Effects of Maternal Smoking on Fetal Organs

Wpływ palenia tytoniu przez matkę na narządy wewnętrzne płodu

Background. Unfavorable effects of in-utero smoke exposure have been shown in several studies.

Objectives. In this experimental study, the authors aimed at showing detrimental effects of cigarette smoke on fetal tissues by assessing apoptosis that is detected by performing TUNEL staining.

Material and Methods. Designed groups were smoke exposed rats before and during pregnancy and control groups. Rat offsprings were sacrificed when they were 12 days old.

Results. Lung, kidney, adrenal and gonad tissues were harvested for histopathologic analysis and assessed by TUNEL (Terminal dUTP Nick End Labeling) staining.


Key words: cigarette smoking, lung, apoptosis.

Streszczenie

Wprowadzenie. Niekorzystne skutki wewnątrzmacicznej ekspozycji płodu na dym papierosowy wykazano w wielu badaniach.

Cel pracy. W badaniu doświadczalnym autorzy mieli na celu pokazanie negatywnego wpływu dymu papierosowego na tkanki płodu przez ocenę apoptozy wykrywanej za pomocą barwienia metodą TUNEL.

Materiał i metody. Do badania włączono szczury narażone na dym tytoniowy przed i w czasie ciąży oraz grupę kontrolną. Potomstwo szczurów uśmiercono, gdy miało 12 dni.

 Wyniki. Pobrano tkanki płuc, nerek, nadnerczy i gonad do analizy histopatologicznej i oceniono za pomocą barwienia metodą TUNEL (Terminal dUTP Nick End Labeling).


Słowa kluczowe: palenie tytoniu, płuc, apoptoza.

Cigarette smoking has many detrimental effects on several organ systems and the leading cause of mortality and morbidity in the world [1]. Smoking has been associated with carcinoogenesis, cardiovascular diseases and other organ disorders [2]. The relationship between cigarette smoking and lung cancer is particularly well established [3]. Ramage et al. pointed out: “Tobacco smoke is a mixture of more than 4000 components including carcinogens, oxidants and aldehydes, all of which have the potential to cause inflammation and damage cells” [4]. Carnevali et al. wrote: “Among the different toxic effects of cigarette smoke on human tissues, oxidation of structural and functional molecules and modulation of cell turnover play a major role” [5].
Prevention (CDC) reported: “Cigarette smoking during pregnancy adversely affects the health of both mother and child. The risk of developing premature rupture of membranes, abruptio placenta, placenta previa, preterm delivery, neonatal mortality, stillbirth, and sudden infant death syndrome is increased by maternal smoking” [6]. The mechanisms how maternal smoking affects the fetus remains unclear.

Carnevali et al. noted: “Apoptosis is a form of cell death that occurs under several physiological and pathological situations, and it represents a common mechanism of cell replacement, tissue remodeling, and removal of damaged cells. Apoptosis may occur spontaneously or in response to specific stimuli such as heat stress, radiation, steroids, and oxidative stress” [5]. Changes in the rate of apoptosis may lead to diseases that are characterized by abnormalities of tissue growth [7, 8]. Reactive oxygen species (ROS), highly reactive and generally nonspecific molecules, are widely reported to trigger apoptosis [7, 9]. In addition, cigarette smoke is a main source of free radicals and it may activate endogenous ROS-related enzymes [10, 11].

The aim of this study was to evaluate the effects of cigarette smoke on fetal tissues by assessing degree of apoptosis in rat offspring exposed to cigarette smoke before and during pregnancy.

Material and Methods

Animals and Study Design

Approval for the study was secured from Gulhane Military Medical Academy Hospital Ethical Committee. Female Wistar rats (weight 200 ± 20 g) obtained from Gulhane Military Medical Academy Research Center. Designed groups were as follows: smoke exposed rats before and during pregnancy (Group 1); smoke exposed rats only before pregnancy (Group 2); and controls without smoke exposure (Group 3). All the groups were consisted of 2 pregnant rats and 4 rat offsprings of every mother were randomly assigned after birth to provide homogeneity and minimize genetic factors.

