INTRODUCTION

Carbon black is a particular matter (PM) which is cytotoxic, genotoxic and most stable in air in the form of carbon monoxide (CO) compounds. The amount of CO in the air approaches 164,383.56 mg/m³ every day (Cassidy et al., 2007). This source of carbon comes from exhaust gas factory, motor vehicle emission and burning domestic waste. Some individuals can be exposed to the presence of carbon black in the work environment. The groups of people who are most often exposed to carbon black are factory workers, motor vehicle repair workers, the fuel industry workers, and the oil and coal industry workers. According to the data received from Kajian Baku Mutu Kualitas Udara Ambien Indonesia (Indonesian Standard Quality Study of Ambient Air Quality), the amount of carbon black exposure received by workers in several regions in Indonesia are amounted up to 10,000 mg/m³ (Ministry of Environment, 2011). The results of investigation of carbon nanomaterial effect on reproductive functions of various systemic groups are

Research Article

Chlorogenic Acid Therapy in Mice (Rattus norvegicus) Exposed to Carbon Black Towards p53 Expression on Placenta and Foetus Growth

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Abstract | This study aims to identify the expression of p53 on placenta and pregnant mice’s foetus growth exposed by carbon black. The experimental units divided into five groups: negative control is pregnant mice without carbon black exposure and without Chlorogenic Acid therapy; positive control is pregnant mice exposed to carbon black without Chlorogenic Acid therapy; treatment 1, 2 and 3 were pregnant mice exposed to carbon black with Chlorogenic Acid therapy at 4.5 mg/kg body weight, 9 mg/kg body weight and 13.5 mg/kg body weight. The stages of the study began by mating female mice before exposing them to 1064 mg/m³ carbon black for 8 hours, then administered with Chlorogenic Acid therapy. The detection of p53 expression was done with immunohistochemical staining and foetus growth with Alizarin red method observation. The results of the study show that chlorogenic acid administration has significant effects (p≤0.05) to the decrease in p53 placental expression compared to the positive control, hence the negative control (7.36 cm± 0.65), the positive control (32.00 cm ± 0.21157), treatment 1 (25.00 cm ± 0.3338), treatment 2 (16.93 cm ± 0.39036) and treatment 3 (11.43 cm ± 0.1397). There were significant differences in the length and weight of foetuses born between treatment groups (p≤0.05), hence the length of the foetus in the negative control (1.50 cm ± 0.04), positive control (0.82 cm ± 0.02), treatment 1 (0.86 cm ± 0.01), treatment 2 (1.01 cm ± 0.98), and treatment 3 (1.44 cm ± 0.03). The foetal weight in the negative control is 4,082 g ± 0,131, in the positive control is 2,022 g ± 0,107, in the treatment 1 is 1,844 g ± 0,406, in the treatment 2 is 3,218 g ± 0,388, in the treatment 3 is 4,009 g ± 0.879. This study concludes that the administration of chlorogenic acid in pregnant mice exposed to carbon black can reduce the p53 expression on the placenta and also improve the placenta’s long and heavy performance.

Keywords | Carbon black, Chlorogenic acid, Foetus, p53

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Carbon black may increase inflammation which indirectly affects cytokine changes in the placenta, which in turn affects the cellular response in the body. This condition will contribute to inflammation and tissue disorders of homeostasis, which will continuously affect the blood vessel system in the placenta. The present of endothelial vascular cells in placenta’s blood vessel channel will experience pathological processes such as angiogenesis, vascular homeostasis, and ischemic (Monique et al., 2001).

Carbon black nanoparticles possess an intrinsic potential to generate reactive oxygen species (Jackson et al., 2012). Nanoparticles can cross the placenta and be taken up by fetal organs (Huang et al., 2015). With the increased MAP Kinase, p53 will cause disruption in the placenta that the implementation will lead to premature birth. Increased cytokines can interfere the differentiation of ectoderm, mesoderm, and endoderm cells during the process of organogenesis. Pro-inflammatory disorders of mesoderm cell differentiation can cause disruption in bone growth (Gilbert, 2000).

Providing antioxidants is one way to suppress free radicals. Robusta coffee has one substance that functions as an antioxidant, namely chlorogenic acid (CGA). Chlorogenic acid (CGA) is a class of esters formed from the bonds between certain hydroxycinnamic acids and quinic acid (Thom et al., 2007). From in vitro studies, CGA can inhibit the formation of free radicals, increase LDL resistance to lipid peroxidation, and inhibit DNA damage (Vitrac et al., 2010).

