

Original Research

The Effect of Motorcycle Fine and Ultrafine Particle Matters on the Mice's Liver

Johan Andoyo Effendi Noor^{1*}, Arinto Yudi Ponco Wardoyo¹,
Unggul Pundjung Juswono¹, Eko Teguh Purwito Adi¹, Arif Budiarto^{1,2}

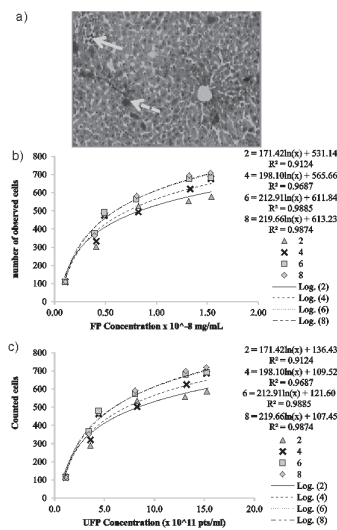
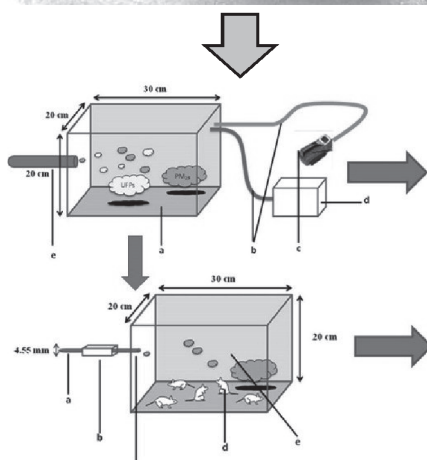
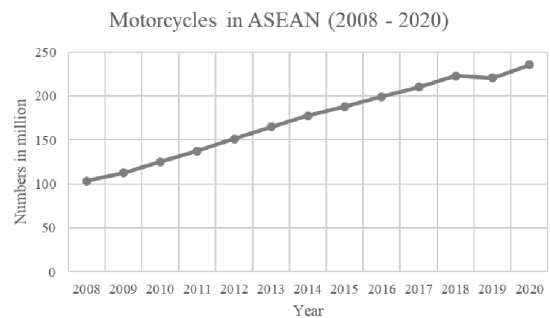
¹Physics Department, Brawijaya University, Jl. Veteran 65145 Malang, East Java, Indonesia

²Physics Study Program, Mataram University, Jl. Majapahit No. 62 83125 Mataram, West Nusa Tenggara, Indonesia

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Abstract



*e-mail: jnoor@ub.ac.id

Liver diseases have been linked to ambient emission exposure, especially fine (FP) and ultrafine particles (UFP). At the same time, the influence of specific emission sources is needed due to the increase in the number of emission sources. In this study, we evaluated the influence of motorcycle FP and UFP emission on mice livers. Motorcycles emission was selected due to the number of motorcycles that were reported to increase significantly, especially in developed countries. The emission was also chosen because no one had yet evaluated the influence of motorcycle FP and UFP exposure on the liver. In order to fill this gap, we evaluated the impact of motorcycle FP and UFP on mice. The mice were grouped into the control and experiment groups. The mice from the experimental groups were exposed to motorcycle FP and UFP particles in different exposure doses: C₁ (20 s), C₂ (40 s), C₃ (60 s), C₄ (80 s), and C₅ (100 s). All of the exposure were conducted twice a day for eight consecutive days. On the last day, all mice were sacrificed, while the Kupffer cells were observed under a digital microscope. In the results, we found that the exposure doses strongly influenced the number of Kupffer cells ($R^2 > 0.9$) with increased exposure doses. The influence of exposure day was also substantial but not as significant as the exposure doses ($R^2 > 0.8$). Furthermore, the Kupffer cells number was raised logarithmically for the exposure doses or exposure day increases. The inflammation was also increased with the Kupffer cell number increase. We observed that the increased exposure dose increases the population with higher inflammation scores. The destructive liver index was raised with increased exposure doses and exposure days. In conclusion, motorcycle FP and UFP exposure significantly impact Kupffer cell activation, liver inflammation, and damage to mice's liver. However, an evaluation of another source of particles, e.g., gasoline or diesel-fueled vehicles, are needed to find the characteristic of each source of emissions.