In group 1 mature female rat was exposed to smoke for one hour twice a day for 12 weeks before pregnancy in a glass cabinet. After they became pregnant, smoke exposure was continued until delivery. In group 2 mature female rats were exposed to smoke for one hour twice a day for 12 weeks before pregnancy in a glass cabinet. Smoke exposure was discontinued when they became pregnant. The 8 pups in each group were sacrificed when they were 12-day-old following anesthesia by using an anesthetic chamber.

Smoke Inhalation System

A tobacco smoke inhalation system was designed by Gulhane Military Medical Academy Research and Biomedical Centers for this study. It was consisting of vacuum motor/blower with a closed system, a leak proof glass cabinet with 50 × 40 × 40 cm dimensions and 0.08 m³ volume, a fish tank motor to evacuate the smoke and a cigarette apparatus with a tube to inhale smoke into the cabinet.

Tobacco Smoke Procedure

The rats exposed to tobacco smoke were put into glass cabinet with individual cages in a period 1 hour in the morning and one hour in the evening every five days of week with a duration of 12 weeks. The rats were kept at room temperature in a natural day/night cycle and were permitted to eat standard rat chow and drink tap water ad libitum when they were out of the glass cabinet.

Histopathologic Examination

The terminal deoxynucleotidyl transferase (dUTP) nick-end labeling (TUNEL) technique was applied, using the “ApopTag Plus Peroxidase In Situ Apoptosis Detection Kit” (Millipore, Billerica, MA, USA), according to the manufacturer’s instructions, to determine the extent of cell death in tissue sections. The fetal tissues were put in 10% formaldehyde for fixation and after routine tissue processing, embedded in paraffin, cut into 5 µm sections and deparaffinized by 3 changes of xylene for 5 min each wash. Sections were next dehydrated in a graded series of ethanol concentrations (100–70%) and washed in one change of PBS for 5 min. For pretreatment, proteinase K (20 μg mL⁻¹) was applied for 15 min at room temperature and washed in two changes of deionized water in a coplin jar for 5 min each wash. Sections were next dehydrated in a graded series of ethanol concentrations (100–70%) and washed in one change of PBS for 5 min. For pretreatment, proteinase K (20 µg mL⁻¹) was applied for 15 min at room temperature and washed in two changes of deionized water in a coplin jar for 2 min each wash. Tissue was quenched in 3.0% hydrogen peroxide in phosphate buffered saline (PBS) for 5 min at room temperature and rinsed twice with PBS for 5 min each time in a coplin jar. After application of equilibration buffer for at least 10 sec at room temperature (RT), tissue was incubated with terminal deoxynucleotidyl transferase (TdT) enzyme in a humidified chamber at 37°C for 1 hour. The sections were then placed in TdT stop buffer for 10 min, washed again with PBS, incubated for 30 min at RT in anti-digoxigenin conjugate solution and washed with PBS. Peroxidase substrate was applied for 5 min at RT monitoring the color development. After washing with deionized water, tissues were counterstained with methyl green. Slides were washed again with deionized water and then 3 changes of 100%
N-Butanol approximately 1 min. The sections were mounted on slides with mounting media and cover slipped.

**Evaluation**

Apoptotic Cell Counting: Apoptotic cell counting was performed in lung, kidney, adrenal and gonads. All of the stained slides for each case were reviewed by two pathologists who were unaware of the characteristics of animals. The nuclear staining was accepted as positive. In evaluating numeric density, first, slides reviewed at low power magnification for the selection of the most intensely stained area. Then, in this area ten randomly selected high power fields were scanned and total TUNEL positive stained cells were counted (at ×400 magnification) under the light microscope. The mean of positive stained cells per high power field was accepted as the apoptosis score for each case.

**Statistical Analysis**

Statistical analyses were performed using the SPSS software version 15. The data were expressed as median (min, max). The Mann-Whitney U test was used to compare apoptosis scores between the groups. A p-value less than 0.05 were considered to show a statistical significant result.

**Results**

There were 13 live births and 3 stillbirths from the mothers of study group. There was no stillbirth in control group. The gas compound levels in the glass cabinet formed by one cigarette smoke resulted in considerable higher amounts than their atmospheric reference values. There was a statistically significant difference between groups for apoptosis scores of lung (p = 0.003) (Fig. 1). However, there was no statistically significant difference between groups for apoptosis scores of kidney, adrenal and gonads of rat offspring (p > 0.05). Exposure to cigarette smoke increased apoptosis in fetal lungs. TUNEL-positive apoptotic cells of lung tissue in each group are shown in Fig. 2.