According to EFSA (2011), CGA dose is to prevent DNA damage, to prevent apoptosis, and to stabilize blood glucose levels which can be used 9 mg/kg body weight. Therefore, this study aims to determine the effective dose and the role of antioxidants contained in Chlorogenic Acid (CGA) in Green coffee bean (Coffearobusta Lindau) in improving foetus’s body weight and length, VEGF levels in the blood, and MAP Kinase expression.

MATERIAL AND METHODS

Producing Pregnant Female Mice in Vivo
According to Widjiati et al. (2017), the procedure of impregnating mice was appropriate. This study used mice 2,5 until 3 months old. Mice were injected with Pregnant Mare Serum Gonadotropin (PMSG) by 10 IU, then 48 hours later injected with the Human Chorionic Gonadotropin (HCG) by 10 IU. For the mating of male mice with mono mating technique is putting one male mouse with one female mouse in a cage. Seventeen hours after mating, vaginal plug is examined. If there is a positive vaginal plug, the mouse is considered to be zero-day pregnant and collected as the main population of pregnant mice. If there is no vaginal plug, it is considered negative and not used as a sample.

Carbon Black Selection
Carbon black is carbon in powder form which has been used extensively as a model for diesel emissions of particles without chemicals and metals. Some of its chemical and physical features are similar to nano-based carbon engineered particles. Carbon black consists of carbon with an impurity of less than 1% organic and inorganic materials (Brown et al., 2000).

Exposure to Carbon Black Procedure
The treatment is given in a maintenance cage placed in a different exposure box with an air temperature monitor. Carbon black is sprayed in the air of exposure box with a flow rate of 5-7.5 km/h (gentle breeze) at local temperature and humidity with an inhalation pressure of one atmosphere. Carbon black exposure is given according to the dose and time of each treatment group (Widjiati et al., 2017).

Population and Sample
Negative control group consisted of 6 normal mice without treatment (CN), positive control group was for pregnant mice administered with carbon black treatment and performed surgery on day 18 (CP). In treatment 1,6 pregnant mice were exposed to carbon black with a dose of 1064 mg/m³ for 8 hours per day at 6-17 days of gestational age and sacrificed on the 18th day and given the CGA supplementation at 6-17 days of gestational age with a dose of 4.5 mg/kg body weight (T1) (Hendrawan et al., 2018). For the treatment 2,6 pregnant mice were exposed to carbon black with a dose of 1064 mg/m³ for 8 hours per day, at 6-17 days of gestational age, sacrificed on the 18th day, and given the CGA supplementation at 6-17 days of gestational age with a dose of 9 mg/kg body weight (T2). In treatment 3,6 pregnant mice were exposed to carbon black with a dose of 1064 mg/m³ for 8 hours per day at 6-17 days of gestational age, sacrificed on the 18th day, and given the CGA supplementation at 6-17 days of gestational age with a dose of 13.5 mg/kg body weight (T3).

Examination of p53 Expression in Placenta with Immunohistochemical Method
The procedure of p53 expression identification by immunohistochemical method was, the preparations of placenta which had been made on glass objects were dipped in xylol twice and in multilevel alcohol (100%, 90%, 80%, 70% and 30%) respectively. Then, those were washed in PBS with a pH of 7.4 three times each for five
minutes and soaked in hydrogen peroxidase (H2O2) 3% for 5–10 minutes. Those were soaked in 1% BSA in PBS for 10-30 minutes at room temperature and added with p53 primary antibody for an hour at room temperature. The preparations were washed in PBS pH 7.4 three times each for five minutes. SA-HRP (Strep Avid in-Horse Radish Peroxidase) was added for 30-60 minutes at room temperature. Those were washed again in PBS pH 7.4 three times each for five minutes, added with Chromogen DAB (3,3-diaminobenzidine tetra hydrochloride) for 10-20 minutes, washed in PBS 3 three times each for five minutes at room temperature, added with counterstain using methyl green for three minutes, and then proceeded to mounting with Entellan. Observations were made using a microscope at 1000 times magnification. The determination of the number of MAP Kinase expressions can be seen from the number of brownish colour changes in placenta cells compared to controls (Robyt and White, 1987; Aulanni’am, 2004).

