

Effect of Acute Exposure of Cigarette Smoke on Endometrium Thickness and Number of Ovarian Follicles in Rats

Izdihar Ramadhanty Abiyasa Putri¹, R Haryanto Aswin², Tri Hartini Yuliawati³

¹Medical Program, Faculty of Medicine, Universitas Airlangga, Surabaya, Indonesia

²Department of Biomedical Science, Faculty of Medicine, Universitas Airlangga, Surabaya, Indonesia

³Department of Anatomy and Histology, Faculty of Medicine, Universitas Airlangga, Surabaya, Indonesia

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ABSTRACT

Background: Cigarette smoke has been associated with many health-related problems especially in long time use. One of many health problems that are affected is the reproductive system that give negative impact in fertility. However, there are limited studies about this in acute exposure of cigarette smoke.

Objective: The aim of this study is to investigate the effect of acute exposure of cigarette smoke on endometrial thickness and number of ovarian follicles in rats.

Methods: Thirty adult female rats were randomly divided into 3 groups: P0, P1 and P2. The P1 and P2 groups were exposed to cigarette smoke for 15 days with dose of 2 and 4 cigarettes daily, respectively. Vaginal swab was done twice, before and after the last treatment, to determine the proestrus phase of the animals. Histological slide of uterus and ovarium using Haematoxylin Eosin staining were observed under light microscop. Number of ovarian follicles were measured, and the thickness of endometrium was measured using CellSens software version 2.3 (Olympus Life Science). Statistical analysis of data was performed using MANOVA in SPSS software version 23 for Windows.

Results: There were no significant differences in both endometrial thickness and number of ovarian follicles between 3 groups ($p>0.05$). However, we found that there were a downward trend of some variables (endometrial thickness, number of primary follicles, de Graff follicles, and corpus luteum) from P0 to P2.

Conclusion: We concluded that acute exposure of cigarette smoke not yet influenced the female reproductive organ, based on histological appearance of endometrium and ovarium.

Keywords : Cigarette smoke, Endometrium, Ovarian follicle, Rats

INTRODUCTION

Approximately there are 250 million female smoker in the world.¹ The distribution of smokers in Indonesia is still very high, according to the data of The Health Ministry of Indonesia in 2007-2013. Smokers tend to increase from year to year, both men and women.² Cigarette smoking is associated with lower fecundity rates, adverse reproductive outcomes and a higher risk of in vitro fertilization (IVF) failure.³

Cigarette smoke consist of polycyclic aromatic hydrocarbon (PAHs) for example: benzo(a)pyrene (B[a]P), aromatic amine, N-nitroso, heavy metal [example: cadmium (Cd)], and other.⁴ Cadmium has been identified as endocrine disruptive chemical and has been implicated to cause impaired folliculogenesis.⁵ In previous study cadmium exposures also cause reduced number of corpus luteum, primary follicles and antral follicles.⁶ Other findings show that N-nitroso-amine that is contain in cigarrate smoke can cause follicle destruction. Liu *et al*, found that N-nitroso amine, one of the component found in cigarette, exposure can reduce the number of primordial and primary follicle.⁷

Cigarette smoke exposure apart from having an effect on the follicles is also thought to have an effect on endometrial thickness. One of the component that is in the cigarrate smoke is BaP. BaP has been studied as endocrine disruptive chemical that can cause effect on estrogen and progesterone balance. In his experimental research, Zhao *et al* found that BaP exposure could disrupt the balance of estrogen and progesterone therefore could influenced on the endometrium histomorphology and receptivity.⁸ In this study we emphasized on the effect of acute exposure

of cigarette smoke on endometrial thickness and number of follicles

METHOD

The experimental study with post test only control group design was conducted in Wistar strain of *Rattus norvegicus*. Thirty adult female rats were randomly divided into 3 groups: P0 as the negative control group; P1 and P2 as experimental groups. The P1 and P2 group were exposed by cigarette smoke for 15 days with dose of 2 and 4 cigarettes daily, respectively. All of the positive control group is expose in the smoking chamber for 15 days. Vaginal swab was done twice, before and after the last treatment to determine the proestrus phase. In this stage, the predominant cells were in small round, nucleated epithelial cells that relatively similar in size and appearance. The cells may stain deeply basophilic. The neutrophil was rarely found in this phase. The animals were sacrificed, then the uterus and ovarium were taken, and processed into paraffin block. The slides from these organs were prepared and stained using haematoxylin eosin for microscopic observation.

