An analysis of the deformed erythrocytes correlated to varied dose of nanoparticles emitted by diesel engine bus

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Abstract. This study was aimed to investigate the morphological changes of the mice’s erythrocytes corresponded to the bus exhaust nanoparticle exposures. Male mice were used as the experimental animals. The exhaust emissions emitted by bus samples B1, B2, and B3 were filtered using a nanoparticulate filtering system consisting of an N95 mask and a sucking pump, and the concentration was measured using a TSI P-Trak Ultrafine Particle Counter. In order to the erythrocyte deformation, we used the unexposed and the exposed mice to the bus exhaust emission with the varied particle concentration of Ld - low dose, Md - medium dose, and Hd - high dose as long as 100 seconds per day in eight consecutive days. Then, all mice were sacrificed for the erythrocytes analysis. We found two deformation types that were most highly increased in the erythrocytes such as: helmet-shaped cells and teardrop-shaped cells. The deformation percentages were in the range of 27% to 47%, depending on the nanoparticle concentration dose. Ld had the lowest deformation percentages: 28% for B1, 34% for B2, and 44% for B3. The highest deformation was found in Hd, with the values of 30%, 37%, and 47%, respectively for B1, B2, and B3.

1. Introduction
PMs (particulate matters) are known as the substance of the air pollutant that are emitted from road transportation sector [1–3], printing activity [4,5], smoking [6], biomass burning [7], and other different exhausts. PMs are believed to have a significant correlation with many human health effects. Several toxicological studies have reported the physical damages in renal cells due to PM exposure in different type and size [8].

Nanoparticles, as known as ultrafine particles (PM$_{0.1}$), are a kind of particulate matters with an average diameter less than 0.1 μm. In terms of the diameter size, nanoparticles are bigger than PM$_{2.5}$ (fine particle) and PM$_{10}$ (coarse particle). Due to the small size of the diameter, nanoparticles have a greater deposition probability in human organs. As confirmed in the previous studies, an exposure to nanoparticles in healthy human has a potential to increase the blood pressure [9]. The deposited nanoparticles are also related to the generation of reactive oxygen species (ROS), inflammatory responses, and oxidative stress in cells [10,11]. In experimental animals, an exposure to nanoparticle can induce inflammatory responses in lung tissue of mice [12]. In addition, it has been studied that inhalation of nanoparticles in a mice model altered Clara cells in allergic lung inflammation [13], including the sustained inflammation and genotoxicity in liver [14].

Although previous studies have provided the information and data about the correlation between exposure to nanoparticles and health, there is very limited data on the nanoparticles emitted from...
diesel engine bus emissions. The data about the potential risk of the exposure to nanoparticles and the erythrocytes deformation has been found in very limited information. Meanwhile, in developing countries, especially in Indonesia, buses still become public transport that are be easily found in the roadway. They have a potential to adverse human health because of their particle emissions. Thus, it is a necessity to investigate the correlation between exposure to diesel engine bus nanoparticles and the cell deformation. In this study, we, therefore, investigated the relationship of the exposure to nanoparticles emitted by the diesel engine buses and the erythrocyte deformation level. The aim was to investigate the impacts on the nanoparticles with the varied dose exposure on the erythrocyte deformation in a controlled system.

2. Materials and methods

2.1. Nanoparticles source

Three diesel engine buses with different engine volumes (cubications): B1 (4570 cm$^3$), B2 (4214 cm$^3$), and B3 (7961 cm$^3$), were used to generate nanoparticles with the diameter of 0.02 – 0.10 μm in different concentration dose exposures: low-dose ($L_d$), mid-dose ($M_d$), and high-dose ($H_d$). The different concentrations were referred to the different times needed to introduce the bus exhaust emission to the measurement chamber: 20s ($L_d$), 40s ($M_d$), and 60s ($H_d$). Our previous simulation indicated that the mice collapsed for the exposure more than 80s using high duty vehicle engine.

