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TELAH MENPELAJARI SECARA SEKSAMA RANCANGAN PENELITIAN YANG
DIUSULKAN, MAKA DENGAN INI MENYATAKAN BAHWA :**

- PENELITIAN BERJUDUL** : Peningkatan Ekspresi (Nuclear Factor Kappa Beta) NF- κ B
Dan Makrofag Pada Plasenta Foetalis Tikus Putih
(*Rattus norvegicus*) Akibat Paparan Carbon Black
- PENELITI UTAMA** : Wahyu Hargiyanto
- UNIT/LEMBAGA/TEMPAT PENELITIAN** : Program Studi S2 Ilmu Biologi Reproduksi
Fakultas Kedokteran Hewan Universitas Airlangga
- DINYATAKAN** : LAJK ETIK

Surabaya, 24 April 2012

Mangestika,
Dokter FKM Unak.



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NIP. 132014464

ISSN:1674-0440

*Journal of International
Pharmaceutical Research*



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Hofbauer Cells and Nuclear Factor of Kappa Beta on Rat's Placenta that Black Carbon Exposure

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Received: 21.11.20, Revised: 20.11.20, Accepted: 18.01.21

ABSTRACT

Increased oxidative stress is closely related to exposure to soot, one of which is through the activation of the Nuclear Factor-kappa Beta (NF-κB) will penetrate the placenta, thereby inducing the activity of macrophages (Hofbauer cells) and NF-κB in placental and fetal cells which results in increased necrosis and apoptosis. This study consisted of 42 pregnant white rats (*Rattus norvegicus*) divided into 3 groups: control (K0) not exposed to soot particulates, group 1 (K1) exposed to soot particulate by 532 mg/m³, group 2 (K2) exposed 1064 mg/m³ for 8 hours with long exposure of 6-11 and 6-17 during pregnancy. Placenta samples were examined by HE staining to count the number of Hofbauer cells and immunohistochemistry to see NFκB expression. The conclusion of this study was the number of Hofbauer cells has a tendency to increase with increasing doses and the length of time of exposure in the pregnancy period. Increased expression of NF-κB indicates inflammation caused by black carbon exposure to the placenta. Increasing the number of Hofbauer cells and NF-κB expression can affect pregnancy development.

Keywords: Nuclear Factor Kappa Beta (NF-κB), Hofbauer cells, placenta, black carbon

INTRODUCTION

Black carbon (BC) is a key component of atmospheric fine particulate matter (PM_{2.5}) and it tends to adsorb various pollutants (e.g., heavy metals and organics) during atmospheric transport [1]. Soot or black carbon is included in the PM component, derived from the residual incomplete combustion product, is cytotoxic and genotoxic, capable of crossing the placental barrier, can increase the number of defects in the fetus due to exposure to a pollutant [2]. Emitted black carbon can be transformed into oxidized black carbon through the photochemical oxidization in the air. How this oxidization process influences the toxicity of black carbon particles is unclear [3]. Previous studies found FBC and OBC could induce oxidative stress and inflammation. Exposure to soot particulates causes an increase in MDA levels and has an effect on pregnancy outcomes in the placenta of white rats [4]. The entry of black carbon or black carbon is known to affect the fetus through several mechanisms including directly entering the placenta, changes in function in the placenta and indirectly through cytokine circulation so that it can

trigger oxidative stress on placental and fetal cells [5].

The entry of particulate exposure includes soot because an exposure is recognized by macrophages in the body especially in the lungs and in other organs when soot is translocated into the blood circulation. Macrophages will produce cytokines TNF-α, IL-1, IL-6 and iNOS and trigger oxidative stress [6]. Increased oxidative stress is closely related to cell inflammation, one of which is through the activation of Nuclear Factor-kappa Beta (NF-κB) transcription factors triggered by TNF-α, IL-1 and T-cell activation, NF-κB transcription factors are inflammatory mediators, molecular adhesion and cytokine molecules [7]. Some cytokines that are activated by NF-κB such as TNF-α and IL-1, are also NF-κB activators in other cells thus providing a potential positive feedback cycle in the inflammatory response [8]. Uteroplacental blood flow will increase during pregnancy, so that there will be many soot particulate or cytokines resulting from inflammation in other organs entering the placenta, thereby inducing macrophage and NF-

κ B activity in placental and fetal cells which results in increased necrosis and apoptosis in that cell. This increase is closely related to the high rate of abnormalities in newborns [2].

