Coal Dust Exposures Change the Spiral Artery Remodeling and Natural Killer Cells Counts in the Uterus of Pregnant Rats

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Abstract

BACKGROUND: Indonesia has numerous coal resources, but the effects of environmental pollution by coal dust to human health, especially the reproductive system, are still less investigated. Chronic coal exposures during pregnancy might cause systemic and uterine inflammation that induces disturbances of spiral artery remodeling.

AIM: This research was conducted to analyze the effect of coal dust exposures to uterine spiral artery remodeling and natural killer (NK) cell counts.

METHODS: There were 42 female adult Rattus norvegicus rats which had been simultaneously mated after synchronization of estrous cycle used as animal subjects. The rats then divided into seven groups, those were K0 (without exposure); K1-1, K1-2, K1-3; and K2-1, K2-2, K2-3 which were exposed to 6.25 mg/m3, 12.5 mg/m3, and 25 mg/m3 dose for 1 h (K1) and 2 h (K2) per day, respectively. The rats were exposed by a dust exposure machine (NKBS-1-2010-0.5) every morning starting from the day-1 to day-19 post-mating and were sacrificed on day-19 afternoon. All uterus lobes of each pregnant rat were taken and histologically processed by HE staining. Five histological slides were randomly taken as samples representing each pregnant rat and were examined for ten visual fields per slide for the measurements of diameter and wall thickness of spiral arteries. NK cells were observed immunobiologically. Data analyses used Kruskal–Wallis.

RESULTS: Result showed that there were significant differences of diameters and wall thickness of spiral arteries and NK cell counts that might influence pregnancy.

CONCLUSION: Coal dust exposures caused the thickening of wall and narrowing of lumen of spiral arteries and NK cell counts that might influence pregnancy.

Introduction

Coal will be a dominant energy source in both developed and developing countries, but the connection between potential environmental problems due to coal with human health is a new field and requires the cooperation of both the geoscience and medical disciplines [1].

Indonesia had potential coal resources that mostly spread in Kalimantan and Sumatra Island [2] as well as in South and East Kalimantan. According to South Kalimantan Statistic Centre Institution, there were 49 coal companies in South Kalimantan Provinces, and the most production of coal was from Tanah Bumbu Regency with 31 million tons per year [3].

Research of Setiawan (2010) stated that coal dust particles measuring <10 µm could enter to lung tissues and blood vessel then gave systemic effects. Coal dust that was deposited in alveolar cells would be phagocytosed by macrophages to produce some potential factors [4]. Active macrophages would produce many reactive oxygen species (ROS) and cytokines such as superoxide radical (-O2·), peroxide hydrogen (H2O2), and nitric oxide [5] that could modulate lung cells and extracellular matrix. Coal dust exposure could also induce cell injury and cause diseases. Oxidative stress and inflammation processes could give effects to many organ systems including the reproduction system as well as in pregnancy. Some researchers showed that there were significant effects of oxidative stress to transcription factors that influenced placental angiogenesis. Increasing of ROS influenced trophoblast extravillious invasion capacity and then affected placental blood vessel development [4].

It is known that the natural killer (NK) cell is one of the immunomodulatory systems that maintain the...
balance of inflammation-anti-inflammation and oxidant-antioxidant in placenta. Decidual NK cells produce pro-angiogenic factors such as VEGF and PIgf which will increase placental vascular development and influence spiral artery remodeling [6]. The spiral artery remodeling process could be observed by measuring their diameters and arterial wall thickness [7].

Due to ethical aspects, experimental research about coal dust exposure effects to health was conducted using animal subjects. Setiawan et al. (2010) studied about pathogenesis of bronchiolar epithelial cell metaplasia in rat’s lung, but not in pregnant rats, by exposing the rats with the doses of 12.5 mg/m\(^3\) of coal dust for 1 h/day with duration of exposure in 1 day, 14 days, and 29 days, respectively [4]. Because the pregnancy period of rats was only 20–21 days, so that the doses in this research were modified to be 6.25 mg/m\(^3\), 12.5 mg/m\(^3\), and 25 mg/m\(^3\) for 1 h/day and the same doses for 2 h/day for 19 days. Hopefully, this research could describe which dose that could influence spiral artery remodeling and NK cell counts.