Keywords: effect, motorcycles, particle emission, liver, alteration

Introduction

Motorcycle usage has increased significantly in ASEAN. In 2008, the total number of active motorcycles was roughly 103 million. In the following years, the number of motorcycles in ASIAN rapidly increased. In 2018, the number of active motorcycles increased to more than 223 million or more than 200% than in 2008. In 2020, the number of motorcycles was reported to be more than 235 million. From all those years, Indonesia was in the first position with 47 million active motorcycles, followed by Vietnam with 24.2 million and Thailand with 16.5 million only in 2008 [1]. In 2020, the number of motorcycles in Indonesia reached 115 million. Meanwhile, in Vietnam and Thailand, the number of motorcycles was reported to be 72 million and 21.5 million in 2020 [2].

In correlation to air quality, the growth in motorcycles' number influenced the air pollutant concentration [3]. A previous study reported that motorcycle emissions contribute more than larger vehicles [4, 5]. On weekdays, the emission was reported to rise due to many active motorcycles [6]. Other studies also stated that the number of active motorcycles influenced the high emission concentration in big cities [7, 8]. The traffic and driving style in the big cities, the age of the active vehicles, engine size, and technology were also linked to the higher emission concentration in big cities [3, 9].

Motorcycle emission was reported to release various types of dangerous gas such as carbon monoxide, NO_x, and hydrogen carbon [3, 5, 10]. In the other studies,

a motorcycle was the main source of Volatile Organic compounds (VOC) emissions [8, 11]. Motorcycles were also linked to ambient BTEX concentration [7, 10]. Polycyclic aromatic hydrocarbons (PAHs) and nitro polycyclic aromatic hydrocarbons (NAHs) were also found in motorcycle smoke [12]. Furthermore, motorcycles also emit various sizes of particulate emission, especially with a size of less than 2.5 μm or fine particle (FP) [13-15]. All stated emissions were categorized as dangerous substances, especially for health.

Many studies reported the impact of FP on health [16-20]. Exposed by this type of emission was known to rise mortality and morbidity in humans [21]. Due to their size [22, 23], UFP can also penetrate deeper into the distal unit of the airways [24] and trigger inflammation responses in lower respiratory systems. As a result, FP exposure was linked to respiratory disease [21, 25]. Moreover, the emission was distributed in the blood circulation system [24] and caused cardiovascular diseases [26, 27]. The distributed FP was also found to cause damage to erythrocytes and kidney cells [28, 29]. Kim et al., in their study, explained how FP reaches the liver in various ways [30] and result in the development of liver fibrosis [18, 31, 32].

The impacts of FP on the liver were reported in previous studies [18, 33, 34]. Ruiz-Lara et al. evaluated the influence of environmental pollution on liver dysfunction. As a result, they stated that air pollutant was strongly related to the increases in lipoperoxidation [35]. In the other study, Tarantino et al. studied the impact of metropolitan city ambient particulate

exposure on liver diseases and reported a strong correlation between the emission and the diseases. They also suggested evaluating the correlation between liver damage and specific ambient particulate [36]. Further studies found a correlation between ambient particulate matter on the development of fibrosis, non-alcoholic steatohepatitis (NASH)-like phenotype, characterized by hepatic steatosis, inflammation, non-alcoholic fatty liver diseases (NAFLD), and liver cancer [33, 36, 37].

This study evaluated the impact of FP produced by motorcycles on the liver. The motorcycle emission was exposed to male mice to find the correlation between the increase in particulate concentration to Kupffer cells in mice. In addition, we also observed liver damage. With this study, we expected to link those observed criteria with liver fibrosis. Furthermore, this study contributed knowledge about motorcycle particulate matter emission exposure's influence on liver damage, which is not yet done.

Material and Methods

Animal Samples and Emission Exposures

In this study, we used male mice aged 10 weeks with an average weight of 23.3 grams. All mice were treated following the animal care ethics provided by the Experimental Animal Ethics Committee of Brawijaya University for the treatment and care of the procedure [29]. This study used a 1200 mL acrylic chamber controlled in terms of temperature and humidity at room conditions as an experimental chamber. The air was filtered in advance to avoid the experimental animal from the ambient particles. Before the experiment, an acclimation procedure was carried out to avoid a psychological impact on these studies [38, 39].

