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Research Article

The Effect of Cigarette Smoke Exposure due to Placental Apoptosis and Gestation Outcomes at Gestation Disorders Mechanism In White Rat (Rattus norvegicus)

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ABSTRACT
The aims of this research to find out the increased incidence of placental apoptosis, abnormal gestation outcomes, and to prove the emergence congenital defects in white rats were exposed to cigarette smoke during gestation. Female white rat (Rattus norvegicus) weighing between 200 and 250 g were used and superovulation used PMSG with a dosage of 10 IU/rat and HCG 10 IU/rat. Female rat were mated, the gestation was indicated by the visible mating plug covering the vulva and then the day was considered the first day of gestation. The animals of the smoking group were exposed to the smoke of five cigarettes per day, from the 6th to the 17th day of pregnancy. The conclusion of this research was the exposure of cigarette smoke can increase the occurence of placental apoptosis on the gestation disorder mechanism on white rat also proved that can cause external congenital disorder on white rat such as body weight loss, foetus body length loss, increasing occurrence of abortus.

Keywords: Cigarette smoke, placental apoptosis, congenital disorder.

INTRODUCTION

Cigarette contains various chemical substances that can jeopardize health, such as nicotine, tar, and other alkaloid substances. Those chemical substances can cause various health disorders on various organs suchas cardiovascular, respiratory, gastrointestinal, reproduction, etc. The disorders can also be fatal, such as susceptibility to infections, heart coroner disease to cancer on various organs [1]. Most of the occurence processes are from oxidative stress. This kind of condition is caused by the disproportion between oxydant rate and antioxidant rate. There's a strong relation between cigarette smoke exposure with the reproduction function degradation. On female reproductive organs, cigarette smoke exposure will cause an acceleration on the lessening of follicle amount in the ovary, degradation of the steroid hormone production process, degradation of ovary follicles maturation, and cilia movement disturbance in the Fallopian tube. While in the infertility cases, cigarette is related to the increasing of conception failure cases and repeated miscarriages on early period gestations [2].

Cigarette is one of the enviromental factors that can cause congenital defect. Smoking habit on pregnant women can cause spontaneous abortus and prenatal foetus death, even meromelia. Though it has been warned that smoking can harm the foetus development, 25% women still smoke in their pregnancy periods. On heavy smokers with 20 or more cigarettes per day, can cause premature birth twice more often compared to non-smoking women, and their babies have lower body weight (less than 2000 gram), that oftenly caused foetus death [3]. Congenital defect is also called malformation congenital or anomalycongenital that is the phrase used to explain structural, behavioral, function, and metabolic disorder found in birth period. Lately, researches showed that 7-10% cause of congenital defect is enviromental factors, 6-15% genetic factors, 20-25% combination of enviromental and genetic factors, and 50-60% is still unknown [3].

Cigarette contains many toxic, as well as stable and unstable free radicals and reactive oxygen species (ROS) in the particulate and the gas phase with the potential for biological oxidative damage [4]. Some of the water-soluble

components of aqueous cigarette tar (ACT) can produce superoxide anion ($O_2^{\bullet -}$) and subsequently H_2O_2 and the reactive hydroxyl radical ($HO^{\bullet}$), which cause oxidative damage to cellular membrane lipids, proteins, enzymes and DNA caused apoptosis [5]. Apoptosis, programmed cell death, is an essential feature of normal placental development but is exaggerated in association with placental disease. Placental development relies upon effective implantation and invasion of the maternal decidua by the placental trophoblast. Placental apoptosis may be initiated by a variety of stimuli, including hypoxia and oxidative stress. In common with other cell-types, trophoblast apoptosis follows the extrinsic or intrinsic pathways culminating in the activation of caspases. Widespread apoptosis of the syncytiotrophoblast may also impair trophoblast function leading to the reduction in nutrient transport seen in Intrauterine Growth Restriction (IUGR) [6]. Intrauterine growth restriction (IUGR), a condition that occurs due to various reasons, is an important cause of fetal and neonatal morbidity and mortality. It has been defined as a rate of fetal growth that is less than normal in light of the growth potential of that specific infant [7]. This became the basic of this research to understand the effect of cigarette smoke exposure which is oxydant on the occurence of placental apoptosis and gestation outcome (congenital defect). A clearer understanding of placental apoptosis and its regulation may provide new insights into placental pathologies, potentially suggesting therapeutic targets.