**Materials and Methods**

This study had got Ethical Approval from Research Health Ethical Committee, Faculty of Medicine Lambung Mangkurat University. This experimental laboratory study was conducted using post-test only with control group design. We used 42 female adult rats (Rattus norvegicus strain) with 2–3 months ages and 15–200 g weights, which one night simultaneously paired mating following synchronization of estrous cycles, by utilizing the Lee-Boot effect, Pheromone effect, and Whitten effect, according to Sardjono et al., 2019 [19]. The presence of vaginal plug on rats following one night caged in pair (1:1) indicated that it had been copulated by the male, and then was considered as day-1 of pregnancy (post-mating). The rats then were divided into seven groups, those were K0 (without exposure); K1-1, K1-2, and K1-3 which were exposed with 6.25 mg/m\(^3\), 12.5 mg/m\(^3\), and 25 mg/m\(^3\) dose for 1 h; and K2-1, K2-2, and K2-3 which were exposed with 6.25 mg/m\(^3\), 12.5 mg/m\(^3\), and 25 mg/m\(^3\) dose for 2 h, respectively. Coal dust exposure started from day-1 post-mating.

Coal dust was exposed every morning start from the day-1 to day-19 post-mating by coal dust exposure machine (NKBS-1-2010-0.5). The size of coal dust was particulate matter 10 (PM\(_{10}\)). Dose of coal dust was appropriate with the groups. The rats were sacrificed on the day-19 afternoon. All uterus lobes of each rat which on the day-19 detected to be pregnant were taken and histologically processed by HE staining with 4 mm thickness of tissue slices. Five slides were taken as representative samples of each pregnant rat for histological examination of the uterine wall thickness and diameter of spiral arteries, also NK cell counts by immune-histochemistry examinations using CD56 as a marker. The slides were blindly examined for ten visual fields per slide by two independent experts (duplo).

**Findings**

**Animal subjects and pregnancy rate**

According to the formula of Federer for calculating the number of samples needed, there were at least four rats per group. By estimation that pregnancy rate is only 75–83% [19] and anticipation of the rats would die because of high dose and long duration of exposures, we added the number of rats became six rats per group, except control (five rats) and K-2-3 group (seven rats), unfortunately one rat from the control group died before the day of scarification. Thus, the number of samples becomes 41 rats left. Rats with enlargement of uterus (dilatation, with intact or retracted/absorbed lobus) were classified to be pregnant (Figure 2).

Among totally 41 rats which had simultaneously mated following synchronization of estrous (Table 1), there only 26 rats were successfully pregnant (pregnancy rate =63.41%). Among six mated rats of the K1-3 group (25 mg dose for 1 h), on the day-19 post-mating, there...
was only one rat detected to be pregnant and fulfill the inclusion criteria so that we did not include this group in further analysis (Table 1).

### Table 1: Data analysis of uterine spiral arteries diameter

| Group | Coal dust exposure | Number of pregnant rats | Mean ± 1SD (µm) |
|-------|--------------------|-------------------------|-----------------|
| K0    | control            | 4                       | 26.6991 ± 2.5102 |
| K1-1  | 6.25 mg dose for 1 h | 6                      | 38.2570 ± 2.5060 |
| K1-2  | 12.5 mg dose for 1 h | 6                      | 51.5805 ± 5.8358 |
| K1-3  | 25 mg dose for 1 h  | 6                      | 35.0018 ± 10.0999 |
| K2-1  | 6.25 mg dose for 2 h | 6                      | 25.5005 ± 2.5021 |
| K2-2  | 12.5 mg dose for 2 h | 6                      | 25.0015 ± 2.5015 |
| K2-3  | 25 mg dose for 2 h  | 6                      | 25.0015 ± 2.5015 |

significant different (p<0.050)

Figure 2: Various pregnancy outcomes after exposure of coal dust PM10 (a) unpregnant, (b) partially absorbed, (c) fully absorbed with dilated uterine, and (d) normal pregnancy

**Effect of coal dust exposures toward spiral artery diameter and wall thickness**

Histological examination to measure the diameter and wall thickness of spiral arteries was done using Optilab program for light microscope with ×100 and ×400. Results of examination of histological slides with HE staining are presented in Table 1 and Figure 3. Based on Table 1 and Figure 3, there were differences of the diameter of the uterine spiral arteries among groups (P = 0.003, ANOVA test). Post hoc tests showed that K0 had different significance with K1-1; K1-1 had significant difference with K2-2 and K2-3; K1-2 had significant difference with K2-3; and K1-2 had significant difference with K2-2 and K2-3, indicated by different notations.