The measurement procedure was done before we exposed the mice to the motorcycle FP emission. We monitored the concentration of FP using Kanomax 3443 dust analyzer. In addition, UFP concentration was also measured during the measurement using the P-Trak 8525 particle counter. The measurement was conducted similarly to the exposure procedures. The FP was filtered using FP filtration systems. Whatman paper for FP was used to filter the smoke before the smoke was injected into the exposure chamber with a flow rate of 2.03 m/s [29].

The mice were exposed to FP with varied concentrations related to the injection duration: 20 s (C_1), 40 s (C_2), 60 s (C_3), 80 s (C_4), and 100 s (C_5) [29]. The mice were exposed for 100 seconds for every exposure dose concentration. The exposure procedure was done twice daily (at 8.00 am and 8.00 pm). The mice's livers were observed on the 2nd, 4th, 6th, and 8th days after the morning exposures. The 8th day was chosen as the observation day due to the hepatic fibrosis initiation phase [40].

The liver was embedded in paraffin and had a 4 μ m thick cut using a microtome. The tissue samples were covered with a glass and a rapid mounting medium to avoid deformation during the observation. The samples were observed by using a BX-51 computer microscope. We took 25 images of each sample for the analysis [28].

Liver Observation Procedure

In order to find the effect of the exhaust emission exposures on the mice's liver, we analyzed the number of Kupffer cells, inflammation, and necrosis. The Kupffer cells were counted from histological images. The inflammation was identified by HE staining and analyzed using a scoring system for non-alcoholic fatty liver disease NAFLD [41]. The necrosis cells were identified to determine the destructive index. The destructive index was calculated by observing the necrotic cell number and the normal cell by using Eq. (1), where Dx is the destructive index of the damaged cells counted, T_d is the necrotic cell, and T_n is the normal cell.

$$Dx = \frac{T_d}{T_d + T_n} \quad (1)$$

Statistical Analysis

The data were analyzed to find the correlation between the Kupffer cell number and the exposure dose concentration. We used a similar method to obtain the correlation between the necrosis, dose concentration, and exposure day. R^2 values were used to determine the correlation [42].

Results and Discussion

The Injection Duration versus Concentration Dose

The particle dose concentration exposed to the mice was varied by setting the different injecting periods. Prolonging the injecting duration increased the dose concentration of FP and UFP. The FP and UFP concentrations are presented in Fig. 1. The concentration measured by injecting the emission for 20 seconds was 2.13×10^{-8} mg/ml for FP particles and 4.03×10^{11} particles/mL for UFP particles. By extending the injection period of 40, 60, 80, and 100 seconds, we found that the FP particle concentration was 2.61×10^{-8} mg/ml, 3.36×10^{-8} mg/ml, 3.90×10^{-8} mg/ml and 5.94×10^{-8} mg/ml respectively. Meanwhile, the UFP particle concentration was 4.0×10^{11} particles/ml, 4.70×10^{11} particles/ml, 8.17×10^{11} particles/ml, 13.34×10^{11} particles/ml, and 15.34×10^{11} particles/ml. The FP and UFP particle concentrations increase linearly to extend the injection duration. The correlation between particle concentration and the injecting time

is significant, with $R^2 > 0.9$. The correlation is valid between FP and UFP particles and the Injecting time with the R^2 of 0.91 and 0.95, respectively. We used the particle concentrations as the exposure doses.

Kupffer Cell Activation versus the Exposure Dose

The exposure dose increase raised the number of observed Kupffer cells. In the C1 exposure, we calculated 307 cells. The cell number was found to be 484 when we exposed the mice to the C2 dose. Increasing the dose to C4 increased the cells to 537. As for the C4 and C5, the number of Kupffer cells was obtained at 559 and 582 cells, respectively. A similar outcome was obtained when we repeated the exposure for 4, 6, and 8 days.

In the influence of exposure days, we observed that the Kupffer cells number was slightly increased by the increase of exposure days. As seen in Table 1, the number of the Kupffer cell was 307 on the 2nd day of exposure. On the 4th day, the Kupffer cells were raised to 331. Then, on the 6th day, the observed cells increased to 379. As for the 8th day, the cell number was reduced to 367 cells. This result was obtained on the observation of the C1 exposure dose. The cell number reduction was also observed on the C2 dose.