Until now there has not been much research on the relationship of oxidative stress due to the effect of particulate soot on the activation of the NF- κ B pathway. Based on the description above, it is necessary to investigate the effect of exposure to particulate matter to an increase in the inflammatory response through the NF- κ B activation pathway to pregnancy and an increase in the number of macrophages in the placenta. This study wanted to determine the exposure of soot particulates at doses of 532 mg/m³ and 1064 mg/m³ with a duration of exposure of 8 hours per day.

MATERIAL AND METHODS

Ethical approval

The study was approved by Faculty of Veterinary Medicine Animal Ethics Committee. All variables after considerations in accordance to Ethics Committee related to animal handling were observed to ensure no discomfort or pain to animal during sampling.

Material

This research was conducted by the Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya. Samples needed in this study were 30 white rats (*Rattus norvegicus*) with the following treatment details: as many as 14 pregnant white rats as a control group (K0) were not exposed to soot particulates during pregnancy, 14 pregnant rats as treatment group 1 (K1) was exposed to soot particulate at 532 mg/m³ for 8 hours per day during pregnancy, as many as 14 female pregnant mice as treatment group 2 (K2) were exposed to particulate jalaga with a concentration of 1064 mg/m³ for 8 hours during pregnancy.

In this study, Pregnant Mare Serum Gonadotropin (PMSG) (Folligon™, Intervet, Boxmeer, Holland) was used for the induction of estrous lust and Human Chorionic Gonadotropin (HCG) (Folligon™, Intervet, Boxmeer, Holland) for ovulation. PMSG is given subcutaneously as much as 10 IU then HCG is given as much as 10 IU given 48 hours afterwards. Each estrous phase can be detected with a vaginal mucosal smear. Smears are made with a cotton bud moistened with sterile aquadest, then inserted in the vagina and rotated, thus the cotton bud already contains vaginal mucosal cells. The swab cotton swab is fed on a glass object and then fixed with 70% alcohol and stained with methylene blue, observed under a light microscope with magnification 100 times or 400 times.

Vaginal smear, the proestrus phase is characterized by the presence of most nucleated epithelial cells and small numbers of leukocytes. In the estrous phase only visible the number of cornification cells with a dominant number, whereas in the metestrus phase is characterized by the number of leukocytes and a little residual cornification cell. This is different from the diestrus phase because in this phase there are a large number of leukocytes while very few nucleated epithelial cells [9].

Methods

Rats are mated gradually according to the treatment group, in a collected way of putting them with one male rat inside a cage. The female rats that already been mated can be known from seeing the vaginal plug on 17 hours after the HCG injection. The vaginal plug consists of gelatin the clotted with the function of the spermatozoa wont spilled out. The vaginal plug was the sign of copulation have happened and at that time it is considered day zero of pregnancy. These pregnant rats then be given numbers with coloring substance according on body parts according to the group.

The Treatment Procedure of Carbon Black Exposure

Carbon black is used as the exposure that sprayed on air of the exposure box. The treatment is given inside different exposure box with air temperature monitor, with the airflow of 5-7,5 km / hour (breeze wind) in local temperature and humidity with 1 atmosphere pressure in a manner of inhalation. The treatment is given gradually according to the group.

The experimental animals are put inside maintenance cage until the treatment started. Acclimatization on the exposure box done before given the treatment, from the pregnancy day-6 until day-17. On day 11 and 17 after the treatment, the experimental animals will be put inside individual cage, and on day 12 and 18 the rats are terminated. The control animal is maintained inside maintenance cage and exposure box with the same treatment with treatment experimental animals except the carbon black exposure.

Hofbauer cell inspection with Hematoxylin-Eosin

Rats placental tissue fixated with buffer formalin 10%. Then staining process using Hematoxylin and Eosin. Then inspected the placental pathological changes and the amount of trophoblast cells with the changes on 10 times field of view.

