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# *Spirulina platensis* effect on oxidative stress of rat's offspring brain exposed to cigarette smoke during pregnancy and lactation

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**ABSTRACT**

**Background**: Maternal exposure to cigarette smoke during pregnancy and lactation might harmful for the fetus. The smoke contains many free radicals that could be eliminated by antioxidant. This study aimed to investigate the effect of *Spirulina platensis* ethanol extract as antioxidant against cigarette smoke exposure during pregnancy until lactation by assessing oxidative stress markers in neonatal brain tissues.

**Methods**: The experimental study used 24 offspring divided into four groups: (C) = offspring of maternal control group; (Cg) = offspring of maternal exposed to cigarette smoke; (CgSp) = offspring of maternal given spirulina and exposed to cigarette smoke; and (Sp) = offspring of maternal given spirulina only group, during gestation and 9 days lactation (30 days). Each group consisted of 6 offspring obtained from 2 adult females mated with male Sprague-Dawley rats. The exposure of cigarette smoke was 4 burn cigarettes/day for 30 days. The dose of extract was 200 mg/kg BW. The offspring were sacrificed, and the brain tissues were taken for MDA, MnSOD activity, as well as catalase activity, carbonyl, and GSH.

**Results**: There was no significant differences in MDA level between groups. The carbonyl, SOD, and catalase activity did not differ between the control and smoked group.

**Conclusion**: Exposure of four burned cigarettes smoke per day during pregnancy, and 9 days of lactation did not trigger oxidative stress. However, the effect of *Spirulina platensis* administration on rat offspring brain could not be analyzed.

**Keywords:** cigarette smoke, offspring brain, oxidative stress marker

## Introduction

Pregnant and lactating women are often exposed to cigarette smoke, whether as active or passive smoker. Passive smokers are more vulnerable to harmful chemical compounds than active smokers [1]. Exposure to cigarette smoke during pregnancy may affect offspring's brain development [2] and can also lead to oxidative stress in maternal tissue. The placenta is the route of nutrition, oxygen, and free radicals to enter fetal circulation, and any substances that pass through can influence the fetus [3].

Free radicals in cigarette smoke are formed from the pyrolytic process, which involves the breakdown of compounds at high temperatures during cigarette burning and the oxidation of compounds in cigarette smoke. These free radicals are divided into two categories: gas-phase and particulate-phase free radicals [4]. Gas-phase free radicals are formed when oxygen or water interact with cigarette smoke and include NO, peroxynitrite, superoxide anions, hydroxyl radicals, and hydrogen peroxide. Particulate-phase free radicals are mostly formed by the combustion reaction of tar, benzo(a) pyrene, polyaromatic hydrocarbon (PAH), aromatic

amines, and phenolic compounds, causing the formation of reactive quinone, semiquinone, and hydroquinone compounds. Furthermore, when these compounds are oxidized, they produce ROS and RNS [4,5]. Free radicals of cigarette smoke can oxidize carbohydrate, lipid, protein, and DNA of maternal tissue may also affect fetal tissue, including the brain tissue.

There are enzymatic and non-enzymatic antioxidants that protect against free radicals. The first enzymatic antioxidant is superoxide dismutase (SOD), that reduces superoxide to produce hydroperoxide that is then eliminated by glutathione peroxidase (GPx) and catalase (CAT). An imbalance of free radicals and antioxidant capacity can lead to oxidative stress [6]. This study aimed to investigate the effect of *Spirulina platensis* against free radicals on the offspring's brain tissue exposed to maternal cigarette smoke during pregnancy and early lactation.

## Methods

### Ethical approval

Ethical approval (no: KET-1476/UN.2F1/ETIK/PPM.00.02/2020) was obtained from the Ethics Committee of the Faculty of Medicine, University of Indonesia - Cipto Mangunkusumo Hospital.

### *Spirulina platensis* ethanol extract preparation

Java Sea *Spirulina platensis* powder (100 grams) was macerated using 95% ethanol and left to stand for 48 hours at room temperature. The solution was then filtered to obtain precipitate, which was further macerated using 95% ethanol. A rotary evaporator was used to evaporate the solvent [7]. The dose of *S. platensis* administered to each rat was calculated to ensure that they received the correct, which was 200 mg/ kg BW.