Further analysis found that exposure days have less impact on Kupffer cell activation than exposure doses, as seen in Fig. 2. In our observation, we found the correlation value between FP exposure concentration higher than exposure day. In the impact of FP concentration, we calculated the $R^2 > 0.9$. Moreover, in the influence of exposure day, we only obtained $R^2 > 0.8$.

Furthermore, we found the number of active Kupffer cells increases logarithmically. In our calculation, exposing the mice with C1-C5 concentration increased the Kupffer cell following the equation of $171 \ln(x) + 531.14$ on day 2 with the R^2 of 0.924. On day 4, exposing the mice in the same manner, we found the cells changed by following the equation of $198.10 \ln(x) + 565.66$ with R^2 of 0.9687. Furthermore, the observation

Table 1. Number of activated Kupffer cells for different dose concentration and day of exposure.

The number of Kupffer cells (cell/ image)					
Exposure days	C ₁	C ₂	C ₃	C ₄	C ₅
Control	126±45				
2	307	484	537	559	582
4	331	451	508	607	687
6	379	478	581	681	691
8	367	445	589	689	705

on days 6 and 8 found that the Kupffer cells number increased by following the $212.91 \ln(x) + 611.64$ and $219.66 \ln(x) + 613.23$, respectively. As for the R^2 , we obtained the value of 0.9885 and 0.9874 for day 6 and day 8 exposures. In the equation, (x) represents FP particle concentration.

Similar behavior was obtained by observing the influences of UFP concentration on Kupffer cells. As seen in Fig. 2, exposing the mice with C1-C5 also raised the Kupffer cells number logarithmically with R^2 higher than 0.9. In our calculation, the cell number was increased following the equation of $171.42 \ln(x) + 136.43$ on day 2. On day 4, the cell number was altered following the equation of $198.10 \ln(x) + 109.52$. As for day 6, the number of cells was raised following the equation of $212.91 \ln(x) + 121.60$. Finally, the cell number followed the equation of $219.66 \ln(x) + 107.45$ for days 8.

Exposure Dose Concentration versus Inflammation

Increasing the exposure dose concentration is found to induce the inflammation response of the liver. We found that the H & E stain number increased significantly in the mice's liver. Fig. 3(a.1-3) shows the presence of the HE stains, defined as the foci (staining/200 x tissue image magnification), indicating

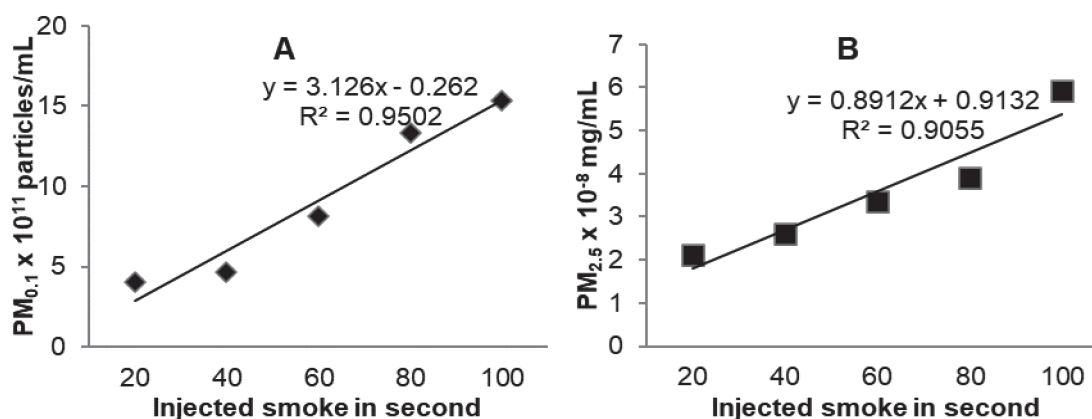


Fig. 1. The concentration of FP and UFP particles as a function of the duration of injection.