MATERIALS AND METHODS

Materials

This research received ethical clearance released by Animal Care and Use Committee, Universitas Airlangga, Faculty of Veterinary Medicine. This study used 30 male and female white rat (Rattus norvegicus) aged 2-3 months obtained from Veterinaria Farma Surabaya. Research material, 30 male and female white rat aged 2-3 months. Hormone used for superovulation Pregnant Mare Serum Gonadotropim (PSMG) (Folligon™, Intervet, Boxmeer, Holland), and Human Chorionic Gonadotropin (HCG) (Chorulon™, Intervet, Boxmeer, Holland), clove cigarette, physiological NaCl. Research instruments: cage, scale, feed, sawdust, syringe, and cigarette smoke exposure cage.

Methods

Female white rat (Rattus norvegicus), weighing between 200 and 250 g, were used. An environmental adaptation was done for 7 days. On the 8th day, PMSG with a dosage of 10 IU/rat was injected into the female rat, and HCG injections with a dosage of 10 IU/rat was performed on the 10th day. Afterwards, the female rat were mated with the 12 weeks old male rat. In the 11th day, gestation examination was carried out. The gestation of the female rat was indicated by the visible mating plug covering the female rat vulva; and then the day was considered the first day of gestation.

Pregnant rats were divided into two groups of 15 animals each: experimental (smoking group) and control (non-smoking group). The animals of the smoking group were exposed to the smoke of five cigarettes per day, from the 6th to the 17th day of pregnancy, while kept inside a special acrylic chamber. The chamber had an entrance hole, where the cigarette was placed to burn, and an exit hole, where a suction bomb was connected to aspirate the smoke inside the chamber. The animals remained inside the chamber until the cigarettes were consumed. The control group was submitted to the same experimental conditions of the experimental group, but without exposure to smoke. The female rats not exposed to cigarette smoke were housed in individual cages in an environmentally controlled room and fed commercial ration and water ad libitum.

On the 18th day of pregnancy, the rats were sacrificed by inhalation of anesthetic ether and the fetuses and placentas were removed through a wide abdominal incision and immediately fixed in a solution of 85 mL 80% alcohol, 10 mL formalin and 5 mL glacial acetic acid. After 24 h of fixation, the fetuses and placenta were blotted dry and weighed in a precision balance. Five fetuses chosen at random from the smoking group and five from the control group were used in this study. The heads, separated from the bodies, were cut in half along the sagittal plane, embedded in paraffin, cut into serial 6 μm-thick sections and stained with Tunel Assay. A total of 25 µg/mL of proteinase K were applied to object glasses for 25 min and the glasses were washed in distillated water. Internal peroxidase activity was blocked by incubating object glasses in 3% H_2O_2 in absolute methanol for 5 min. The glasses were washed in PBS and incubated for an hour at 37 °C in digoxigenin-containing solution labelled with deoxy-UTP and terminal deoxynucleotidyl transferase. Then the glasses were washed in PBS and incubated for 30 min in solution containing anti-digoxigenin peroxidase. The glasses were washed in PBS for 5 min, incubated in diaminobenzidine solution (3, 3 '-Diaminobenzidine tetrahydrochloride, Sigma-Aldrich, Saint Louis, Missouri, USA), and counterstained with methyl green. Three slices of each sample were observed and examined by

microscope (Olympus® CX-41). The average number of syncytiotrophoblast cells expressing of apoptosis under a light microscope with 1000 magnification. The expression of apoptosis were identified by the color reactions that arise which was yellowish brown. Expression data of apoptosis and gross morphology of foetus (body weight, body length and foetus absorbtion) were analyzed using Mann-Whitney's nonparametric test. In facilitating statistical calculations, Statistical Product and Service Solution (SPSS) version 17.0 is used.

