cm$^3$. The concentration of the particles contained in the chamber was written as the number of particles contained in 1200 cm$^3$ chamber (Particles/cm$^3$) (Wardoyo et al., 2017).

The alveolar was observed by using a morphometric method (Stinn et al., 2013). The mean core line length $L_m$ was measured in order to find the geometrical alteration of the alveolar (Muñoz-Barrutia et al., 2012). The mean chord was measured based on the intercept distribution, and this data was used to analyze the geometrical damage in alveolus (Sørensen et al., 2010; Ishii et al., 2012). The geometrical alteration was counted as the numerical data to obtain the alteration level together along with the depleted septum alveolar (DSA) and the inflammation. The inflammation was identified for all histological images by observing the H&E stain. The damages of the lung were counted and presented in the percentage as seen in the Eq. 2.

$$DgP\ (\%) = DgA/TA \times 100\% \quad \text{.....(2)}$$

Where; DgP is the damage (in %), DgA is the total damage of alveoli, TA is the total of alveoli. Data are presented as the mean value of n observations ± standard deviations (SD). The correlation between the total of $PM_{0.1}$ and the lung damage percentages was investigated by using R-square analysis (Yu et al., 2016).

### Experiment Setup

The motorcycle smoke exposures to Wistar mice (*Mus musculus)* were conducted by injecting the smoke into a 1200 cm$^3$ chamber with the pump rate of 2 m/s as long as 60 seconds (1-minute). The smoke was exposed to the Mice twice a day for 100-seconds. The exposure was repeated for ten sequence days. The automatic transmission motorcycles were chosen randomly as their popularity in Indonesia, and the brand was hidden to avoid the conflict of interest in this research. The setup of the measurement is shown in figure 1. Male mice were used as an experimental animal in this research. The mice were put into the chamber and were isolated from any ambient particulate matters, under the static room temperature, were provided with water, and food ad libitum by the following animal cares for an acclimation process. The acclimation process was carried out for 3 days to avoid a stressed and evaded mistreated condition. The mice then were divided into 2 different groups where each group contained 10 mice. The first group acted as the control group. The control group was unexposed by the smoke while kept in the quarantined condition. The second group was exposed to the smoke with the dose concentration that was set by introducing the smoke into the chamber for 60 seconds. The mice lung was observed for every 2 days. In order to make the sample preparation, the mice lungs were cleaned using *NaCl* solution (0.9%) and soaked with formalin solution (10%) in order to examine the histological image in a week (Jia *et al*., 2012). The lungs were rinsed using alcohol and *xylol* three times for the duration of 20 mins. The results of these histological steps then were put on the object glasses and covered with *entellan*-cover glasses. Finally, they were observed using computer microscope (400x-magnification) in five random areas (Brandenberger et al., 2015) to generate the histological image. All animal treatments used based on the international standard guide for animal treatment and the guide of *Ethic Committee* of *Experiment Animal of the University of Brawijaya* (Ethical Clearance No: 541-KEP-UB).

**Table 1.** $PM_{0.1}$ concentration in particles /cm$^3$

| Exposure day | C | Concentration x $10^5$ particles/cm$^3$ | | |
|---|---|---|---|---|
| Single exposure | $C_0$ | 4.4 | ± | 0.1 |
| days 2 | $C_2$ | 64.5 | ± | 4.5 |
| days 4 | $C_4$ | 67.1 | ± | 2.1 |
| days 6 | $C_6$ | 71.9 | ± | 3.7 |
| days 8 | $C_8$ | 72.2 | ± | 1.9 |
| days 10 | $C_{10}$ | 76.8 | ± | 4.3 |