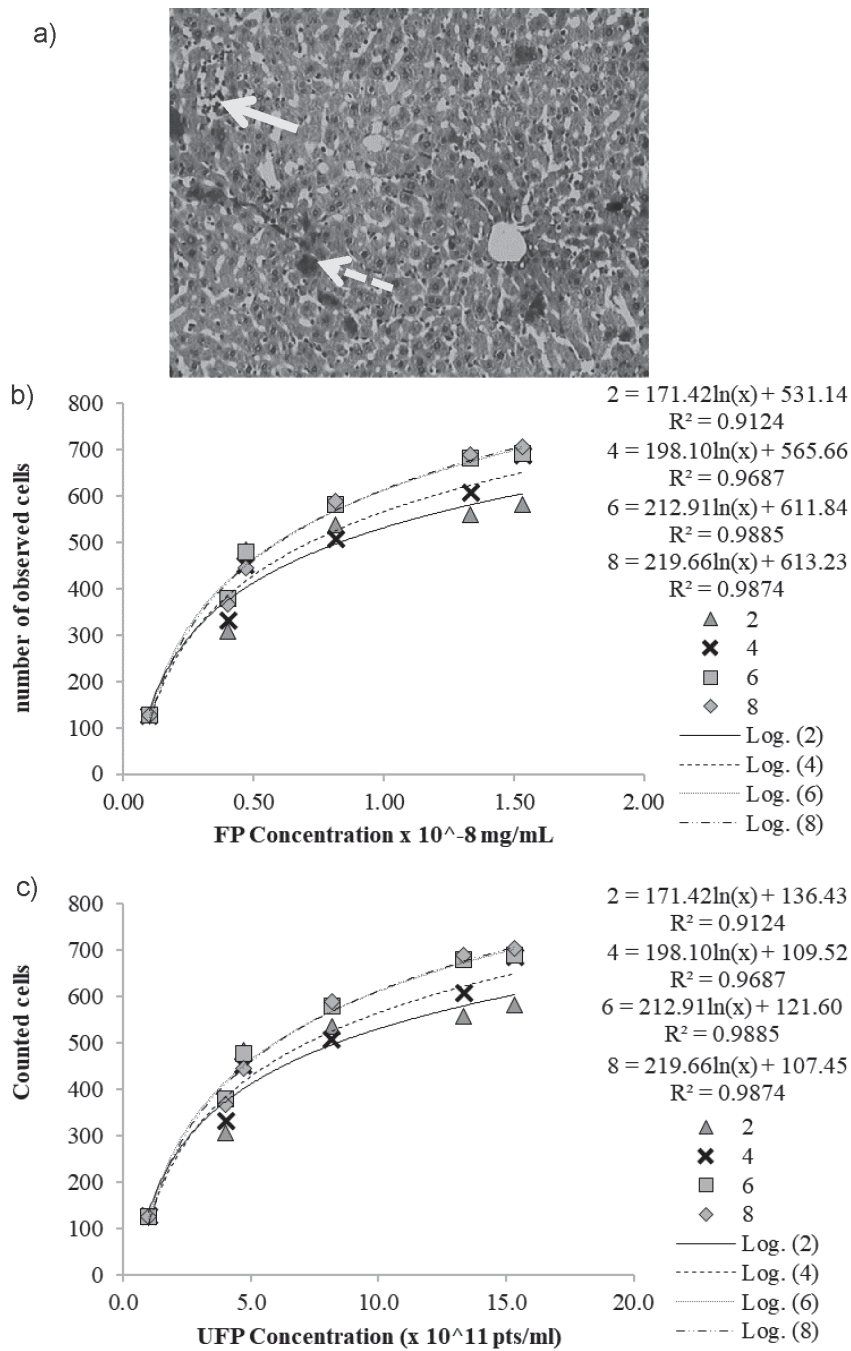


Fig. 2. a) Kupffer cells (arrow) and hemorrhages that show inflammation (dotted arrow) are identified in the representative histological image. b) the average Kupffer cell number increases more strongly for the higher dose concentration. c) The number of Kupffer cells increases less significantly for the different day of exposure.

inflammation in the liver tissues. In Fig. 3(a.1), the counted inflammation increases from score one foci to two foci in Fig. 3(a.2) and more than five foci in Fig. 3(a.3). Based on this result, we define the score of inflammation as score 1, score 2, and score 3 for Fig. 3(a.1-3. a.3), respectively. By analyzing the samples' images, we compare the damages in Fig. 3(b.1-b.4) [41].

We find that the inflammation rate increases with the higher exposure dose concentration. For example, on the 2nd day, we observed the C₁ exposure dose concentration resulting in 15 images with a score of

zero and 10 images with a score of one. The detailed exposure-response of liver inflammation is shown in Table 2.