The bus samples were in a good condition and had standard engines. The exhaust emission was filtered using a nanoparticle cyclone system containing an N95 mask (3 M 8210 Particulate Respirator), cylinder tube, and a suction pump (constant flow rate = 33.05 cm$^3$/s), and calibrated using a HEPA filter. The concentrations of the filtered nanoparticles were measured using a P-TRAK Ultrafine Particle Counter (TSI, Model 8525). Detailed protocols for the performance of nanoparticle exposure have been previously published [8].

2.2. Animal treatment

Animal experiments were performed following the guidance of the Animal Care and Use Committee of Brawijaya University, Malang, Indonesia (Ethical Clearance No: 541-KEP-UB). Wistar mice (N = 50) were kept under controlled relative humidity (57.8 – 58.8%), temperature (26 – 29°C), and light (12:12 dark-light cycle). They were treated humanely and provided with foods and water ad libitum, and acclimated in acrylic cages for three days of acclimation procedure. The mice were arranged into some groups related to table 1.

Table 1. An experimental model in mice (within the different groups, mean ± SD).

| Mice Groups | Abbreviation | Number of Mice | Nanoparticles [x10$^5$ particles/cm$^3$] |
|-------------|--------------|----------------|------------------------------------------|
| B1          | B1$L_d$      | 5              | 3.13±0.13                                |
|             | B1$M_d$      | 5              | 3.40±0.03                                |
|             | B1$H_d$      | 5              | 4.58±0.38                                |
| B2          | B2$L_d$      | 5              | 4.70±0.06                                |
|             | B2$M_d$      | 5              | 7.08±0.23                                |
|             | B2$H_d$      | 5              | 11.84±0.54                               |
| B3          | B3$L_d$      | 5              | 9.08±0.41                                |
|             | B3$M_d$      | 5              | 12.36±0.56                               |
|             | B3$H_d$      | 5              | 14.19±0.85                               |
| Control     | CTRL         | 5              | -                                        |
The control mice (CTRL) were not exposed to nanoparticle at all. The mice from the B1 group were exposed to \textit{Ld, Md}, and \textit{Hd} emitted by B1 for eight consecutive days, respectively (100 seconds per day, constant flowrate 33.05 cm$^3$/s). The mice of B2\textit{Ld}, B2\textit{Md}, and B2\textit{Hd} were exposed to nanoparticle emitted by B2 for eight consecutive days (100 seconds per day, constant flowrate 33.05 cm$^3$/s) with the doses of \textit{Ld, Md}, and \textit{Hd}. Similarly, the B3 mice were exposed to nanoparticle emitted by B3 with the dose of \textit{Ld, Md}, and \textit{Hd}. In the day-9, the control and exposed mice from B1, B2, and B3 groups were sacrificed using a cervical dislocation technique. For the fixation process, the blood drops were placed onto object glasses (1.0 - 1.2 mm of thickness) and dripped with 70% methanol solution. After the fixation process (5 minutes), all samples were colored using 1:3 Giemsa – buffer pro Giemsa solution. When they came dried, they were rinsed using fresh water [15].

2.3. Manual analysis
Each slide was evaluated by light microscopy (Olympus, model BX-51). The number of erythrocytes (\textit{TE}), including the normal (\textit{NE}) and deformed erythrocytes (\textit{DE}), was counted from 25 different areas of each mouse sample (covering an area that was 162.53 mm x 121.41 mm). Deformation percentage (\textit{Def}) was attributed as noted in equation (1) [15]. Mean values were used for comparison of the different groups. The digital image samples were processed using an ImageJ software to investigate the existence of deposited nanoparticles in the erythrocytes.

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\textit{Def} = \frac{\Sigma \textit{DE}}{\Sigma \textit{TE}} \times 100%
\]

2.4. Statistical analysis
Values were interpreted as mean±SD (standard of deviation). Statistical analysis was performed by a Student \textit{t}-test and a one way ANOVA (\textit{p} < 0.05 showed a significant difference) using a Microsoft Excel 2016 software. The correlation between nanoparticle exposure dose and deformation percentage was evaluated using regression analysis ($R^2 > 0.80$ was considered to be statistically correlated).