### Experimental

In this experimental study, 24 offspring were used and divided into four groups. Each group consisted of 6 offspring that were obtained from 2 adult females mated with male of Sprague-Dawley rats. The four groups were: (C) = offspring of maternal control group; (Cg) = offspring of maternal exposed to cigarette smoke; (CgSp) = offspring of maternal given spirulina and exposed to cigarette smoke; and (Sp) = offspring of maternal given spirulina only group.

All groups were treated during gestation and 9 days lactation (30 days). The exposure to cigarette smoke was four sequentially burned cigarettes a day for 30 days in smoking chamber. The dose of *S. platensis* extract was 200 mg/kg BW per day for 30 days, given through intubation using a gastric tube [7,8]. After birth, the offspring were separated from their parents during cigarette smoke exposure, but were still be lactated after maternal exposure to cigarette smoke. Each female rat delivered 9-10 offspring, and six offspring were chosen randomly to obtain the required number of each group. On the $10^{th}$ day, the offspring were sacrificed, and brain tissues were collected and stored at -80°C before the measurement of markers.

### Homogenate preparation

One hundred milligrams of offspring brain tissue were put into a microtube and mixed with 0.5 mL 0.1 M PBS pH 7.4. The mixture was then homogenized using micro a pestle on icebox to maintain cold condition. After homogenization, 0.5 mL 0.1 M PBS pH 7.4 was added into the microtube to reach a total volume 1 mL. Then, microtubes were centrifuged at 5000 g for 10 minutes.

### MDA concentration measurement

Malondialdehyde concentration was measured using the Wills method, by adding 200 µL of 20% trichloroacetate (TCA) into 400 µL homogenate. The sample was then centrifuged at 5000 rpm for 10 minutes, and the supernatant was collected in a new microtube. After that, 400 µL of 0.67% thiobarbituric acid (TBA) was added to the supernatant and heated at 96°C for 10 minutes. After cooling to room temperature, the absorbance was measured at 530 nm using a spectrophotometer [9].

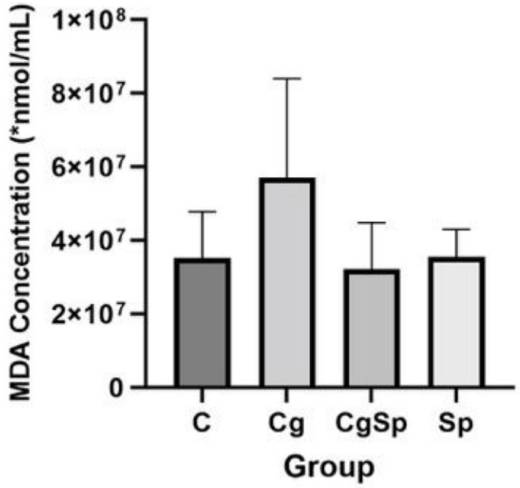


**Figure 1.** MDA levels of offspring brain. There was no significant differences in MDA levels activity between groups (ANOVA, p = 0.062)

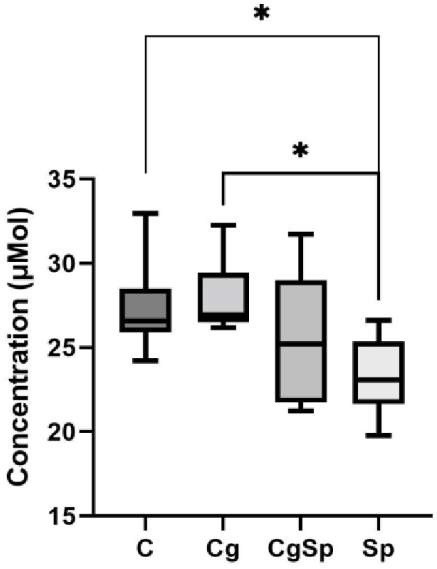


**Figure 2.** Carbonyl concentration in offspring brain. There were significant differences in carbonyl levels between groups (Kruskal walis, p = 0.027). The post hoc study showed that there was a significant difference in group C and Cg compared with the Sp group (Benjamini, Krieger, and Yekutieli, p = 0.007 dan p = 0,003)