Emission Exposures versus Liver Damage Index

The relationship between the necrosis, the dose concentration, and the exposure day are also shown in Fig. 4b). Fig. 4 illustrates the mice's liver condition for the exposure with the dose concentration C₁ for 8 days. In Fig. 4.a, the necrosis is identified as a pale

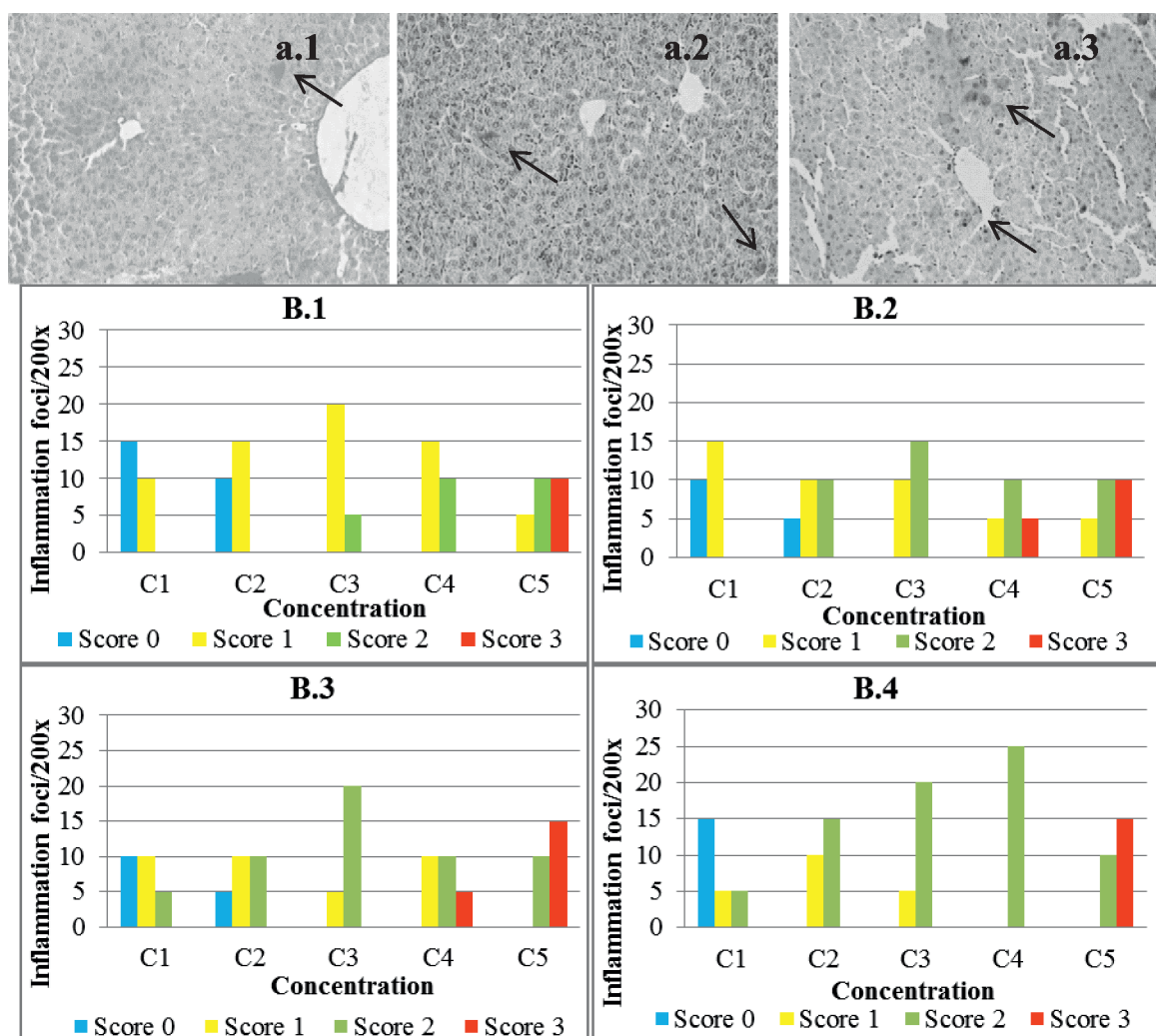


Fig. 3. a) The increase of the score and the hemorrhage (Arrows) by the longer exposure is shown in representative histological image (a.1-a.3). b) The inflammation score of the liver for the different exposure dose concentration and the different exposure day by motorcycle emission

cell without any nucleus, shown by an arrow. In the same figure, we recognize the presence of binucleated cells shown in the dashed arrow. The particle concentration impacts the destructive index as presented in Fig. 4b). Fig. 4b) presents the destructive index for exposure day 2 as a double dot line. The dashed line in the second lowest place represents the destructive index after the four exposures day. The line and dotted line represent the destructive index after the 6th and 8th exposure days.