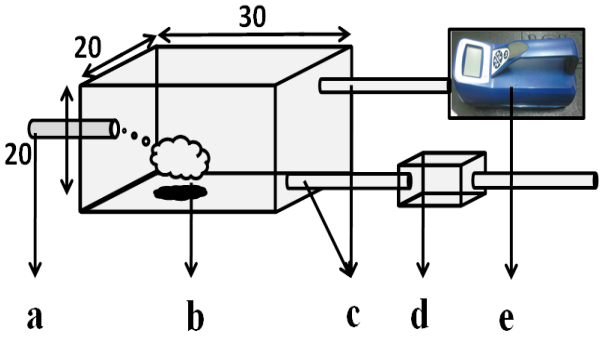


**Figure 1.** The particle measurement setup ((a) exposure chamber; (b) rubber tube; (c) P-Trak; and (d) sucking pump).

## Results

### $PM_{0.1}$ Concentration

The mean and standard deviation of the total concentration of $PM_{0.1}$ was measured of (4.40 ± 0.1) x$10^5$ particles/cm$^3$. The concentration was used as a single dose exposure concentration. The $PM_{0.1}$ exposure dose concentration is presented in table 1 and figure 2.

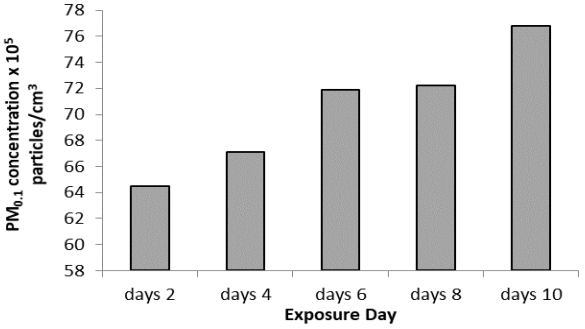


**Figure 2.** The graph of the measured $PM_{0.1}$ concentration for the different exposure day.

**Alveolar Size Enlargement**

The alveolar enlargement was identified in the histological image. The enlargement of the line length may be caused by the repeated exposure. It is also found that the change of geometrical properties of the emphysema. This can be used to identify the geometrical damage. The change of the core length is presented in table 2.

**Table 2.** Average line core length in mm for 10 days

| Average Core Length | C1 | |
|---|---|---|
| | Mean | Stdev |
| $C_0$ | $5.7 \times 10^{-3}$ | $1.4 \times 10^{-3}$ |
| $C_2$ | $8.5 \times 10^{-3}$ | $3.7 \times 10^{-3}$ |
| $C_4$ | $9.8 \times 10^{-3}$ | $5.1 \times 10^{-3}$ |
| $C_6$ | $9.6 \times 10^{-3}$ | $4.6 \times 10^{-3}$ |
| $C_8$ | $10.2 \times 10^{-3}$ | $4.1 \times 10^{-3}$ |
| $C_{10}$ | $11.5 \times 10^{-3}$ | $6.0 \times 10^{-3}$ |

For the control group, the core line length is (5.7 ± 1.4) $x10^{-3}$ mm that increases to 8.5 ± 3.7 $x10^{-3}$ mm after the exposure for 2 days. For the exposure day of 4, 6, and 8 the core length is found of (9.8 ± 5.1) $x10^{-3}$ mm, (9.6 ± 4.6) $x10^{-3}$ mm, and (10.2 ±4.1) $x10^{-3}$ mm respectively. In the days 10, the core length becomes (11.5 ± 6.0) $x10^{-3}$ mm. The alveolus alteration depends on the exposure day. The comparison is presented in the figure 3.

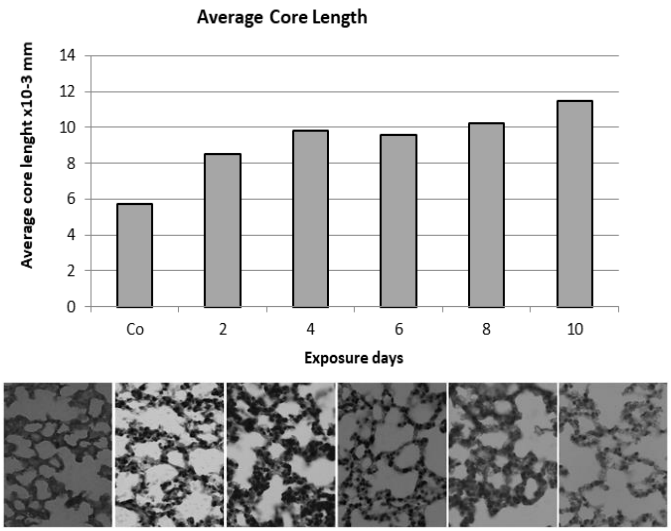


**Figure 3.** The comparison of the alveolus core length is found increasely for each exposure day. The tissue histological images illustrate the alveolar alter.