RESULT

Based on the result of the calculation with scoring on placental tropoblast cell using semiquantitative modification method Remmele Index Scale (RIS) which is a result of multipication between positive cell percentation score (A) with color reaction intensity score (B) [8], the result of analysis calculational Mann Whitney test on overall treatment obtained significantly different result 0.031<0.05 (p<0.05). In the One Sample T Test the weight appears significant 0.000<alfa score 0.05 means significantly different, the length of the body also appears significant 0.000<alfa score 0.05 means significantly different, while the foetus absorbtion showed significant score 0.006<alfa score 0.05 means significantly different.

Table 1: Average and Standard Deviations Variable of Length, Body Weihgt, Fetal Absorbtion and Placental Apoptosis

Variable	Control (non- smoking group) (means±SD)	Treatment (smoking group) (means±SD)
Foetus Body Weight	3,03 ± 0,59[a]	1,53 ± 0,52[b]
Foetus Body Length	3,47 ± 0,40[a]	2,08 ± 0,57[b]
Foetus Absorbtion	0,00 ± 0,00[a]	5,78 ± 3,60[b]
Placental Apoptosis	1,33 ± 1,22[a]	3,22 ± 2,28[b]

Note: Superscript which is different in the same row is significantly different (p ≤ 0,05)

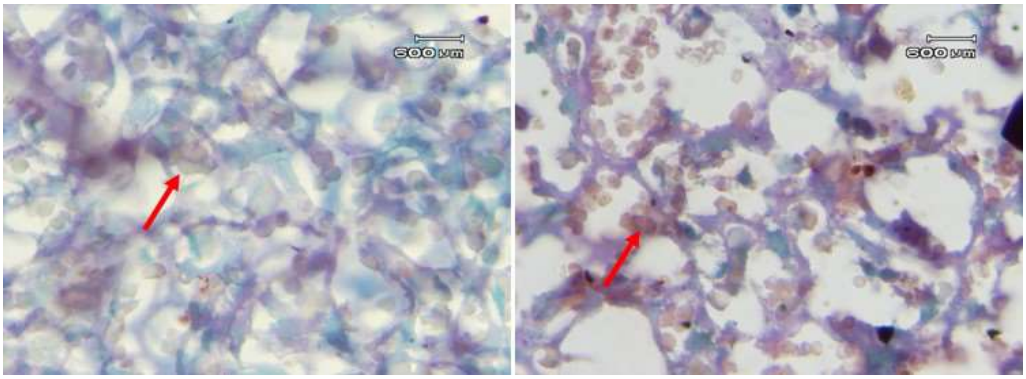

Fig.1: Apoptotic expression is shown by light brown to dark brown in immunoreactive cells. On the left slide (non-smoking group) showed apoptosis with moderate intensity found in syncytiotrophoblast cells (arrows) with medium brown color and the right slide (smoking group) showed apoptosis with strong intensity found on syncytiotrophoblast cells (arrows) with color dark brown (Tunel Assay; 1000x magnification; Olympus (Olympus® CX-41). Pentax optic 230; megapixel digital camera 2.0).

DISCUSSION

One way to see the disturbance of gestation caused by cigarette smoke exposure is by looking at the cell apoptosis degree on placenta and one of the indicators are the presence of apoptec antibody expression in placental cell. The presence of apoptec antibody can prove the occurenceof apoptosis on placental celland is done using the immunohistochemical technique. Cigarette smoke contains nicotine, cadmium, bensopiren, oxidant, aceton, mercury thatcan induce the occurence of Reactive Oxygen Species (ROS). The side effect of cigarette usage not only happens to the smoke, but also to the bystanders also known as passive smoker. So many health disorders occured and the pathophysiological of those disorders implicate oxidative stress triggered by cigarette.

One cigarette contains 10^{17} molecule of oxydant. Oxydative stress can be acknowledged from various symptoms, also using oxydative agent direct measurement such as ROS on peripheral

blood cell, or using oxydative stress effect measurement on targeted molecule (peroxidation lipid product and oxidatedprotein) [9]. On healthy condition ROS and antioxidant is always on a balanced condition. But if that balance is disturbed, increasing of ROS will occur that outnumber the antioxidant and there will be oxidative stress condition. This increasing is also caused by the lacking of ROS elimination. Oxidative stress acts in causing reproduction process disturbance from the oocyte maturation until conception, even from embryo developmentuntil fertilization. Oxidative stress is also related to the increasing of infertility related to someone's reproductive age. Oxidative stress can also happen on gestation and birth period. Thus causes an increase on placental apoptosis on white rat exposed to cigarette smoke. In normal condition, placental apoptosis happens in the end of gestation period, but since getteing the cigarette smoke exposure and increasing amount of ROS in the body so apoptosis occurs faster. Balanced placental apoptosis plays a crucial role in pregnancy continuation, apoptotic p53 protein over-expression decreased anti-apoptotic Bcl-2 expression in placental tissue during early pregnancy, could lead to pregnancy failure [10]. This is also consistent with the research previously that mentioned about the pathogenesis of abortus [11].