NF- κ B Expression Identification with Immunohistochemical Method

Immunohistochemical coloring with the avidin-biotin complex method to determine NF- κ B expression on rats placenta in a way : The rats placenta fixated on object glass, then fixated on

object glass, then rehydrating process with multilevel alcohol, and washed with PBS, soaked in 3% hydrogen peroxide H₂O₂ (inside DI water) 20 minutes, 1 % BSA inside PBS 30 minutes on room temperature, Primary antibody (Anti TGF) 1 : 1000 for one night, at 4°C, secondary antibody labeled biotin (Anti Rat IgG biotin Labelled) and primary antibody anti NF-κB, for one hour inside room temperature, SA-HRP (Strep Avidin-Biotin Horseradish Peroxidase) for 60 minutes in room temperature, Counterstain (Aceto-orcein) for 3 minutes, inside room temperature then inspected by microscope. Every changing step are washed with PBS for cleaning the remaining attached substance.

Observation data were analyzed using the Kruskal-Wallis test, if there were significant differences it would be followed by the Mann-Whitney test. It calculated using Statistical Program for Social Scientific (SPSS) version 21.

RESULTS

The number of Hofbauer cells

The analysis result with one-way ANOVA test on every treatment showing the level of significance less than 0.01, so that it could be said that there is a significant change between the pregnancy time and the dose on Hofbauer cells. The Hofbauer cell count on normal condition, without exposure from

between the two control and treatment IIK0 and IIIK0 not having significant different. The average of Hofbauer cells tend to decrease in time of pregnancy. The highest average shown by treatment IIIK2 (day 6-17 pregnancy and dose 1064 mg/m³). The Hofbauer cell count by the carbon black exposure having tend to increase in time of pregnancy.

HSD test show that the exposure of pregnancy age 6-11 days, with dosage of 532 mg/m³ (IIK1) and 1064 mg/m³ (IIK2) giving a significant difference to the Hofbauer cell count, while the exposure on pregnancy age 6-17 days, with dosage of 532 mg/m³ (IIIK1) dan 1064 mg/m³ (IIIK2) giving a less significant difference to Hofbauer Cell count.

The exposure time difference on pregnancy age 6-11 days (IIK1) and 6-17 days (IIIK2) with the same dosage that is 532 mg/m³ showed significant difference on exposure time of pregnancy age 6-11 (IIK2) and 6-17 days (IIIK2) with the same dose 1064 mg/m³ show significant difference with the average Hofbauer cells getting an increase.

Statistical analysis shown that the Hofbauer cell count normally tend to stagnant with the increasing pregnancy age, but having exposed to the carbon black, the Hofbauer cell count tend to increase following the increase of dosage and the exposure time at pregnancy period.

Table 1: Hofbauer Cells Averages and Standard Deviation

Treatment	Mean ± SD
GD 6-11 days, without exposure (IIK0)	5.20 ± 1.304 ^a
GD 6-11 days, exposure 532 mg/m ³ (IIK1)	7.60 ± 1.140 ^b
GD 6-11 days, exposure 1064 mg/m ³ (IIK2)	10.80 ± 1.304 ^c
GD 6-17 days, without exposure (IIIK0)	4.40 ± 1.140 ^a
GD 6-17 days, without exposure (IIIK0)	12.60 ± 1.517 ^{cd}
GD 6-17 days, exposure 1064 mg/m ³ (IIIK2)	14.00 ± 0.707 ^d

Superscript with the same notation indicates that there is no significant difference (p > 0.05)

NF-κB Expression on Placenta

The effect of carbon black on placental NF-κB through immunohistochemical staining based on the pregnancy age and dosage of exposure, counted by semi-quantitative, can be seen from table 2. The sample score including inside nonparametric, all treatment tested with Kruskal-Wallis test and got the result that clearly different (p < 0.01), then do the Mann-Whitney test for comparing 2 treatment.

