Effects of paternal exposure to tertiary cigarette smoke on fetal morphometry and cognition of the offspring and its impact towards pollution

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Abstract. The aim of this study was to determine the effect of paternal exposure to tertiary cigarette smoke on fetal morphometry and cognition of the offspring. This research is an experimental study with a complete randomized design. Twenty adult male mice used and randomly divided into control group and treated group which exposed to tertiary cigarette smoke for 14 days. After the smoking period, each male mated with two adult female mice. One group of female mice sacrificed and dissected to isolate the fetus on the 18th day of gestational. Fetal morphometry observed immediately. An other group of female mice allowed to give birth naturally. Then on the 30th day, novel object recognition (NOR) test performed to assess the cognitive function of the offspring. The results showed that the weight and length of the fetus from the treated group significantly smaller than that of the control group. Furthermore, offspring of the control group showed better performance significantly during NOR test. These results indicated that paternal exposure to cigarette residues affect the fetal development so that the fetus has smaller size and worse offspring cognition.

1. Introduction

World Health Organization data shows that there were around 967 million or almost one billion active smokers in 2012 who consumed around 6.25 trillion cigarettes [1]. Meanwhile, Basic Health Research of Indonesia Ministry of Health data shows that the prevalence of smokers has increased by 34.7% in 2010 to 36.3% in 2013 [2]. The increasing prevalence of active smokers is not only detrimental to the health of active smokers (first-hand smoker) and passive (second-hand smoker), the danger of smoking also threatens tertiary smokers (third-hand smoker). Cigarette smoke exhaled from active smokers will leave residues attached to various surfaces of objects in the room or environment, such as clothing, hair, furniture, carpets, and others. The rest of cigarette smoke will not just disappear, this dangerous compound can last for months or even years and causing negative effects on health. Individuals exposed to cigarette residues are referred as tertiary smokers. Cigarette residues can react with other compounds in the air, including nicotine which can react with nitrogen in the air to produce compounds called tobacco-specific nitrosamines (TSNAs). These compounds are known to have mutagenic and carcinogenic manner. In addition, TSNAs compounds will also decrease children intelligence, causing...
children at high risk of experiencing neurological disorders, and interfere fetal lung development. Cigarette residues that react with nitric acid can also produce harmful carcinogetic compounds, including arsenic, carbon monoxide, and hydrogen cyanide. Exposure to these substances can also trigger changes in liver metabolism that leads to type 2 diabetes mellitus [3,4].

The adverse effects of cigarette smoke exposure on adult people are not only felt by them, but also by their offspring. Many studies show that pregnant women who smoke or exposed to cigarette smoke have a greater risk of having an abortion or giving birth offspring with teratogenic disorders, impaired respiratory system, increased risk in developing cardiovascular disease, decreased cognitive function, and exhibiting hyperactivity [5-7]. However, smoke exposure and cigarette residues must also be avoided by men or prospective fathers because some studies have shown that toxicity to cigarettes can accumulate in semen and sperm thereby reducing sperm motility, sperm concentration, and causing infertility in men [3,8,9]. Exposure to cigarette smoke also causes apoptosis and disruption of the seminiferous tubules so that impairing spermatogenesis and decreasing sperm quality. Paternal smokers also correlate with infertility, birth defects, diabetes, and cancer in offspring [3,10].

Although tertiary cigarette smoke is known to increase the risk of various diseases, the exact mechanism and pathogenesis pathway remain unclear. The specific implications if someone is exposed to smoke for the long-term period is also still unknown. In addition, the limited data and research related to the mechanism of tertiary cigarette smoke in causing pathological conditions in the body of non-smokers encourage researchers to study this topic further. Therefore, the main problem studied in this research is how the effect of paternal exposure of tertiary cigarette smoke on fetal morphometric and cognitive functions of the first-generation offspring.