**EXAMINATION OF ALIZARIN RED FETAL BONE STAINING METHOD**

The examination procedure for Alizarin Red foetal bone staining method is by carrying out foetal collections and sacrifice, taking internal organs, skin and fur. Fixation on the foetus was done in 96% ethanol solution for 1 week and soaked with 2% KOH solution for 24 hours. This soaking is intended, so that the muscles appeared transparently. After that, it was transferred to 2% KOH solution which was mixed with 0.005% Alizarin Red S and soaked in 1% KOH, then purified in a solution of 2% KOH mixture and glycerine with the ratio of KOH: Glycerine 3: 1 for 24 hours, KOH: Glycerine, 1: 1 for 24 hours, and KOH: Glycerine, 1: 3 for 24 hours. Next, it was stored in 100% glycerine plus thymol to prevent mould growth.

**STATISTICAL ANALYSIS**

This research was conducted in the laboratory using a completely randomized design model and using mice as experimental animals consisting of 5 groups, with 6 replications per group based on the following calculations: t (n-1) ≥ 15; with t=number of treatments and n= number of replication (Kusriningrum, 2008).

**RESULTS**

**P53 EXPRESSION ON PLACENTA**

The expression of p53 in each sample was assessed semi quantitatively according to the modified Remmele method (Nowak et al., 2007). Remmele scale index (IRS) is a multiplication between percentage scores of immunoreactive cells that express p53 with a score of colour intensity produced in cells. The effect of carbon black exposure on the expression of p53 on the placenta through immunohistochemical staining based on chlorogenic acid therapy and the analysis of expression of p53 was examined using the Kruskall Wallis method demonstrated in Table 1.

The low immunoreactive score of p53 in the placenta showed less light brown intensity in placental immunoreactive p53 score in the placenta showed a dark brown colour. The expression of p53 on the placenta in various treatment groups can be seen in Figure 1.

**FOETUS GROWTH**

Cytotoxic and genotoxic carbon black exposure can affect the process of division and formation of the extremities of mice during day 6-17 of gestational age. Table 2 below indicates the average length and weight of foetus in the treatment group (CN, CP, T1, T2 and T3).

Based on Table 2 above, the length and weight of foetus in the negative control do not indicate significant results compared with the other treatments (T3) and in both groups the development of complete extremities can be seen in photos of foetus that have been stained with alizarin red; while in the positive control and T1 shows lower length and weight of foetus along with some tarsal extremities compared to negative controls, T3, and T2. The T2 group showed higher foetus length and weight and complete extremity compared to the positive control group and T1, but the percentage of immunoreactive cells p53 in T2 was higher compared to T3 and negative controls. The images showing the length and completeness of foetus extremities in various treatment groups can be seen in Figure 2.
Table 1: The Expression of p53 on pregnant mice placenta exposed with carbon black without therapy and with chlorogenic acid therapy.

| Treatment                                             | Positive Percentage score (a) | Color intensity scores (b) | Index of irs (axb) | Mean rank ± standard deviation |
|-------------------------------------------------------|-------------------------------|-----------------------------|--------------------|--------------------------------|
| CN                                                    | CN-1 1                        | 1                           | 1                  | 4.64 ± 0.4561^e               |
| Pregnant mice without carbon black exposure           | CN-2 1                        | 1                           | 1                  |                                |
|                                                       | CN-3 1                        | 2                           | 2                  |                                |
|                                                       | CN-4 2                        | 1                           | 1                  |                                |
|                                                       | CN-5 1                        | 1                           | 1                  |                                |
|                                                       | CN-6 1                        | 1                           | 1                  |                                |
|                                                       | CN-7 2                        | 2                           | 2                  |                                |
| CP                                                    | CP-1 3                        | 2                           | 6                  | 32.00 ± 0.21157^a              |
| Carbon black exposure of 1064 mg/m³ in pregnant rats aged 6-17 days for 8 hours/day | CP-2 3                        | 3                           | 9                  |                                |
|                                                       | CP-3 3                        | 3                           | 9                  |                                |
|                                                       | CP-4 2                        | 3                           | 6                  |                                |
|                                                       | CP-5 2                        | 3                           | 6                  |                                |
|                                                       | CP-6 1                        | 3                           | 3                  |                                |
|                                                       | CP-7 3                        | 2                           | 6                  |                                |
| T1                                                    | T1-1 2                        | 3                           | 6                  | 25.00 ± 0.3338^b               |
| Carbon black exposure of 1064 mg/m³ in pregnant rats aged 6-17 days for 8 hours + CGA dose 4.5 mg/kg body weight | T1-2 1                        | 3                           | 3                  |                                |
|                                                       | T1-3 2                        | 3                           | 6                  |                                |
|                                                       | T1-4 3                        | 2                           | 6                  |                                |
|                                                       | T1-5 2                        | 2                           | 4                  |                                |
|                                                       | T1-6 3                        | 1                           | 3                  |                                |
|                                                       | T1-7 3                        | 3                           | 9                  |                                |
| T2                                                    | T2-1 1                        | 3                           | 3                  | 16.93 ± 0.39036^c              |
| Carbon black exposure of 1064 mg/m³ in pregnant rats aged 6-17 days for 8 hours + CGA dose 9 mg/kg body weight | T2-2 1                        | 3                           | 3                  |                                |
|                                                       | T2-3 1                        | 2                           | 2                  |                                |
|                                                       | T2-4 3                        | 1                           | 3                  |                                |
|                                                       | T2-5 3                        | 1                           | 3                  |                                |
|                                                       | T2-6 3                        | 1                           | 3                  |                                |
|                                                       | T2-7 3                        | 1                           | 3                  |                                |
| T3                                                    | T3-1 1                        | 2                           | 2                  | 11.43 ± 0.1397^d               |
| Carbon black exposure of 1064 mg/m³ in pregnant rats aged 6-17 days for 8 hours + CGA dose 13.5 mg/kg body weight | T3-2 1                        | 2                           | 2                  |                                |
|                                                       | T3-3 2                        | 1                           | 2                  |                                |
|                                                       | T3-4 1                        | 3                           | 3                  |                                |
|                                                       | T3-5 1                        | 2                           | 2                  |                                |
|                                                       | T3-6 2                        | 1                           | 2                  |                                |
|                                                       | T3-7 1                        | 2                           | 2                  |                                |