The exposure day influences the destructive index. For example, for the exposure with the C₁ dose concentration, we find the destructive index of 0.21, 0.23, 0.25, and 0.27 for the 2nd, 4th, 6th, and 8th exposure days, respectively. The increase of the destructive index is 0.02 for the two days longer exposure. The C₂ exposure results in the destructive index of 0.23, 0.26, 0.27, and 0.29 for the exposure days: 2, 4, 6, and 8. The destructive index also increases, about 0.02 for the two days longer exposure. The trend of the destructive index as a function of the exposure day is also found for

the exposures conducted with the concentration of C₃, C₄, and C₅. The destructive index increases by 0.01 per exposure day. The correlation between the destructive index and the concentration dose is found exponentially with $R^2 > 0.96$. The increase in the destructive index due to the longer exposure may be because of the effect of long-term inflammation.

Discussion

Gasoline-fueled vehicles produce fine and ultrafine particles as one of its emission [43, 44]. The particles are mainly formed from carbonaceous compounds, water-soluble ions, e.g., Ca²⁺, SO₄²⁻, NO₃⁻, etc., and metal elements such as Na, Ca, Fe, Zn, and Al [43]. Due to the fuel, all those compounds also form fine and ultrafine particles on motorcycles emission.

FP and UFP are linked to various diseases due to their ability to trigger the development of reactive

Table 2. Inflammation score for different dose concentrations (C1 - C5) and day of exposure.

Day of Exposure	Score	Control	Number of image with score 0-3				
			C1	C2	C3	C4	C5
2	Score 0	25	15	10	0	0	0
	Score 1	0	10	15	20	15	5
	Score 2	0	0	0	5	10	10
	Score 3	0	0	0	0	0	10
4	Score 0	25	10	5	0	0	0
	Score 1	0	15	10	10	5	5
	Score 2	0	0	10	15	15	10
	Score 3	0	0	0	0	5	10
6	Score 0	25	10	5	0	0	0
	Score 1	0	10	10	5	10	0
	Score 2	0	5	10	20	10	15
	Score 3	0	0	0	0	5	10
8	Score 0	5	15	0	0	0	0
	Score 1	0	5	10	5	0	0
	Score 2	0	5	15	20	25	10
	Score 3	0	0	0	0	0	15

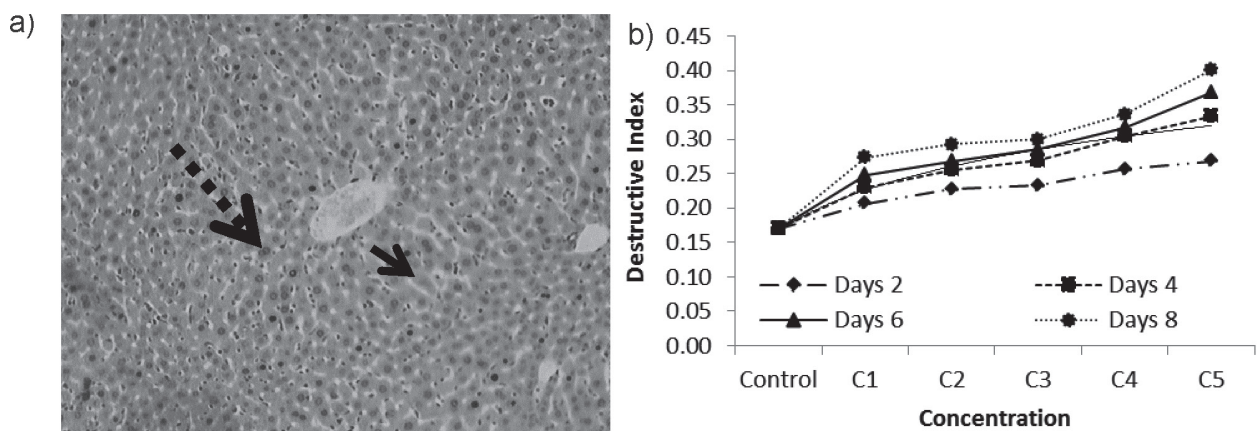


Fig. 4. a) The representative image shows the presence of necrosis and bi-nucleate in liver tissues after C1 exposure for 8 days. b) necrosis increases with higher concentration.