**Discussion**

Cigarette smoke induces oxidative stress and inflammation in many tissues and cells, both in vitro and in vivo. It contains over 4,000 chemical species, including high concentrations of oxidants [5]. Authors’ goal was to examine the effects of cigarette smoke exposure on apoptosis in the fetal organ systems. For this purpose, the effects of cigarette smoking on different tissues were assessed by evaluating the degree of apoptosis in lung, kidney, adrenal and gonad tissues of rat pups. In the present study, cigarette smoke significantly increased the degree of apoptosis in fetal lung tissue. The authors could not detect any evidence of increased apoptosis in fetal tissues such as kidney, adrenal, and gonads exposed to cigarette smoking.

Sufficient evidences indicate that ROS play an important part in apoptosis under various conditions [7, 9]. There are many free radicals in gas phase of fresh smoke, including nitric oxide (NO), nitrogen oxides (NOx), hydrogen peroxide (H2O2), carbon monoxide (CO), and others [12, 13]. It has been proven that ischemia is a main source of ROS.
in vivo [14]. There are several mechanisms related to harmful effects of maternal cigarette smoking. It may have an indirect effect on deterioration of blood flow of umbilical vessels and placenta and transfer of chemical toxins to fetus directly by placental flow [15]. It has been shown that cotinine concentrations in placental tissue, amniotic fluid and fetal serum were similar to corresponding maternal serum levels [16]. The authors proposed that placental transfer of chemicals in cigarette smoke causes an increase in free oxygen radicals in fetal tissues which may lead to an increase in fetal lung tissue apoptosis. Wang et al. detected an increase in apoptotic cells in gastric mucosa of rats exposed to cigarette smoke [1]. They reported: “Activation of ROS may play an important role in this caspase-3-mediated apoptotic process”. Carnaval et al. reported increased apoptosis in human lung fibroblasts after exposure to cigarette smoke associated with an increase in oxidative stress [5]. They speculated that development of pulmonary emphysema may be the result of both cigarette smoke related apoptosis and oxidation of fibroblasts in lung. Majority of the studies on fetal exposure to cigarette smoke have investigated the atherosclerosis associated changes in fetal blood vessels. In most of these studies fetal exposure to cigarette smoke has been shown to cause preatherosclerotic changes in fetal coronary and other arteries [17–20].

There are few studies evaluating the effects of cigarette smoke exposure on apoptosis in fetal tissues. Izzotti et al. reported: “Transplacental extract of cigarette smoke (ECS) can induce genotoxic damage in mouse fetus liver along with a variety of alterations in gene expression” [21]. In another study it is shown that ECS causes human umbilical vein endothelial injury by inducing excessive apoptosis mediated through a p53-independent caspase-3-activating pathway [22, 23]. In the present study, exposure to cigarette smoke significantly increased apoptosis in fetal lungs. However, there was no significant increase in apoptotic cells of fetal kidney, adrenal and gonads.

Chemicals in cigarette smoke are known to reach fetal plasma and amniotic fluid through transplacentally [16]. Especially acute hypoxia during smoking can lead to fetal gasping which may
cause fetal lungs to directly contact with amniotic fluid. During this process, lung tissue may be more vulnerable to oxidative damage that may lead to increased apoptosis than other organ systems. Kidneys may be preserved from damage throughout intrauterine period, because of low renal blood flow and less glomerular filtration rate. Gonads were protected from cigarette related damage by the way of blood-testis barrier and adrenals may also be protected by a similar mechanism. Cigarette smoke may affect fetal lung tissue by two different mechanisms. One of them is fetal pulmonary circulation and the other is direct contact with amniotic fluid. However it is well known that only 10% of pulmonary artery blood flow passes through the lungs in intrauterine life. Therefore the authors suppose that intrauterine lung damage may be originated from direct contact of lungs with amniotic fluid rather than pulmonary artery circulation.

The authors concluded that increased apoptosis in lung tissue suggest that particularly fetal lung is more susceptible to the effects of intrauterine exposure to smoking. Lack of significant damage on kidney, adrenal and gonads in terms of apoptosis may indicate that intrauterine cigarette smoke exposure may have effects of different degrees on fetal organs.

References


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