The analytical result of carbon black exposure to the immunoreactive NF-κB expression using Kruskal-Wallis test show a very real difference on every treatment with significant level less than 0.01. Comparing between 2 treatment with Mann-Whitney test showing that on treatment IIK0 (GD 6-11, dosis 0 mg/m³) towards (GD 6-11, dosis 532

mg/m³) didn't show significant difference, while toward IIK2 (GD 6-11, dosis 1064 mg/m³) having a significant result.

On treatment (GD 6-18, dosis 0 mg/m³) if compare to IIIK1 (GD 6-18, dosis 532 mg/m³) show a significant result, while on IIIK2 (GD 6-17, dosis 1064 mg/m³) giving a very significant result toward NF-κB expression on placenta. This thing showed that the exposure on the same pregnancy time, increasing the carbon black dose can increase the significance level of NF-κB expression, and NF-κB expression have the tendencies to rise.

The Exposure on different pregnancy age with the same carbon black, IIK1 (GD 6-11, dosis 532 mg/m³) and IIIK1 (GD 6-17, dosis 1064 mg/m³) show a real difference, and by the IIK2 (GD 6-11, dosis 1064 mg/m³) and IIIK2 (GD 6-17, dosis

1064 mg/m³) treatment showed a very real difference. This Showed that the more time of exposure with the same dosage have a tendency to rise signification NF-κB expression, and NF-κB expression tend to increase, especially on dosage 1064 mg/m³.

Table 2: Expression of NF-κB Immunoreactive Cells in the Placenta

Treatment		Positive Percentage Score (A)	Color Intensity Score (B)	IRS Index (AXB)
GD 6-11 days, without exposure (IIK0)	IIK0-1	0	0	0
	IIK0-2	1	1	1
	IIK0-3	0	0	0
	IIK0-4	0	0	0
	IIK0-5	0	0	0
average				0,2
GD 6-11 days, exposure 532 mg/m ³ (IIK1)	IIK1-1	0	0	0
	IIK1-2	1	1	1
	IIK1-3	1	1	1
	IIK1-4	1	1	1
	IIK1-5	1	2	2
average				1
GD 6-11 days, exposure 1064 mg/m ³ (IIK2)	IIK2-1	1	1	1
	IIK2-2	1	2	2
	IIK2-3	1	1	1
	IIK2-4	1	2	2
	IIK2-5	1	2	2
average				1,6
GD 6-17 days, without exposure (IIIK0)	IIIK0-1	0	0	0
	IIIK0-2	1	2	2
	IIIK0-3	0	0	0
	IIIK0-4	0	0	0
	IIIK0-5	0	0	0
average				0,4
GD 6-17 days, exposure 532 mg/m ³ (IIIK1)	IIIK1-1	2	2	4
	IIIK1-2	1	2	2
	IIIK1-3	1	2	2
	IIIK1-4	2	3	6
	IIIK1-5	2	2	4
average				3,6
GD 6-17 days, exposure 1064 mg/m ³ (IIIK2)	IIIK2-1	3	3	9
	IIIK2-2	3	3	9
	IIIK2-3	-	-	-
	IIIK2-4	-	-	-
	IIIK2-5	-	-	-
average				9

Table 3: Mann-Whitney test of Expression NF-κB of Placenta

Treatment	IIK0	IIK1	IIK2	IIIK0	IIIK1	IIIK2
IIK0	1	0.065	0.011	0.881	0.007	0.004
IIK1	0.065	1	0.166	0.174	0.013	0.005
IIK2	0.011	0.166	1	0.044	0.033	0.005
IIIK0	0.881	0.174	0.044	1	0.012	0.004
IIIK1	0.007	0.013	0.033	0.012	1	0.005
IIIK2	0.004	0.005	0.005	0.004	0.005	1

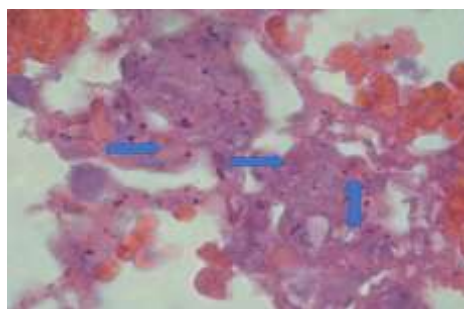


Fig.1: Hofbauer cell (arrow) of White Rat Placenta (*Rattus norvegicus*) With HE staining, magnify 1000x