## Carbonyl concentration measurement

A total of 100 µL of homogenate was mixed with 400 µL of 2,4-Dinitrophenylhydrazine (DNPH) in 2.5 M HCl. The sample was incubated at room temperature for 1 hour in a dark room. Then 500 µL of 20% TCA was added, vortexed, and incubated for 5 minutes at 4°C. The sample was centrifuged at 10,000 G for 10 minutes at 4°C. After that, the supernatant was discarded. The pellet was resuspended with 500 µL of 10% TCA solution and incubate for 5 minutes. Then it centrifuged at 10,000 G for 10 minutes at 4°C. After that, the supernatant was discarded, followed by resuspension with 500 µL of ethanol-ethyl acetate mixture, and continued with centrifugation at 10,000 G for 10 minutes at 4°C. The supernatant was discarded. Resuspension and centrifugation were repeated 2 more times. The last step was adding 250 µL of 10 M urea solution, and it centrifuged again at 10,000 g for 10 minutes at 4°C. For measurement the optical density, 110 µL of supernatant was transferred to a cuvette and measure the absorbance at a 375 nm wavelength [10].

## GSH concentration measurement

As much as 50 µL homogenate mix with 200 µL 5% TCA, and centrifuged at 5000 rpm for 10 minutes and then the supernatant was move to new microtube. Then, as much as 1750 µL phosphate buffer and 25 µL DNTB were added into the supernatant, and was incubated in the dark place at room temperature for 1 hour. After that, the absorbance was measured at 412 nm using a spectrophotometer [9].

## SOD activity measurement

SOD activity was measured using the RANSOD kit. SOD activity was measured using spectrophotometer UV-Vis. Measurement was based on the amount of red formazan dye. The absorbances showed the degree of inhibition of reaction between superoxide radicals, and 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride [11].

## Catalase activity measurement

Measurement of catalase activity was run using the Mates method. Fifty µL homogenate was taken into the cuvette, then added with 950 µL $H_2O_2$ into the cuvette, and mixed. The absorbances was read in the first 30 seconds ($t_0$) and 2 minutes later ($t_1$) with a spectrophotometer at wave length 210 nm [12].

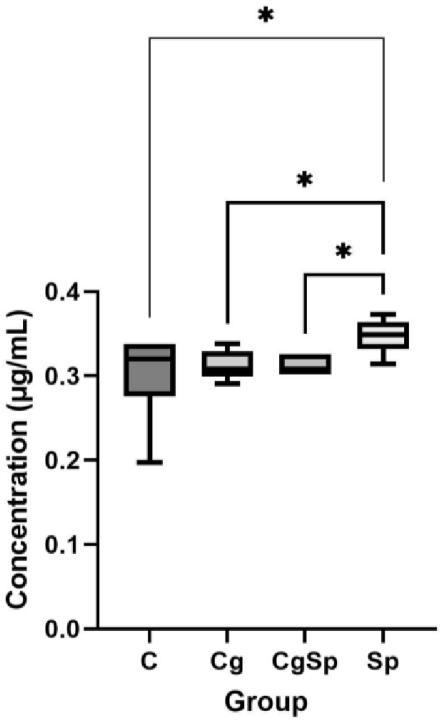


**Figure 3.** Glutathione concentration of offspring brain. There were significant differences in GSH levels between groups (Kruskal-walis, p = 0.027) with post hoc results there were significant differences between groups C, Cg, and CgSp compare with the Sp group (Benjamini, Krieger, and Yekutieli, p = 0.01; p = 0.01; p= 0.03).

## Data analysis

We used SPSS version 23 software to analyze statistical tests and GraphPad Prism 9 to draw the graphs.

# Results

## MDA concentration

Results showed that MDA was not significantly different between groups (ANOVA, p>0.05). Based on the results of the study, there were no significant differences in MDA levels between groups (ANOVA, p = 0.062). It can be seen that there is only a tendency to increase MDA in the Cg group compared to other groups (Figure 1).