Endometrial thickness was measured using 100 magnifications and the number of follicles was measured using 100x, 400x, and 1000x magnifications on Olympus CX10 microscope. The endometrial thickness was measured by drawing a line perpendicular from the base to the peak of the endometrium. Six points were used to represent the endometrial thickness, three points to represent the thinnest part and the others for the thickest part of the endometrium, and the average was calculated. The number of follicles was measured in one of the ovarium using 100 magnifications first and then using 400 and 1000 respectively. The number of follicles was measured using three

different magnifications because there were some follicles that we can see using 100 magnification such as corpus luteum and some other follicle such as the primary follicles can only be seen using the 1000 magnifications. The direction of counting was done consistently from the top left to the right and then

downwards until all of the ovary was explored.

Statistical analysis was performed using SPSS software version 23 for Windows. Normality test was performed using Shapiro Wilk whereas the Levene's test was used to determine the homogeneity of the data. The Multivariate Analysis of Variance (MANOVA) was performed to analyze the differences between groups. The significant difference was set in $p < 0.05$.

RESULT

Table 1 showed decrease in endometrial thickness of the experiment group but statistical test showed no significant differences in MANOVA.

Table 1. Effects of cigarette smoking on endometrium

Groups	N	Endometrial Thickness (mean \pm SD)	p
P0	10	327.21 \pm 96.88	0,201
P1	10	317.88 \pm 57.75	
P2	10	310.43 \pm 51.77	

Table 2. Effects of cigarette smoking on number of ovarian follicles

Kel	N	Average \pm SD number of ovarian follicles											
		Primary follicle	p	Growing follicle	p	Follicle de graf	p	Corpus luteum	p	Corpus albicans	p	Atresia follicle	p
P0	10	3.3 \pm 1.8	0,075	7.6 \pm 2.9	0,904	0.6 \pm 0.8	0,393	7 \pm 3.3	0,930	0.9 \pm 1.5	0,928	0.7 \pm 1.3	0,115
P1	10	1.6 \pm 1.5		8.2 \pm 3.0		0.2 \pm 0.4		6.6 \pm 3.3		1 \pm 0.9		0 \pm 0	
P2	10	2.2 \pm 1.4		8.2 \pm 4.2		0.3 \pm 0.7		6.4 \pm 4.2		1.1 \pm 0.9		0.8 \pm 1.0	

Number of ovarian follicles is in mean \pm SD

Shapiro Wilk test is used to determine the normality of the data followed by Lavene test to determine whether the sample is homogen or heterogen and the last step is MANOVA test to see the significance of the data. The p value under 0.05 is considered as significant, statistical analysis shows that the p value is 0,201 therefore there is no significant different between the P0 group, P1 and P2 group.

The following are pictures of the endometrial thickness of the P0 group, P1, and P2 group:

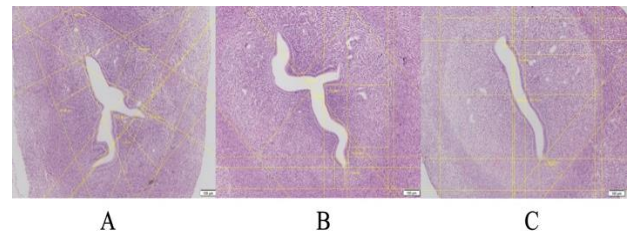


Figure 1. The picture above is endometrial thickness 100x, Hematoxylin Eosin staining; a: P0 group; b: P1 group with 2 cigarettes; c: P2 group with 4 cigarettes daily

Table 2 showed decrease in the total of primary follicle, follicle de graf, and corpus luteum from control group to experiment group, while the total of growing follicle, corpus albicans, and atresia follicle is increasing.