Body weight is a important parametre to find out the effect of foreign subtance due to the foetus, it showed by decreasing body weight of the foetus.The result of analysis test for body weight variable and lenght variable of the white rats's foetus obtain significantly means cigarete smoke exposure treatment can provide a significant diference at average of body weight and the lenght of the foetus on the treatment group. The result of this research consistent with Wilson's opinion, that external congenital deffect can caused decreasing the body weight of the foetus, because the decreasing body weight of the foetus showed the abnormality of growth either in human or laboratory animals. Decreasing body weight and lenght of the foetus are the lightest form of teratogenic effect and a sensitive parametre individual developmental disorder in the uterus caused several deffect, such as abnormally body weight birth. Decreasing body wight and the lenght of the foetus are the indication of fetal growth retardation. Fetal growth retardation can occur if the agent affecting cell proliferation, cell interaction,and decreasing of biosyntesis rate related with nucleic acid syntesis inhibition, protein, or mucopolysaccarida [12]. Analysis test between control group and treatment group has significant result ($p<0.05$) this is means that cigarete smoke exposure provide significant diference the amount of absorbed foetus. The result of this research consistent that higher the level of dose on the embriotoxic dose range, will result in higher level response, ranging from growth retardation, malformations, until intrauterine death and resorption [13].

Results external congenital defect data in the group treated with the control group there were significant differences in fetal weight, fetal body length, and fetal absorption, but there is no difference in the eyes and finger abnormalities. This is not in line with the opinion of [14] that the 10-day gestation in mice (Mus musculus) is a critical period of organogenesis, palate, and a member of sense organs (the formation of the eyes, ears, and nose) as well as axial, and skeleton [15].

The results are consistent with the [16], which is absorbed and the dead fetus in the womb has not finished experiencing growth and confirms that exposure to cigarette smoke in rats parent will be entered into the fetus through the placenta after the parent and is metabolized in the liver into the blood circulation has embryotoxic effects. Embryotoxic effects of cigarette smoke exposure in this research is fetal resorption.

Absorption fetus was found in the form of early embryonic death as indicated by the presence of residual embryonic tissue in the uterus. Fetal death, fetal resorption and that life does not happen to any parent dealing with the different abilities of each parent to metabolize cigarette smoke exposure. This is an indication of growth disturbance. From the observations in the field, proved that individuals who experience malformations are generally smaller when compared to normal individuals. Weight of the animal must be compared with the controls used to ensure that the growth inhibition reflects the general growth inhibition.

This is consistent with a study published by the U.S. Centers for Disease Control and Prevention noted that while the rate of smoking among pregnant women in the Western world have fallen in recent years, smoking remains the leading cause of newborn death, premature birth and birth with low birth weight. The researchers found that smoking was associated with: 5-8% premature birth, 13-19% cases of low birth weight in babies carried to full term, 5-7% related premature deaths, 23-34% deaths from sudden infant death syndrome (cot death) [17]. In the UK, smoking during pregnancy causes up to 5,000 miscarriages, 300 peri-natal deaths and about 2,200 premature births each year. Conclusion this research that giving cigarette smoke exposure

can increase the incidence of placental apoptosis in gestation disorder mechanism in white rats (Rattus norvegicus). Giving exposure to cigarette smoke is proven to cause external congenital defects in white rats of weight loss, fetal body length, increased incidence of abortion.

CONCLUSION

This research is the exposure of cigarette smoke can increase the occurence of placental apoptosis on the gestation disorder mechanism on white rat (Rattus novergicus) also proved that can cause external congenitaldisorder on white rat such as body weight loss, foetus body length loss, increasing occurence of abortus.

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