**Inflammation, (DSA), and Hemorrhage.**

The inflammation is shown by the appearance of the erythrocytes that covered in certain lung sample as present in figure 4. The reactive substances in the smoke may be responsible for these. The area covered by the erythrocytes also is found randomly in the inflamed area. The *DSA* are identified in the image by the loss of the alveolus wall. The septum alveolus depletion permits the erythrocytes to flood the area around the inflammation due to the opening of the blood vessel into the alveolus. The appearance of the erythrocytes is seen in the area closest into the inflammation and is used to identify the presence of inflammation. The erythrocytes are inside the alveolus (Bianca et al., 2016) and around the inflamed sites indicating the open access of the blood vessel which is caused by the depleted of epithelium (Chu et al., 2016).

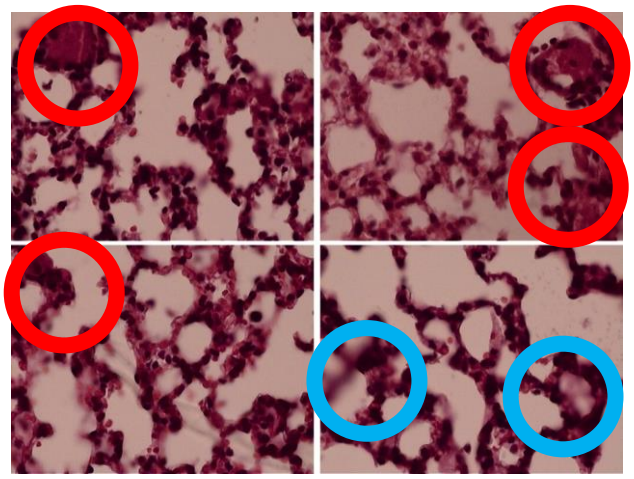

**Figure 4.** The H&E stain indicates the presence of the inflammation. The appearance of inflammation (red circle) is found in the various level and random location indicating the reactive substance that spreads unevenly. The inflammation also leads to the increase of lung liquid that is shown in blue circles.

**Lung Alteration**

The lung damages of the repeated exposures are shown in figure 5. The damages are identified as *DSA* and alveolar alteration. The damages increase for the longer exposure day means that the repeated exposures give more effects in the mice. Consequently, more lung damages are as the result. The correlation of the repeated exposures to the lung damage is calculated by plotting the lung damage versus repeated exposures. The best fitting approach has been applied to a linear equation. The $R^2$ is found more than 0.95. This indicates that a very strong correlation between the repeated exposures and the lung damage as shown in figure 5.

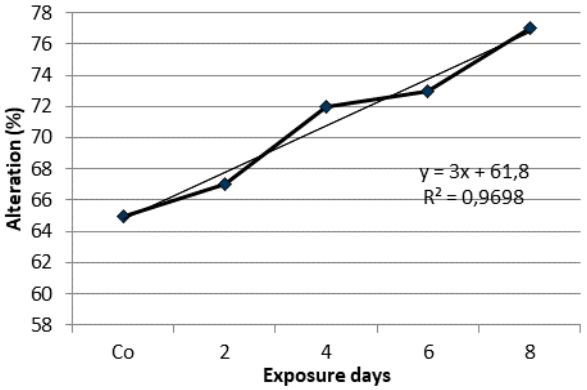


**Figure 5**. The lung alteration is found higher for the longer exposure day. The $R^2$ value is calculated equal to 0.9698 that shows a strong correlation between the repeated exposure and the lung alteration