2. Method
This study is pure in vivo laboratory experimental research with a completely randomized design and carried out in January to August 2018 at the Medical Biology Laboratory, Universitas Islam Bandung, Jl. Tamansari No. 22. The experimental animals used in this study were mice (Mus musculus L.) with inclusion criteria: adult males aged 8-10 weeks, weight 35-40 g, never mating before, health, no injuries, move actively, and responsive. Before the treatment period, an acclimatization period was done for one week. During the research period, animals placed in cages (size: 40x28x25 cm). Each cage filled with ten experimental animals. Cages are also equipped with food and drink so that animals can get access to food and drinks ad libitum. Wood sawdust is placed on the base of the cage to absorb animal urine and waste. A total of 20 male mice divided into two groups randomly: (1) the control group that was not exposed to tertiary cigarette smoke and (2) the treated group that was exposed to tertiary cigarette smoke for 14 days. During the smoking process, the animal of the treated group transferred to another cage. Tertiary cigarette smoke exposure is done by smoking the animal cage with one cigarette in a separate area with maintenance space so that the animals were not exposed to cigarette smoke directly. After the treatment period completed, each male mice mated with two female mice. The breeding process did naturally by uniting male and female mice in one cage in the afternoon. Pregnancy testing is done by observing the presence of a vaginal plug in the next morning. When the vaginal plug is found in female mice, the day counted as day 0 of gestation. The criteria of female mice used in this study were 8-10 weeks, weight 30-35 g, never mating and pregnant before, health, no injuries, move actively, and responsive.

One set of females sacrificed on the 18th day of gestational by dislocation and dissected to isolate the fetus. Furthermore, observation of the fetal morphometric (length and weight) was carried out immediately. One group of female mice was left to give birth naturally, the offspring that were born would not interfere until the weaning period for 21 days. On day 22, the female parent separated from its offspring. Then on the 30th days, a test performed to assess the cognitive function of the offspring. The test conducted in this study is a novel object recognition test. At the end of the study, all experimental animals were sacrificed and parts of the body of the mice that were not used in the study were collected and buried.
3. Results and discussion
The data in Table 1 shows that the weight of the treated group’s fetus was significantly lower than that in the control group. The length of the fetus in the treated group was also significantly lower compared to that of the control group fetus. These results indicate that the treatment given in this study can affect the fetal morphometric (fetal weight and length). However, there was no observed external congenital abnormality in the fetus from both the control and treated groups.

Table 1. Fetal morphometric observations.

| Parameter Assessed | Control (N = 45) | Treated (N = 42) | p-value |
|--------------------|-----------------|-----------------|---------|
| Fetus weight (g)   | 0.87±0.25       | 0.60±0.39       | 0.000*  |
| Fetus length (cm)  | 2.60±0.36       | 2.2±0.55        | 0.000*  |

Data is presented in mean±SD (standard deviation). Statistical analysis for each parameter used Mann Whitney test at 95% confidence interval (α = 0.05); N = number of samples; * indicates significantly different.

This study also observed cognition function of the first-generation offspring, data in Table 2 shows that mice offspring from the control group spent significantly less time in exploring old objects. There was no significant difference in terms of the duration in exploring new objects of both offspring from the control group and treated group. Descendants of the control group spend a longer time exploring new objects than old objects that introduced previously at the habituation stage. While the offspring of mice from treated groups spend relatively the same time in exploring both new objects and old objects. This indicates that the offspring of the control group have a better ability to recognize and memorize the old objects. Rodentia will generally spend a longer time exploring new objects compared to objects that it has recognized. This result indicates that offspring of the control group were able to recognize objects that have been previously introduced and remember it so that they spend more time exploring new objects. These results also indicate that the treatment given in this study influences the cognitive function of the first-generation mice.

Table 2. Memory test results of the first-generation offspring with NOR test.

| Parameter Assessed | Control (N = 57) | Treated (N = 33) | p-value |
|--------------------|-----------------|-----------------|---------|
| Tob (second)       | 13.75±6.42      | 12.36±6.57      | 0.333   |
| Tol (second)       | 8.14±4.17       | 12.88±7.76      | 0.0013* |

Data is presented in mean±SD (standard deviation). An independent T-test used to analyse Tob and Mann Whitney test for Tol at 95% confidence interval (α = 0.05); N = number of samples; Tob = duration in exploring new objects; Tol = duration in exploring old objects; * indicates significantly different.