Treatment Mean Rank ± Standard Deviation

| Treatment           | Mean Rank ± Standard Deviation |
|---------------------|--------------------------------|
| Negative Control    | 4.64 ± 0.4561^e                |
| Positive Control    | 32.00 ± 0.21157^a              |
| Treatment 1         | 32.00 ± 0.21157^a              |
| Treatment 2         | 25.00 ± 0.3338^b               |
| Treatment 3         | 16.93 ± 0.39036^c              |

Notes: Different superscripts in the same column show significant differences between treatment groups (p≤0.05); Negative Control: Negative control, pregnant mice without carbon black exposure and without Chlorogenic Acid therapy; Positive Control: Positive control, pregnant mice exposed to carbon black without Chlorogenic Acid therapy; Treatment 1: Treatment 1, pregnant mice exposed to carbon black with Chlorogenic Acid therapy with a dose of 4.5 mg/kg body weight; Treatment 2: Treatment 2, pregnant mice exposed to carbon black with Chlorogenic Acid therapy with a dose of 9 mg/kg body weight; Treatment 3: Treatment 3, pregnant mice exposed to carbon black with Chlorogenic Acid therapy with a dose of 13.5 mg/kg body weight; Based on Table 1. above, the expression of p53 in the negative control showed significant results compared to other treatments (T3) indicated by a low percentage of score p53 immunoreactive cells. Whereas in the positive control and T1 showed a higher percentage of immunoreactive cells compared to negative controls, T3, and T2. T2 showed that the percentage score of immunoreactive cells p53 was lower than the positive control and T1, but the percentage score of immunoreactive cells p53 in T2 was higher than T3 and negative controls.
Table 2: The Average of Foetus Length and Foetus Weight.

| Treatment        | Foetus Length (cm±SD) | Foetus Weight (g±SD) |
|------------------|-----------------------|----------------------|
| Negative Control | 1.50 ± 0.04<sup>d</sup> | 4.082 ± 0.131<sup>c</sup> |
| Positive Control | 0.82 ± 0.02<sup>a</sup> | 2.022 ± 0.107<sup>c</sup> |
| Treatment 1      | 0.86 ± 0.01<sup>a</sup> | 1.844 ± 0.406<sup>c</sup> |
| Treatment 2      | 1.01 ± 0.98<sup>b</sup> | 3.218 ± 0.388<sup>c</sup> |
| Treatment 3      | 1.44 ± 0.03<sup>d</sup> | 4.009 ± 0.879<sup>b</sup> |

Notes: Different superscripts in the same column show significant differences between treatment groups (p<0.05); Negative Control: Negative control, pregnant mice without carbon black exposure and without Chlorogenic Acid therapy; Positive Control: Positive control, pregnant mice exposed to carbon black without Chlorogenic Acid therapy; Treatment 1: Treatment 1, pregnant mice exposed to carbon black with Chlorogenic Acid therapy with a dose of 4.5 mg/kg body weight; Treatment 2: Treatment 2, pregnant mice exposed to carbon black with Chlorogenic Acid therapy with a dose of 9 mg/kg body weight; Treatment 3: Treatment 3, pregnant mice exposed to carbon black with Chlorogenic Acid therapy with a dose of 13.5 mg/kg body weight.