The lung damage caused by the repeated exposures is found a significant increase. Longer smoke exposure results in a worse lung damage. The lung damage for longer exposure day of 2, 4, 6, 8, and 10 days is calculated of (65 ± 5) %, (67 ± 2) %, (71 ± 5) %, (72 ± 2) %, and (77 ± 4) %. The correlation between the lung damage and the repeated exposure day is fitted by a linear equation of y = 3x + 61.8 with $R^2$ = 0.9698 where y is the lung alteration,

and x is the repeated exposure day. The $R^2$ more than 0.95 shows the strong correlation between the repeated exposure to the damage in the mice lungs (Eeftens et al., 2015).

**Table 3.** Lung damage after exposed by C1 and C2 for 10 days

| Damage (%) | C1 | | C2 | |
|---|---|---|---|---|
| | D | ±D | D | ±D |
| $C_2$ | 6 | 5 | 6 | 3 |
| $C_4$ | 6 | 2 | 7 | 3 |
| $C_6$ | 7 | 4 | 7 | 4 |
| $C_8$ | 7 | 2 | 7 | 2 |
| $C_{10}$ | 7 | 4 | 7 | 2 |

## Discussion

The smoke concentration affects the inhaled dose of particles. The higher concentration of the smoke causes more inhaled particles. Especially during the busiest hour, the smoke concentration was reported high and also the inhaled particles (Argyropoulos et al., 2016). Inhaled particles with high dose would result in the development of various diseases including the respiratory diseases such as: as asthma (Bråbäck & Forsberg, 2009), influenza (Feng et al., 2016) and COPD (Giannadaki et al., 2016). And another study reported inhaled reactive substances containing motorcycle smoke that leads to the serious damage to the lung (Oravisjärvi et al., 2014). In this study, we exposed the smoke to the mice with the determined concentration. We repeated the exposure to investigate the influences on the lung alteration.

The results show that the repeated exposures cause the lung damage in terms of enlargement of the emphysema core size, hemorrhage, DSA (Depleting of Septum alveolar) and inflammation. The particulate matter emissions especially $PM_{0.1}$ are distributed by the cardiovascular system to blood. Meanwhile, the undistributed particles could react to lung epithelium causing the damage of lung epithelium (Manzo et al., 2012; Capasso et al., 2014). The substances of particles play a role in the reaction. They may reactive oxygen species produced by burning of fuel lead to a lung inflammation (Borm et al., 2006; Tuet et al., 2016).

For a high exposure dose concentration, the particles may damage alveolus instantly causing the lung failure (Stanek et al., 2011) due to high oxidative stress. Meanwhile, for the low concentration, the particles reach the lung and trigger the pro-inflammatory releasing (Arunachalam et al., 2010). The pro-inflammatory leads to inflammation response in the lung epithelium that is identified by the appearance of the erythrocytes inside alveolus (Araujo & Nel, 2009). In the same time, the lung produces mucous known as lavage fluids covering the inflamed area as the responses of the inflammation process (Landsiedel et al., 2014). In the mucous contain anti-inflammatory cytokines such as: as IL-4 (Jaspers et al., 2009), IL-6 (Lee et al., 2014), IL-10, IL-11, and TGF-$\beta$ (Li et al., 2016) was released by the macrophages (Wang et al., 2005). The result is the development of emphysema having a strong correlation with the inflammation in the lung (Arunachalam et al., 2010). Emphysema is simply called as the unrecovered of alveolus destruction (Kurimoto et al., 2013) that leaves the cell in the condition after the damage. The geometrical change of the emphysema shape is related to the length of the repeated exposure. At the first exposure, the alveolus overcomes the inflammation and then recovery to become emphysema. At the next exposures, the emphysema becomes the worse condition in terms of the morphometric term and function.

The total damages become larger for the longer exposure as the result of the contact area enlargement. The inflammation response gets worse and results in the increase of the septum alveolar depleting number. This phenomenon was observed in this study.

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