DISCUSSION

Hofbauer cell (placental macrophage) came from the mesenchyme tissue, are a type of macrophage with micropinocytic and the phagocytosis ability especially breakdown pathogenic agent from the parent to child (vertical transmission), Hofbauer cell can produce cytokine pro-inflammatory like IL-1, IL-2 and TNF- α . Hofbauer cell are included in the process of vasculogenesis and angiogenesis with expressing angiogenic growth factor (VEGF). On rat placenta, Hofbauer cell first appear in pregnancy in the age of day 9 (GD 9), as macrophage with a primitive shape. On day 11-12 pregnancy Hofbauer cells are located in villi stroma placenta and all primitive macrophage have differentiated. On day 12 (GD 12) Hofbauer cell count are at the highest count and that quantity are linear state until pregnancy age 16-18 days, and will go down then disappear following partus [10].

Hofbauer cells phagocytosis activity shown with expressing Major Histocompatibility Complex (MHC) Class I and II antigens. Cytokine that produced by Hofbauer cell were known to have the ability to affect communication between cell in placenta, including Hofbauer cell itself, trophoblast cell and uterine Natural Killer (uNK) cell. An infection or entering some pathogen to the placenta from blood circulation maternal-foetus increase the sensitivity of Hofbauer cell on secreting cytokine Pro-inflammation. Exposure to carbon black in the pregnancy period is known to affect fetuses including shortening of pregnancy time, reduction of placental and fetal weight and fetal disability. The entry of carbon black classified as ultra-fine particles in the placenta can affect the placental immune system including placental macrophages (Hofbauer) [11].

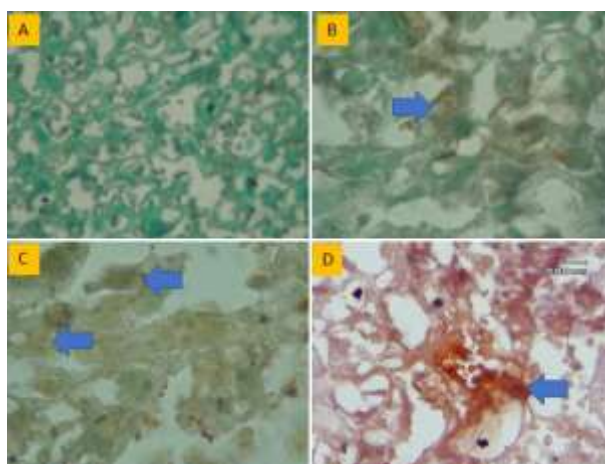


Fig.2: immunoreactive expression NF- κ B on White Rat Placenta (*Rattus norvegicus*). Slide A showed no immunoreactive expression. (B) showed NF- κ B expression with weak color intensity on trophoblast cell (arrow). (C) showed NF- κ B expression with mild color intensity on trophoblast cell (arrow). (D) showed NF- κ B expression with strong color intensity on trophoblast cell (arrow).

The effect of carbon black exposure increases the number of Hofbauer cells when compared to controls/without exposure. The increase in the number indicates an increase in the placental barrier to the presence of pathogenic agents

(carbon black). [12], mentioned an increase in the number of Hofbauer cells because a pathogenic agent would increase the expression of inflammatory cytokines. According to [13], hydrocarbon exposure affects the morphological

state and physiological response in rat placenta, especially in the placental labyrinth where the parent and fetal blood circulation meet, the presence of hydrocarbon exposure causes high ROS in placental cells including endothelial blood vessels, high ROS causes necrosis and apoptosis in these endothelial cells. Damage to blood vessels triggers physiological responses to create new blood vessels (angiogenesis).