### Table 2: Data analysis of uterine spiral arteries wall thickness (µm)

| Group | Coal dust exposure | Number of pregnant rats | MEAN ± 1SD (µm) |
|-------|--------------------|-------------------------|-----------------|
| K0    | control            | 3                       | 27.1822 ± 11.5502 |
| K1-1  | 6.25 mg dose for 1 h | 4                      | 33.0058 ± 2.4572 |
| K1-2  | 12.5 mg dose for 1 h | 5                      | 35.1161 ± 0.8716 |
| K1-3  | 25 mg dose for 1 h  | 5                      | 22.3378 ± 4.2513 |
| K2-1  | 6.25 mg dose for 2 h | 4                      | 25.5148 ± 3.6491 |
| K2-2  | 12.5 mg dose for 2 h | 4                      | 20.1578 ± 2.0629 |
| K2-3  | 25 mg dose for 2 h  | 5                      | 22.3378 ± 4.2513 |

significant different (p<0.050)

Figure 4: Data analysis of uterine spiral artery wall thickness suggests that there is an effect of coal dust exposure on the wall thickness of the uterine spiral artery (different notations means significant different (P < 0.050)).

There are differences among groups (different notations means significant different (P < 0.05)). There were differences of the thicknesses of the uterine spiral arteries among groups (P = 0.010, Kruskal–Wallis test) and between groups (Mann–Whitney U-test). K1-1 had significant differences with K2-1, K2-2, and K2-3; K1-2 had significant differences with K2-1, K2-2, and K2-3; K2-2 had significant differences with K2-3. The different notations indicated the significant difference.

Some sections of uterine wall showing the diameters and wall thickness of uterine spiral arteries, as shown in Figure 5.

There were wider diameters and thicker walls in the groups with lower dose of exposures. Furthermore, the uterine spiral artery diameters then tended to become smaller, and wall thickness tended to become thinner relevant with increasing of coal dust doses and duration of exposures.

**Uterine NK cell counts**

The uterine NK cells were counted on immunohistochemistry slides using Optilab light
microscope. Data of NK cells were analyzed by normality test before ANOVA test. Data were not in normal distribution with \( P < 0.05 \). It was continued by the Kruskal–Wallis test with 95% confidence interval. Statistical analysis is in Table 3.

| Table 3: Data analysis of NK cell number |
|-----------------------------------------|
| Group Coal dust exposures Number of pregnant rats Mean ± SD (\( \mu \)m) |
| K0 (control) 3 7.32a ± 3.08 |
| K1-1 6.25 mg dose for 1 h 4 14.74b ± 6.10 |
| K1-2 12.5 mg dose for 1 h 5 12.32b ± 2.50 |
| K2-1 6.25 mg dose for 2 h 4 20.28bc ± 11.35 |
| K2-2 12.5 mg dose for 2 h 4 33.46c ± 22.52 |
| K2-3 25 mg dose for 2 h 5 14.62b ± 4.31 |

There were differences in the number of NK cells among groups (\( P = 0.008 \), Kruskal–Wallis test) and between groups (Mann–Whitney U-test). K0 had significant difference with K1-1, K1-2, K1-3, K2-1, K2-2, and K2-3; K1-1 had significant difference with K2-2; K1-2 had significant difference with K2-2; and K2-2 had significant difference with K2-3.

Visualization of NK cells is shown in Figure 7.

Discussion

In this study, we prepared an animal model (pregnant rats) by one-night simultaneous paired mating following estrous synchronization utilizing natural phenomenon. From the remaining 41 rats, we mated (1:1) for one night, on the day, the rats were sacrificed (day-19 post-mating) we got 26 rats that were pregnant or showing the dilatation or absorbed uterus that we considered as pregnant (Figure 2). It means that the total pregnancy rate was 63.41% (Table 1). This result was a little bit lower than the previous study (73.3–83.3%) [19]. Pregnancy rate in the six exposed groups (62.16%) was lower than the control group (75%). Individual pregnancy rates of each exposed group were lower than the control group, except the K1-2 group. It seems that exposure to coal dust has negative effects on pregnancy.

There are specific natural phenomenon found in rodents, as well as in rats and mice, that were used to synchronize the estrous cycle of rats. (1) Housing of females with the only females in groups and separated from the males for a certain period (about 2–3 estrous cycles), will suppress the female’s estrous cycle, and the females become unestrous (Lee Boot Effect) (2). Exposing male’s urine (or dirty bedding from a male’s cage) to the females’ cage will restart the estrous cycle (Pheromone effect), and (3) within 3 days or about 72 h after exposure with the males’ thing, will induce the females to become estrous (Whitten effect). These natural phenomena are useful to be utilized for obtaining a group of females with the same estrous condition without hormonal interferences. In general, if we put a male into the cage for one night, at least 75% of the females will become pregnant [8].