Shapiro Wik test is used to determine the normality of the data followed by Lavene test to determine whether the sample is homogen or heterogen and the last step is MANOVA test to see the significancy of the data. The p value under 0.05 is considered as significant, statistical analysis shows that the p value is 0,201 therefore there is no significant different between the P0 group, P1 and P2 group.

The following are pictures of the ovarian follicles of the P0 group, P1, and P2 group:

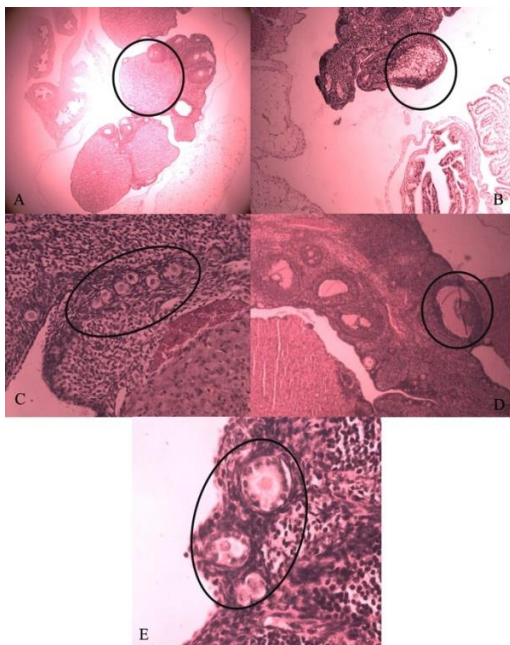


Figure 2. Ovarian follicles in control group (a: corpus luteum, 100x; b: corpus albicans, 400x; c: primary follicles, 1000x; d: follicle de Graaf 100x; e: growing follicles, 400x) with Hematoxylin Eosin staining

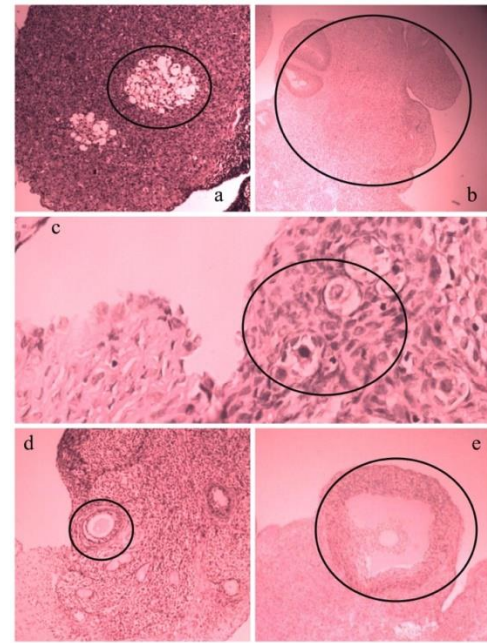


Figure 3. Ovarian follicles in rats exposed in 2 sticks of cigarette a day (a: corpus albicans, 400x; b: corpus luteum, 100x; c: primary follicles, 1000x; d: growing follicles, 100x; e: follicle de Graaf, 400x) with Hematoxylin Eosin staining