Table 1 shows an increase in Hofbauer cells due to carbon black exposure. Increasing the dose of exposure affects the number of Hofbauer cells. Especially in the middle of pregnancy, the second week of pregnancy in rats, is the peak of the proliferation of promonocytes into macrophages in the placenta. Immune response is the interaction between antigens and the body's defense system, the presence of antigens or foreign bodies will trigger the body's immune system. Besides having a role in the placental immune-system the proliferation of Hofbauer cell numbers has a function in producing angiogenic growth factor (VEGF) together with Trophoblast cells [10]. The peak formation of rat placental blood vessels took place on the 12th day of pregnancy until the 16th day, during which phase the placental labyrinth experienced the formation of new blood vessels. Damage to maternal and fetal blood vessels due to carbon black exposure during this period will trigger Hofbauer cells and Trophoblast cells in producing angiogenic growth factor (VEGF) [14].

In the last pregnancy (IIIK1 and IIIK2) the effect of exposure to different doses did not give significant results on the number of Hofbauer cells, normally the Hofbauer cells in the late pregnancy period experienced a decrease in number because the formation of blood vessels had been completed at the end of the pregnancy period. The presence of carbon black exposure triggers Hofbauer cells to continue to respond to pathological agents so that the number of Hofbauer cells tends to remain static [15].

Indirectly the macrophage response is influenced by inflammation elsewhere in the body, inflammation in other parts will produce pro-inflammatory cytokines due to oxidative stress due to carbon black exposure. Inflammation in other organs will produce cytokines TNF- α , IL-2, IL-6 and IFN- γ , these cytokines can induce other macrophage cells to respond to the presence of antigens. The presence of these cytokines is positive feedback toward the immune response [16].

Hofbauer cells can be invasive, the presence of Hofbauer cells normally in the placental villi. The movement of Hofbauer cells out of the placental villi indicates a response to pathological agents. In this study, the presence of Hofbauer cells was not

only found in the placental villi but was found at the border of the junction zone (spongio) and the labyrinth, the endothelial maternal blood vessels [17].

Increased Immunoreactive NF- κ B Due to Carbon Black Exposure.

Activation of Nuclear Factor Kappa Beta (NF- κ B) in the placenta indicates oxidative stress, and increased expression of pro-inflammatory cytokines due to systemic endotoxin exposure [18]. The placenta can secrete different pro-inflammation mediators, these mediators are known to play an important role in the normal and abnormal inflammatory processes [19]. Continuous exposure during pregnancy can have an effect on the reproductive system, placental cells and the fetus. These effects can include mitochondrial damage, inflammation of the trophoblast, and inflammation of the endothelium of placental blood vessels. According to [20] carbon black exposure in the white pregnancy period can increase levels of MDA (Malondialdehyde) and TNF- α expression in the placenta.

NF- κ B expression in placental cells can occur in cytotrophoblast, decidua cells, and blood vessel endothelial cells [21]. In this study the effect of carbon black exposure effects the expression of NF- κ B on cytotrophoblast cells and blood vessel cells. The higher the dose of NF- κ B expression exposure became stronger, characterized by an increase in color intensity. The effect of pregnancy time GD 6-11 and GD 6-17 gives effect on NF- κ B expression, increasing pregnancy, NF- κ B expression also increases, NF- κ B expression is shown in IIIK2 treatment (GD 6-17, exposure dose 1064 mg/m³). Increasing the dose affects the expression of NF- κ B, the increasing dose also affects the increasing expression of NF- κ B.

The effect of carbon black exposure can cause oxidative stress on placental cells, so that the placenta cells will swell, swelling cells indicate the cells become inflamed [22]. The products of inflammation are cytokines such as TNF- α , IL-1 and IL-2. The cytokines will activate NF- κ B in placental cells. Besides being excreted by cells that have swelling pro-inflammatory cytokines, they are excreted by Hofbauer cells due to the presence of pathogenic agents entering the placenta. Placenta that expresses NF- κ B greater than normal indicates an inflammatory process in the placental cells.

ACKNOWLEDGEMENT

The authors are grateful to the authorities of Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya and Department of Veterinary Reproduction, Faculty of Veterinary

Medicine, Brawijaya University, Malang, East Java, Indonesia.

CONFLICT OF INTERESTS

The authors declare that there is no conflict of interests regarding the publication of this article.

SOURCE OF FUNDING

The authors express sincere thanks to the Ministry of Research, Technology and Higher Education of the Republic of Indonesia and Universitas Airlangga for funding research (Number 1408/UN3/2019).

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