3. Results
3.1. Deformed vs. normal erythrocytes
Cell deformations have been typically using as the first step of the observation of the structural membrane components changes. According to the digital images (figure 1), we found normal cells and some of the erythrocyte abnormalities regarding the cell morphology. Generally, a normal erythrocyte, as known as normocyte or discocyte, has a diameter between 6.7 – 8.2 \textmu m with a biconcave form [16,17]. The abnormality of the erythrocytes (figure 2) can be examined from the deformation, and observed as sickle-shaped (SC), teardrop-shaped (TD), helmet-shaped (HS), saddle-shaped (SD), elliptocyte (EL), and stomatocyte (ST) [18,19].
According to the calculation, $B_3Ld$, $B_3Md$, and $B_3Hd$ had more than sixty deformed erythrocytes ($80 \pm 7$, $77 \pm 7$, and $63 \pm 12$ cells, respectively). The observed deformed erythrocytes in the B2 group were still more than the ones found in the B3 group. $B_2Ld$, $B_2Md$, and $B_2Hd$ had $110 \pm 12$, $103 \pm 26$, and $107 \pm 11$ deformed erythrocytes. Meanwhile, B1 group had $72 \pm 18$, $60 \pm 7$, and $45 \pm 7$ deformed erythrocytes, respectively for $B_1Ld$, $B_1Md$, and $B_1Hd$. However, these values were still higher than the one calculated for the CTRL. The erythrocyte morphology did not obviously change in the CTRL group. The CTRL only had $20 \pm 5$ deformed erythrocytes, with $263 \pm 10$ of healthy erythrocytes. The most observed abnormalities were HS and TD. HS and TD were shown as the disruption in the cell-wall structure and morphology which looked like a helmet and teardrop [16].

Figure 1. Digital images of mice erythrocytes exposed to nanoparticles with the concentrations of $Ld$, $Md$, and $Hd$, and CTRL group. All scale bars represent 20 μm.

Figure 2. Healthy (NE) and deformed erythrocytes: SC, TD, HS, and EL. Scale bar: 20 μm.
3.2. Deformation levels

Figure 3(a) shows the deformation level of the erythrocytes subjected to the exposed and unexposed mice. As seen in the graph, the deformation level in each group under study has different values ($p < 0.05$). The deformation level in the CTRL is presented for the mice excluded from the exposure. The mean deformation level of the CTRL is under 10% (7 ± 1%). In all exposed mice from the B1 group, the level of erythrocytes deformations is higher than the value found in the CTRL. The B1 group has 28 ± 2% (Ld), 29 ± 1% (Md), and 30 ± 1% (Hd) of the erythrocyte deformation levels. However, these values are still lower than the deformation level counted in the B2 group. The deformation levels in the B2 group are 34 ± 1%, 35 ± 3%, and 37 ± 2%, respectively for the doses of Ld, Md, and Hd. Interestingly, the B3 group, in which having the highest nanoparticles concentration, has the most deformation level. Compared to CTRL, the deformation level counted in B3 group are up to 37% higher, resulting 44 ± 1% (Ld), 45 ± 2% (Md), and 47 ± 3% (Hd).

![Graphs showing deformation levels in CTRL and exposed groups](image)

**Figure 3.** (a) Deformation level of the mice erythrocytes in CTRL and exposed groups. The correlation between nanoparticles concentration and deformation level in the group of: (b) B1; (c) B2; and (d) B3.

The correlation between the dose of nanoparticle concentrations and the erythrocyte deformation level is interpreted in figure 3(b)-(d). The erythrocytes deformation value in each exposure group is reduced by the deformation percentage calculated in the CTRL group to generate a value that mimics the exposure dependency. According to the graphs above, the exposure to nanoparticles within the concentration ranges from $3.13 \times 10^5$ to $14.19 \times 10^5$ particles/cm$^3$ has a significant correlation with the deformation level. The similar pattern is found in all groups. A higher dose of nanoparticles concentration has more deformed erythrocytes, as seen as a higher deformation level. Meanwhile, the lowest dose shows the least deformation percentage ($R^2 > 0.80$).