oxygen series (ROS), reactive nitrogen series, etc. FPs and UFP are linked to various diseases, from allergy responses to cancer [37, 45-49]. Emissions exposure is strongly linked to oxidative stress [24], especially in the lungs, skin, or other directly exposed organs [16, 48-50]. Furthermore, FPs and UFP also impact internal organs, especially the liver [27, 36, 51-53]. At the cellular level, FP and UFP exposures are linked to necrotic dead cells [54, 55].

The mechanism of FP and UFP translocation to the liver was explained before. At least three scenarios were proposed to explain how the emissions reach the liver. First, the emission penetrates through the skin

into the cardiovascular system. Secondly, the particles are translocated into the liver from the lung through the respiratory process [30]. Both mechanisms involve systemic circulation to translocate the particles into livers [56]. Lastly, the particles penetrate orally during mouth breath, food, or mice case when they ingest the particles as they lick their fur [57].

Kupffer cells are known to be liver-derived macrophages that are responsible for immune systems. The cell contributes up to 90% of body tissue macrophages [58, 59]. Furthermore, the cell has an important role as the protection system against intruders such as bacteria or foreign materials such as FP

and UFP. When foreign material enters the liver, Kupffer cells express pattern recognition receptors (PRR) such as Toll-like receptors (TLRs), NOD-like receptors (NLRs), and mannose receptors [60]. This behavior is caused due to the cell, identified danger-associated molecular patterns – DAMPs or pathogen-associated molecular patterns – PAMPs. In the case of DAMPs, it is known as the metabolism alteration due to necrotic cells. As for PAMPs, it is associated with the presence of a pathogen. These are also supported by the fact that the primary immune function of the Kupffer cells is to phagocytose any pathogen from the portal vein and arterial circulation [61-63].

As stated in a previous study, FP and UFP trigger lipopolysaccharide (LPS) release [62]. LPS plays the most important role in Kupffer cell activation since it can directly activate the macrophages through TLR4 signaling pathways. At this moment, the FP and UFP particles act as TLR4 ligands in the proinflammatory cytokine released [30,64]. TLR4 signaling pathways also lead to the upregulation of various cytokines such as CCL2, CCL3, Gro1, IL-1 β , IL-6, IL-8, IL-10, IL-12, IL-18, IFN- γ , TNF- α , and TNF β 1 [33, 64, 65]. The previous study [33] reported that cytokine genes are raised in the mice's liver after FP exposure. The increase in cytokine indicates that more Kupffer cells were activated due to FP's presence. This statement agrees with our result, where higher exposure doses raised the number of Kupffer cells.

Kupffer cell activation contributes to proinflammatory cytokine releases [56]. As explained before, the presence of various cytokines, such as IL-1 β , IL-6, IL-8, etc., resulted in the liver's inflammation response. During the inflammation, the macrophages release TNF and TGF- β [62, 66-69]. Increasing the inflamed area in the liver would lead to higher TGF- β release, advancing liver fibrosis. This result was found for the effect of alcohol, virus, and bacteria on the liver [70-72]. Long-term inflammation activates the hepato-stellate cells (HSC), which promote hepatic fibrosis [73, 74].

Conclusions

It can be concluded that there is a significant correlation between exposure to FP and UFP particle concentrations emitted from motorcycles and the Kupffer cells level. The correlation is >0.90. The alteration or deformation of the mice's liver cells can be seen from the Kupffer cells as the biomarker. A higher dose concentration has more biomarkers and inflammation. A longer exposure day also has more deformation indicated by the Kupffer cells, inflammation, and necrosis. The activated Kupffer cell number was proportional to the particle dose concentration. Meanwhile, the Kupffer cell was obtained logarithmically to the exposure day. Since the impact of motorcycles on liver was found significant, in

the future, another study to characterize each source of ambient emission is needed.

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Conflict of Interest

The authors declare no conflict of interest.

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