Uterine spiral artery remodeling was the key to normal fetal growth and pregnancy. The uteroplacental artery remodeling consists of three steps, those are (1) vascular changes because of trophoblast invasion, (2) vascular remodeling that was induced by interstitial trophoblast, and (3) trophoblast infiltration in blood vessel wall [9]. This process changed blood vessels that supply the placental from muscle to artery, making blood vessels become wider 5–10 times. The worsening of spiral artery remodeling might be signified by inhibition of spiralis artery formation and lesion on artery [10], [11], [12], [13], [14].

This study showed that at the initial phase, where the exposures were still low, the diameter of
spiral arteries was wider than control and tended to be narrower related to time and increasing the dose. It is relevant with the previous study [6], where the wider arterial diameter at the early remodeling process would increase the placental blood supply, but if the lumen of vessels becomes narrower than normal, would disturb the normal development of pregnancy.

Table 2 and Figure 4 showed the same trends, where coal dust exposures caused the spiral arterial walls to become significantly thicker than control, especially Groups K1-1 and K1-2. Although the longer duration and higher exposures (K2-1, K2-2, and K2-3) showed the thinner wall thickness, the total end results were narrowing of lumen of blood vessels, relevant by duration, and the doses (Figure 4). It means that the coal dust exposures caused disturbance in spiral artery remodeling, by both increasing the wall thickness and decreasing the diameter of the vessels, resulting in the decreasing placental blood supply and disturbing the pregnancy. In this study, the wider diameters followed by increasing pregnancy rate in Group K2-3 are relevant but need further explanation.

Exposure to coal dust is a source of inflammation in the mother. Inflammation in the mother will impair the remodeling of the spiral arteries. The inflammatory process will result in impaired vascular permeability, neutrophil activation, and enter the extravillous trophoblast invasively [14]. This condition will damage the tunica media of the spiral arteries, even though this layer is indispensable in the remodeling process of the spiral arteries. Damage to this layer will interfere with the remodeling of the spiral arteries [14].

Table 3 and Figure 6 showed that there were significant differences of NK cell counts in all groups. The lowest mean of NK cell count was in the control group and the highest count was in K2-2. The data showed that coal dust exposure could increase NK cell counts, except the K2-3 group. It was possibly caused by inflammation from coal dust exposure. Inflammation can induce NK cell activation.

NK cells were lymphoid cells in innate immunity, and it was early body defense to avoid inflammation and foreign substances NK cell was activated by abnormal cell initiation. It was signed by excreting inflammatory cytokines because of the infected cell. Active NK cells could induce T-cell response and stimulate maturation of dendritic cells. NK cells could induce T-cell immune response through regulatory T cells to kill agents [15]. In pregnancy, NK cells were the most lymphocytes in the maternal layer and involved in uterine spiral artery remodeling. In normal condition, the NK cell in early pregnancy was still low but increased at the end of pregnancy [16].

In this research, the number of NK cells increased because of inflammation due to coal dust exposure (Figure 7). Coal dust particles activate
macrophage and then produce many inflammation cytokines [17]. The NK cell was recognized by abnormal cell initiation from the inflammation process [15]. The previous studies showed that NK cells produced pro-inflammatory cytokines such as IFN-γ and TNF-α that gave negative effects to implantation and trophoblast invasion. The negative effect was apoptosis stimulation to trophoblast and block fetus growth [15], [18].

Table 3 also showed that the control group was significantly lower compared with all of the exposure groups. It meant that coal dust exposure in all doses and duration could affect NK cell number. This research was similar to Hertz-Picioti that stated that NK cells were higher in the higher dose of air pollution group than smaller dose of air pollution group [19].

There was relationship among NK cell, diameter, and wall thickness of spiral arteries and coal dust exposure. Coal dust exposure induced inflammations. Inflammation activated NK cell. This inflammation damaged caused remodeling spiralis artery and changed diameter and wall thickness of spiral arteries.

Conclusion

This research concludes that coal dust exposures might influence the pregnancies through the thickening of wall and narrowing of lumen of spiral arteries and the increase of NK cell counts in the uterus.

Ethical Clearance

This research has been declared ethically worthy by the ethics committee of the medical faculty of the Lambung Mangkurat University No. 229/KEPK-FK UNLAM/EC/VIII/2019.

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