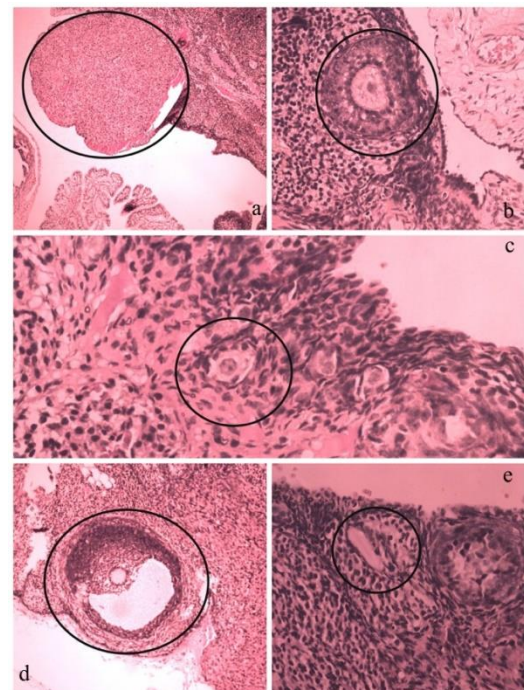


Figure 4. Ovarian Follicles in rats exposed with 4 cigarettes sticks a day for 15 days (a: corpus luteum, 100x; b: growing follicle, 400x; c: primary follicle, 1000x; d: follicle de Graaf, 100x; e: atretic follicle, 400x) with Hematoxylin Eosin staining

DISCUSSION

Endometrial thickness

In previous study, Ding *et al.*, using whole body mainstream smoke exposure found that endometrium and myometrium in the experimental group is thinner than in the control group. They expose mice in an exposure chamber twice daily for 30 days.⁹ Other studies, we found that statistical test result from table 5.1 shows that the decrease of endometrial thickness was not significant. We think the longer the period of exposure and the higher the dose of cigarette sticks may contribute to the results. Cigarette smoke contains so many different types of chemicals, these different types of chemicals might also come into play with the results of our study.

Cadmium, one of the chemicals contained in the cigarette smoke has been researched that can disturb endocrine by its estrogenic and anti estrogenic effects.⁵ This phenomenon can lead to disturbance with the maturation process of the endometrium. Nesatyy, *et al* revealed that cadmium can mimic the effects of estrogen by interacting with estrogen receptors and lead to estrogen receptors activation.¹⁰ Based on previous study, it seems that the effect of cadmium exposures varies based on the dose and routes of administration.¹¹ Zhang, *et al.*, conducts research on rat's endometrium given intraperitoneal cadmium and found thickening of the endometrium and myometrium, the study also stated that intraperitoneal cadmium causes inflammation in the endometrial stroma of the rats.¹² Da Costa *et al.*, also conducted an experimental research on cadmium exposures by oral routes but they did not found any significant difference in the thickness of endometrium, however they found decrease of myometrium thickness in the experimental

group.⁶ Nasiadek *et al.*, also conduct a study with oral cadmium and found that cadmium in small doses tend to express estrogenic effect and in higher doses cadmium tend to express antiestrogenic effect. They also found that lower dose cadmium significantly increased the thickness of endometrium but in higher dose cadmium they found noticeable endometrial atrophy, therefore it concluded that cadmium can have both anti-estrogenic and estrogenic effect.¹¹ In this study, we found no significant difference in the control and experimental group, we theorize that this might be because we used lower dose and short amount of exposure time only. We also use whole smoke exposure thus there are many different chemical that might influenced the result of this study.

Benzo(a)pyrene is also found to be a disrupting agent towards steroid hormone. BaP has been reported to have estrogen and anti-estrogen features, to activate aromatic receptors and through the estrogen receptor⁸. Benzo(a)pyrene can interfere with the cytochrome that is involved in estrogen metabolism. The other chemical that is in cigarette smoke is nicotine. Soghomonians *et al.*, found that nicotine can inhibit the decidualization, motility and migration of endothelial cell in the uterus.¹³ Khorram *et al.*, did a research with human endometrial culture cell administered with 10% concentrated cigarette smoke. This research found that nicotine and B(a)P in the cigarette smoke inhibited endometrial cell proliferation via nitrite oxide pathway.¹⁴ Zhao *et al.*, also did a research using animal model and found that BaP could disrupt the balanced of estrogen and progesterone, this leads to the changes in endometrial receptivity and reduce implantation sites.⁸ There are many component of cigarette

smoke that is both has estrogenic and anti-estrogenic effect, this could have an effect in the results of the endometrial thickness after exposure to cigarette smoke.