Fetal morphometry observation showed that the treated group had fetuses with lower body weight and shorter length than that of the control group significantly. These results indicated that paternal exposure to tertiary cigarette smoke can affect the development process of the embryo and fetus. Amount of literature shows that toxic substances of cigarettes can affect sperm quality. Nicotine, heavy metals, and other substances from cigarettes and residues detected in the male reproductive organs, these substances can accumulate in semen and sperm so that can cause genetic material damage in sperm and reduce sperm quality [3,8,9]. It is known that cigarette smoke can interfere with sperm sumoylation process so that disrupting spermatogenesis and decreasing sperm quality produced [11]. Research by Esakkya et
al. showed that cigarette smoke condensate (CSC) exposure was genotoxic to spermatocytes, causing apoptosis and disruption of tubules seminiferous through oxidative stress [4]. Dai et al. states that heavy metals contained in cigarettes include cadmium (Cd) and lead (Pb) [12]. Cadmium has a long half-life in the human body (20-40 years) so it has the potential to accumulate in the body. Increased cadmium levels in semen detected in smokers and resulted in decreasing sperm count and motility caused by DNA fragmentation and sperm morphological abnormalities. In serum and semen smokers also found an increase in lead levels causing a decrease of alkaline phosphatase activity and Na-K ATPase which has implications for decreased sperm motility.

Dai et al. also states that toxic compounds of cigarettes also play role in activating the oxidative stress mechanism so that an increase free radical in the body occurs [12]. Free radicals’ enhancement is positively correlated with DNA fragmentation that triggers DNA damage. PHA and benzene in cigarettes can also trigger DNA adducts formation, which is a segment of damaged DNA due to binding to certain carcinogenic compounds. Increasing DNA adducts formation is also an important factor that causes apoptosis. In addition, the formation of DNA adducts results in an increase of point mutations that affects synthesized proteins function. DNA adducts also contribute to enhancing DNA fragmentation, termination of double chains and single-chain DNA so that increase the chromosomal aberration and/or changes in sister chromatids [13]. Moreover, benzo (a) pyrene (B[a]P) found in cigarette smoke is a mutagen and carcinogen. This compound will bind covalently with DNA to form DNA adducts called benzo (a) pyrene diol epoxide-DNA (BPDE-DNA). It is known that BPDE-DNA increases in smokers and significantly decreases the percentage of acrosome halo formation and contributes to causing DNA damage in smoker sperm [14].

Kovac et al. showed that smokers had lower zinc content than nonsmokers [15]. Zinc is known to play an important role in spermatogenesis, a decrease in zinc levels is positively correlated with a decrease in sperm concentration, motility, and normal sperm morphology. Nicotine is also mentioned as a compound that plays a role in causing sperm quality decrease in smokers. However, further research is needed to find out the pathogenesis of nicotine in reducing sperm quality. Damage caused by cigarettes toxicity not only affects spermatogenesis directly but also affects Sertoli cells and Leydig cells functions. Exposure to free radicals and toxins results in decrease those two cells’ functions, causing sperm quality decrease [12,14]. Overall mechanisms that described before, oxidative stress mechanisms, DNA fragmentation, DNA adducts formation, accumulation of toxicity in semen, decreased testicular function play a role in reducing sperm concentration and motility, decreasing the ability of sperm to fertilize and increasing the risk of miscarriage [13].