4. Discussion

This study aimed to investigate the deformation responses resulting from low (Ld), mid (Md), and high (Hd) inhalation exposure to the nanoparticle in the mice erythrocytes (red blood cells). The published studies investigating the effect of the exposure to particulate matter in erythrocytes have been conducted in limited information. Especially, the responses of erythrocytes due to the exposure to
nanoparticle PM$_{0.1}$ from the heavy motor vehicle exhaust emission (e.g., car, bus, and truck) are still unclear. The red blood cell becomes the main cell in circulation and has an important function in the body transport system, such as oxygen delivery and waste removal functions [20]. It is a need to investigate the abnormality of the blood regarding erythrocyte morphology that may influence the whole body transportation system. Thus, we conducted a whole body exposure system scenario with the relevance toward human exposures that mimic the scenario of human inhalation [12]. We found that those concentrations are more than our previous study using motorcycle exhaust emission [1]. Also, all concentrations measured in this study were above the measured concentrations of nanoparticle in a real human environmental.

Data plotted in figure 3 gives us information that there is an interesting correlation between the erythrocyte deformation levels and nanoparticle concentrations. Figure 3 indicates that there is a significant correlation between nanoparticle concentrations and the erythrocyte physical damages, with the $R^2$ values of more than 0.80. It is estimated that the level of the erythrocyte deformations is positively correlated by the inhaled nanoparticles. Short-term exposure to ultralinite particles from indoor sources, such as toasting bread, frying sausages, and candle burning, has potential in increasing the blood pressure [9]. An *in vitro* study showed that the silica nanoparticles (SiNPs) have induced genetic and cellular toxicity, where the level of the toxicity was influenced by the particle size [21]. However, the mechanism of inhaled nanoparticles in altering the erythrocytes is still unclear. Possible changes of the erythrocytes deformation levels in response to the varied nanoparticle exposure (312,533 to 1,419,200 particles/cm$^3$) were related to the deposited nanoparticle in the blood system.

As shown in Fig. 3, the deformed erythrocytes, including teardrop-shaped, helmet-shaped, schistocytes, and elliptocytes, have lost their membrane integrities. This assay enables identification of both early and late deformed erythrocytes or apoptotic cells. No significant effects on these parameters are observed in the control group in response to an unexposed condition (0 particles/cm$^3$). No agglomerates of nanoparticles are observed in the control mice.

Erythrocytes deformation is related to some factors, such as cellular viscosity, elastic properties of the membrane, the geometry itself [22,23]. The deformation is also related to the interaction with any pathogen or inflammatory agent [24]. When a strange particle deposits deeper into the blood system, it may cause disruption. The signal of this disruption can be investigated by the level of the morphological change, as the response to the deforming force [22]. The deforming force may be related to the triggered inflammatory response. As reported by Silva-Herdade et al. [24], there is a high correlation between acute inflammatory response and the erythrocytes deformation. Therefore, the exposed nanoparticles in this present study may cause changes in the physical structure of the erythrocyte membrane. As the consequence of the inflammatory, the body system has two mechanisms, anti- and pro-inflammatory substances, and is related to the production of reactive oxygen species. Reactive oxygen species become key mediators of tissue injuries and are corresponded to the stress responses [25,26]. The production of reactive oxygen species has been known to have a correlation with oxidative stress, in the case of nanoparticles [10]. Reactive oxygen species production, such as hydrogen peroxide, superoxides, and other oxygen radicals, can be linked to the mitochondrial dysfunction and DNA damage, as well as in the apoptosis case and inflammatory responses [25,26]. As the impact, the oxidation of hemoglobin may alter the physical form of the erythrocytes and disturb the normal metabolic processes and the depletion of oxygen in human tissues, including the change in the blood velocity [24].

5. Conclusion
In conclusion, diesel engine bus nanoparticles significantly affect the mice erythrocyte deformation. More nanoparticles exposed to the mice resulted in more deformation effects. The correlation between nanoparticle concentration and the erythrocyte deformation was obtained proportionally with the $R^2 > 0.80$. 


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Conflict of interest
All the authors declare that there are no conflict of interests.

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