Dechanet, *et al.*, conducted a systematic review on the effects of cigarette smoke with reproduction and in the study, it stated that the doses in the cigarette smoke and the length of exposure also affect the results on experimental studies. Experimental studies tend to have acute exposure rather than chronic exposure as in humans.³ This study can be categorized as acute exposure therefore the length of exposure might have effect on the result of this study. The doses of exposure also might not be adequate enough for acute exposure and that might have effect on the results in this experimental study.

Number of ovarian follicles

Result showed that primary follicles, graafian follicles, and corpus luteum have a downwards trend, although that result have been proven insignificant by the statistic test. Different than other research before, this research has found that the downward trend in follicle number after being exposed by cigarette smoke was not significant. This might have been caused by the different in the length and dose of exposure.³ Paixão, *et al* use 15 days as the length of exposure but with the dose of 8 hours cigarette smoke exposure per day.¹⁵ Tuttle *et al.*, also has done similar study with this one and use 8 weeks as length of exposure and 2 sticks of cigarette per day. Both of their studies have found a significant decrease in number of follicles.¹⁶ Lee *et al.*, did a study with animal model using whole body inhalation for 4 weeks straight and 10h exposure of cigarette smokes per day. They found a significantly decrease primary and secondary follicles.¹⁷

Sobinoff *et al.*, use nose only exposure cigarette smoke for 1h twice a day for a total of 12 weeks, they found a significantly reduced primordial and primary follicles.¹⁸ Gannon *et al.*, use whole body exposure in 8 weeks old mouse for 4,8,9, and 17 weeks and found reduced number of primordial follicle as early as in 4 weeks of exposure. The length of exposure in this study is acute length of exposure by only 15 days, we theorize that this length of exposure can also influenced on our result. We only 2 and 4 sticks of cigarette in this experiment which can be seen as a small dose, the dose we use might not interpreted well in the result, therefore we might suggest higher dose on future research.

Based on the results of previous studies, different type of administration or administration with more specific substance in cigarette might have influence the numbers of follicles. Sadeu *et al.*, conduct experimental research with isolated mice follicle with BaP and found that BaP might be responsible for signaling apoptosis pathway and delaying the follicular growth.¹⁹ Mohammadghasemi *et al.*, did a study with intraperitoneal nicotine and found similar results of decrease in number of pre-antral and antral follicles and also found decrease in estradiol hormones in rats.²⁰ Madden *et al.*, use dimethylbenz[a]anthracene (DMBA) exposure, one of the component in the cigarette smoke, in cultured rat ovaries and found large primary follicle loss in both the high dose and small dose DMBA.²¹

Steroid hormones concentration in rats can also influence in the decrease in number of follicles. As explained above cigarette smoke has many different component that can cause estrogenic so does anti-estrogenic effect, this can influence the folliculogenesis. A cohort studies that is consist of pre menopausal

smoking and non smoking women shows significant difference in hormone levels, which the premenopausal women that smoke shows higher level of FSH in their early follicular phase and also higher level of LH in their menstruation.²² Kapoor and Jones showed that cigarette smoke causes a decrease in the concentration of steroid hormones.²³ da Costa *et al.*, using oral cadmium exposure found that high basal serum LH in mouse with cadmium exposure. They also found an abnormal proestrous cycle in the experimental group, this could cause reduced number of ovarian follicles and can also cause disruptive development on ovarian follicle. da Costa *et al.*, found reduced number of corpus luteum in experimental group.⁶ Sadeu and Foster also did a research with cigarette smoke condensate (CSC) and found that estradiol output significantly decrease after CSC exposure, this could lead to the disruption on the follicular development.²⁴ This research does not measure the rats steroid hormones level, even so it might have influence the results in this study. Further research is needed on the effect of cigarette smoke on steroid hormone concentrations.

CONCLUSION

We concluded that acute exposure of cigarette smoke does not influenced the female reproductive organ, based on histological appearance of endometrium and ovarium. We suggest using higher dose and longer time exposure in order to see the true association of cigarette smoke with endometrial thickness and number of ovarian follicles.

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