DISCUSSION

The contamination of carbon black in the lungs can trigger a strong inflammatory response in the lungs. Carbon black gets deposited in the lungs and is eliminated slowly, thus it causes tissue hypoxia. After that, carbon black will translocate the nanoparticles from the lungs into the running blood circulation slowly and continuously throughout the tissues, especially in pregnant individuals. This will indirectly affect placental function (Jacobsen et al., 2008). Exposure to carbon black in pregnant mice can cause foetus in the womb to produce higher levels of carbon monoxide in the body. The carboxy haemoglobin bond causes hypoxia in tissue and placenta by expressing Hypoxia Induced Factor-1 alpha (HIF-1α). The occurrence of tissue hypoxia can lead to an increase in free radicals which will translocate nanoparticles from the lungs into the blood circulation, and then travel to the placenta so that it can trigger an increase in proinflammatory cytokines. The result of the assessment of nanosized carbon black effect on the mice breed show the absence of statistically significant difference in offspring quality from exposed females. However, in malfunction in gene expression of the offspring from mouse females exposed in pregnancy period were reported (Vasyukova et al., 2015).

The inflammatory response that occurs in all organs including the placenta triggers the production of various cytokines from inflammation and increases into the placenta through the bloodstream and particulates. The smaller the size of the nanoparticles, the more cytokines and inflammatory responses, and the greater the number of distributions through the blood vessels will be (Claude et al., 2008). As a result of the inflammatory response secreting cytokines and particulates along with hydroxyl radicals, what is carried by blood on the placenta affect the permeability of the cell membrane. This can cause an increase in blood viscosity which results in a decrease in oxygen supply in foetus. If trans placental oxygen supply is disrupted, it can cause hypoxia in the foetus (Cunningham et al., 2001).
Chlorogenic acid is an antioxidant which inhibits the increase of ROS in the tissue and prevents tissue hypoxia, thus it can reduce the expression of p53 on the placenta indirectly and make the expression of p53 almost as low as in the negative control group. At a given dose of chlorogenic acid, 13.5 mg/kg body weight can significantly reduce the expression of p53 compared to other treatment groups. This states that the dosing of chlorogenic acid at 13.5 mg/kg body weight can be used to prevent the occurrence of early abortion and low birth weight and can improve the performance of pregnant mice.

Exposure to hydrocarbons affects morphological conditions and physiological responses in mice placentas, especially in the placental labyrinth which is the meeting place of the mother and foetal blood circulation. Carbon black exposure causes high ROS in placental cells including endothelial blood vessels. High ROS causes necrosis and apoptosis in cells the endothelium. Vascular damage triggers a physiological response to make new blood vessels (angiogenesis). At the placental level, it is suspected that oxidative stress that occurs during pregnancy will induce macrophages to produce pro-inflammatory cytokines by modulating the transcription of the proinflammatory gene until it causes necrosis during the cell division process at 6–17 days of gestational age (Monique et al., 2001). Therefore, giving carbon black exposure at the gestational age of 6–17 days can cause bone formation in foetus, which includes shorter foetus bone length in the group which was given carbon black but not given CGA therapy. But the group which was given exposure and chlorogenic acid therapy can improve foetus bone length compared to the positive control group.

Giving chlorogenic acid as an antioxidant can inhibit the increase of ROS in the tissue and prevent the occurrence of tissue hypoxia, hence it can indirectly reduce the occurrence of necrosis in the cleavage process during the process of forming extremities to improve foetus performance and foetus body length. At a dose of 13.5 mg/kg body weight chlorogenic acid can improve the performance of pregnant mice by preventing the occurrence of necrosis to improve the performance of pregnant mice through significant improvement in foetus length compared to other treatment groups. This states that the dose of giving chlorogenic acid therapy at 13.5 mg/kg body weight can be used to prevent the occurrence of early abortion and low birth weight and can improve the performance of pregnant mice.

CONCLUSION

The conclusion of this study is the administration of chlorogenic acid in pregnant mice exposed to carbon black give effect toward expression of p53 on the placenta. Chlorogenic acid to pregnant mice exposed to carbon black can improve the weight and length of the foetus compared to the positive control group.

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AUTHORS CONTRIBUTION

VFH and W designed the study, interpreted the data, and drafted the manuscript. DW, YO and AF were involved in collection data and also contributed in manuscript preparation.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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