## Carbonyl concentration

Results showed that Carbonyl was significantly different between groups (ANOVA, p<0.05) (Figure 2). Based on the results of the study, there were significant differences in carbonyl levels between groups (ANOVA, p = 0.032), after transforming the data. Study showed that there was a significant difference in group C and Cg compared with the Sp group. Only Sp group had decrease carbonyl concentration, but did not significant between Cg compared to C and CgSp. Though there was no increased of protein damages. It meant there was no oxidative stress.

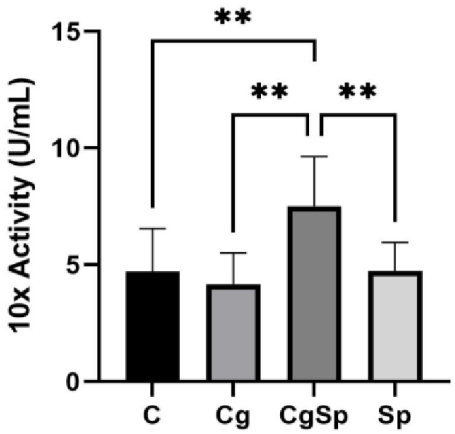


**Figure 4.** SOD activity of Offspring brain. There were significant differences in SOD activity between groups (ANOVA, p = 0.01) with post hoc results there were significant differences between groups C, Cg, and Sp compare with CgSp group (LSD, p = 0.01; p = 0.008 ; p= 0.009).

## Glutathione concentration

Based on the results of the study, there were significant differences in GSH levels between groups (Kruskal-Wallis, p = 0.027). The differences only between C, Cg, CgSp with Sp, but there was no significant different between C and Cg, also between Cg and CgSp (Figure 3.). It considered that there was no stress oxidative in all group exposed to cigarette smoke.

## Superoxide dismutase activity

The results showed that there was a significant difference in SOD activity between groups (ANOVA, p = 0.01) with post hoc results there was a significant difference between the C, Cg, and Sp groups compared with the CgSp group (p = 0.008; p = 0.002; p= 0.009). It also showed that there was no significant difference between C and Cg (Figure 4). It considered that in cigarette smoke

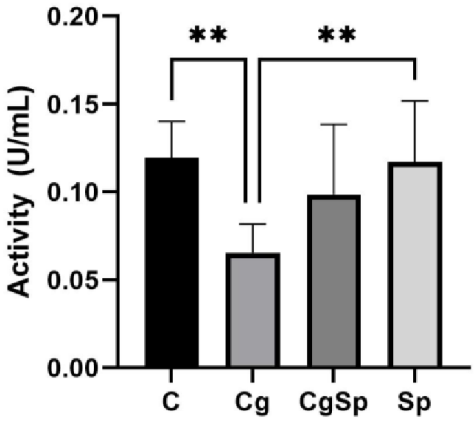


**Figure 5.** Catalase Activity of Offspring brain. There were significant differences in catalase activity between groups (ANOVA, p = 0.012). The post hoc study showed that there was a significant difference in group C and Cg compared with the Sp group (LSD, p = 0.005, p = 0,007).

exposure with 4 cigarette a day did not lead to oxidative stress in offspring brain.

## Catalase activity

There was a significant difference in catalase activity between groups (ANOVA, p = 0.03) with significant difference between groups C and Cg compare with the Sp group (p = 0.004 and p = 0.02) (Figure 5). Conversely, there was no significant different between Cg and CgSp. It means that there the dose of S platensis could not recover Catalase activity in the offspring from maternal exposure with cigarette during pregnancy and 9 days lactation.

## Discussion

The damages caused by cigarette smoke can be performed as MDA, Carbonyl protein, and 8-OHDG [13]. In this study, MDA and carbonyl protein were measured. MDA did not show a significant difference, but carbonyl showed it (Figure 1 and Figure 2.). The difference between control and *S. platensis* treatment shows that it has the capacity to reduced oxidative stress that is exposed to brain lipids, proteins, and carbohydrates. The *S. platensis* treatment on CS could not decease MDA and carbonyl concentration. It also be considered that maybe the dose of *Spirulina platensis* should be increased. Another study observed *S. platensis* protective effect against nicotine, with the dose of nicotine was 0,5 mg/kg BW/day and *S. platensis* was 500 mg/kg BB/day for 4 weeks, showed that rats were treated with nicotine only had high MDA significantly, and the *S. platensis* could reduce MDA level [14].