Research on animal models and epidemiological studies in humans shows that sperm epigenome is passed on to the next generation so that it can affect the health status of the offspring. Not only lifestyle and maternal nutritional intake will affect offspring quality. Paternal lifestyle, nutrition intake, exposure to certain substances play an important role in influencing sperm epigenome and these changes will be inherited across generations [16]. Esakky and Moley stated that cigarette smoke can affect the genome and epigenomic components of sperm [10]. Children born to male smokers have a higher risk of cancer in childhood. Congenital abnormalities, such as anorectal malformations, cardiac anomalies, congenital heart disease, cleft lip, hydrocephalus, urethral stenosis, spina bifida, decreased kidney volume and father smokers and cigarette smoke [17,18]. Paternal exposure to cigarettes contributes to implantation failure. Godschalk et al. shows that B[a]P can induce hypomethylation in DNA which results in inherited mutations in offspring [19]. Research in mice shows that sperm DNA mutations caused by cigarette smoke cause decrease chance of pregnancy after conception, impaired blastocyst implantation, and no embryonic development [20].

Nicotine is known to have neuroteratogenic effects on brain development in several ways, such as causing neuronal death, upregulating acetylcholine receptors so that disrupt brain maturation, decreasing acetyl cholinergic activity patterns, disrupting regular patterns of cholinergic activity, and influencing intracellular signaling pathways [21]. Nicotine causes DNA synthesis suppression which causes cell death in the brain. This is presumably due to a decrease of choline transporters expression in the nervous system. Cholinergic synapses are functionally played important role in brain morphogenesis. Nicotine
exposure in the prenatal period causes a permanent decrease in receptor function and noradrenergic and dopaminergic neurons and causes the fetus unable to release catecholamines after birth. Nicotine exposure in prenatal and perinatal periods is also known to increase adenylyl cyclase expression, increasing the potentiation of excitatory postsynaptic potentials (EPSPs) mediated by the N-methyl-D-aspartic acid (NMDA) receptor in the auditory cortex, decreasing the EPSP mediated by 2-amino-3-(5-methyl-3-oxo-1,2-oxazole-4-yl) recanoic acid (AMPA) receptor in the hippocampus [22].

Dai et al. show that paternal nicotine exposure can induce changes in the behavior of the first-generation offspring [23]. The exposure to nicotine induces downregulation of the mmu-miR-15b gene of rodensia’s spermatozoa. The epigenetic modification is thought to mediate the decrease in neuropsychological abnormalities induced by nicotine. Decrease expression of the mmu-miR-15b gene in spermatozoa will cause increase expression and synthesis of Wnt4 protein in the thalamus of first-generation offspring. This will be followed by activation of the Wnt4 pathway in the hippocampus and cause inhibition of GSK3 phosphorylation in that area of the brain. GSK3 is a protein associated with depression and anxiety. Inhibition of this protein synthesis induces hyperactivity and decreases in offspring. This is in line with earlier research by Figueiró et al. their study shows that exposure to tertiary cigarette smoke causes hyperactive behavior, the experimental animals try to run longer and faster in the open field test [6].

Hyperactive behavior was also observed in offspring from the treatment group in this study. The offspring that came from the treated group tended to run and jump around during the cognition test session. The objects placed in the test area were more widely used to jump over and try to get out from the test area, not exploring it. The experimental animal looks uncomfortable and tries to find a way out of the test area. Different from the descendants of the control group who are calmer when placed in the test area and interested in exploring new objects that placed in the test area. These results are consistent with the studies that have been described that nicotine in cigarettes or cigarette residues left on cigarette residues has potential to interfere brain maturation process so that it can affect the cognitive function of mice's descent in this study. The offspring that came from the treatment group were not able to distinguish between old objects and new objects and showed hyperactivity.

4. Conclusion
The results showed that paternal exposure to tertiary cigarette smoke could affect embryonic development and fetal growth. Toxic compounds left on cigarette residues could interfere with those processes so that the fetus of treated group had a smaller size (lower weight and shorter birth length). Disruption during fetal development also contributed to impairing the cognitive function of the offspring so that the first-generation offspring of treated group showed worse performance during NOR test.

5. Acknowledgments
We would like to thank the Institute for Research and Community Services, Universitas Islam Bandung, for funding this research. We also thank you to Biomedical Laboratory, Faculty of Medicine, Bandung Islamic University for facilitating this research.

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