Cigarettes contain approximately 7000 dangerous compounds that are free radical generators. Tar is the main source of free radicals and nicotine is the main neurotoxic agent in cigarette smoke [15]. Free radicals will attack the double bonds in the lipid structure of the membrane causing lipid peroxidation and oxidative stress. The body has settings to deal with endogenous oxidative stress. The defense mechanism is through the activation of endogenous antioxidant action [16]. If endogenous antioxidant activity is not able to overcome oxidative stress, it is necessary to add exogenous antioxidants [17]. Antioxidants can protect from oxidative stress that can make cell damage and apoptosis in the case of nerve cells.

Our study showed the level of glutathione was kept high by *S. platensis* (Sp). Conversely, the control (C), cigarette smoke exposure (Cg), and also a combination of cigarette smoke exposure with *S. platensis* (CgSp) did not show GSH differences among them. Catalase decreased significantly in the Cg group compared to the C and Sp groups. In the CgSp group, there was no significant increase in catalase activity compared to the Cg group. It consider that the doses of cigarette smoke was not enough to produce oxidative stress, or, maybe the dose of *S. platensis* did not sufficient to recover catalase activity. SOD activities were increased significantly in the group exposed to cigarette smoke and given spirulina extract compared to groups C, Cg, and Sp. Only the combination of cigarette smoke and *S. platensis* could increase significantly the SOD activity, although *S. platensis* alone could increase the SOD activity. It considered to explore the phenomena further.

In line with the result of this study, other studies show that nicotine induction leads to increase oxidative stress [18] by reducing the antioxidants[19]. Treatment with *S. platensis*

recovers oxidative stress by reducing the MDA level, increasing GST and GSH. The protection effect was through inhibition of inflammation through (NF-κB).[14] A study using *S. platensis* at the doses 500 and 1000 mg/kg BW could reduce liver and kidney injury, increase glutathione levels. Brain damage caused by Pb could be reduced by *S. platensis,* also reduce the effect of acrylamide. It showed that *S. platensis* reduced the levels of MDA, NO, and increase GSH, GPx, catalase, and SOD in the brain [20].

Cigarette smoke exposure during pregnancy caused harmful effects to offspring's brains. Otherwise, it is also harmful to the placenta that transfers toxic substances. It leads to brain tissue damage and degeneration [21]. The material that damaged the brain was nicotine that was absorbed firstly by maternal blood and flow pass through the placenta entering offspring circulation. Besides nicotine could accumulate in breast milk, three times higher than plasma [22–24]. In this study, the amount of cigarettes did not yet trigger oxidative stress in the offspring brain.

The *S. platensis* treatment (200mg/kg BW/day) did not show effectiveness to overcome cigarette smoke exposure radicals during pregnancy and lactation. Even though the dose chosen in this research was already proven in the other study. According to the results, it is considered to increase the *S. platensis* dose of treatment and use positive control. The ingredient of *S. platensis* that consider to have an effect is c-phycocyanin, β-carotene, and other antioxidants [8]. Phycocyanin one of the ingredients could repress NO synthase, inhibit lipid peroxidation, an inhibitor for cyclooxygenase-2 (COX-2) [8,25]. Therefore. further study should be conducted to explore the dose of cigarette and the dose of *S. platensis.*

## Conclusion

Offspring brain tissue did not suffer oxidative stress from maternal 4 a day cigarette smoke exposure during pregnancy and lactation, according to no significant difference in MDA, GSH, SOD, GPx, and catalase between control, cigarette smoke exposure, and combination cigarette smoke with *S. platensis* groups. Though, the effect of *S. platensis* could not be analyzed yet.

## Acknowledgment

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## Author contributions

KCA conducted data collection, wrote the manuscript; FCI, SWAJ, F supervised study, contributed to the completion of the manuscript, ARP guided to study design, supervised study, reviewed and finalized the manuscript, reviewed and finalized the manuscript, contributed to the completion of the manuscript.

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