Effects of Maternal Nicotine Exposure on Expression of Collagen Type IV and its Roles on Pulmonary Bronchogenesis and Alveolarization in Newborn Mice

Mehdi Jalali1, Mohammad Reza Nikravesh1, Abbas Ali Moeen2, Shabnam Mohammadi1, and Mohammad Hasan Karimfar2

1 Department of Anatomy, School of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran
2 Department of Anatomy, School of Medicine, Zabol University of Medical Sciences, Zabol, Iran

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ABSTRACT

Nicotine is one the chemical substance with high level of toxically. It crosses the placenta and accumulates in the developing organs of fetus. Our previous investigations indicated that collagen type IV plays a key role in basement membrane of various embryonic organs. In this study we evaluated the effect of maternal nicotine exposure pre and postnatal period on collagen IV expression during bronchogenesis and alveolarization in the lungs of newborn mice. Female Balb/C mice were mated and Sperm positive in vaginal smear was designated as embryonic day zero. Pregnant mice were divided into 2 experimental and 2 control groups.

Experimental group 1, received 3 mg/kg nicotine intrapritoneally from day 5 of gestation to last day of pregnancy. Experimental group 2 received the same amount of nicotine during the same gestational days as well as 2 first week after birth (lactation). The control groups received the same volume of normal saline during the same periods. At the end of exposure times, all of newborns were anesthetized and their lungs were removed for immunohistochemical method.

Our finding indicated that collagen reaction in the bronchial basement membrane and extra cellular matrix of lung parenchyma in experimental groups increased significantly compared to control groups. Our results also showed alveolar remodeling and abnormal bronchogenesis were observed in experimental group especially group 2.

These data indicate that maternal nicotine exposure may induce abnormal collagen IV expression and cause defects in bronchopulmonary development.

Key words: Aveolarization; Bronchogenesis; Collagen IV; Mouse; Nicotine

INTRODUCION

Unlike other body systems, the lung development is important as a gas exchanger. The lung should to develop in uterus and prepared to function at birth but similar to other mammals, final stages of its development do not complete until after birth. The lung development is divided into several stages. The natural process of lung development is important due to its future role as a system of gaseous exchange.
Disturbance the developmental stages of the lung may affect the lung maturation and resistance to disease in future life.\textsuperscript{1-4} Mesenchyme cells direct the cytoskeleton and growth tissue by growth factors and differentiation.\textsuperscript{5}

Investigation have been shown a direct correlation between blood-gas-barrier and mechanical stretch due to breathing movements.\textsuperscript{6-7} The previous studies also showed that mechanical stretch prevents from the proliferation of fibroblasts and induces apoptosis during the canalicular stage and causes defects in lung development.

Hence, any changes in extra cellular matrix of alveolar may affect cell differentiation.\textsuperscript{8-10} Extracellular matrix contains various parts such as reticular fibers, glycoprotein, proteoglycans and glycosaminoglycans. Collagen especially collagen type IV is the most important protein of the basement membrane. As nicotine crosses the placenta barrier and occurs in the mother’s milk offspring rats during lung development it causes adverse effects on connective tissue of lung. It is necessary to study the effects of nicotine exposure on lung connective tissue development of the offspring especially collagen type IV.

**MATERIALS AND METHODS**

**Nicotine Administration and Tissue Preparation**

After purchasing from the Razi institute, 24 female Balbc/c mice were randomly divided into 2 experimental and 2 control groups. Sperm positive in vaginal smear was designated as embryonic day zero. The environmental conditions were 22±1°C and 12 hr light-dark cycle with free access to water and food. The experimental group1 injected daily intraperitoneal dose of 3 mg/kg of nicotine from day 5 of gestation to last day of pregnancy\textsuperscript{11} and experimental group 2 were received nicotine for two weeks postnatal.

The control groups were received nicotine solvent (Normal saline). Finally, the animals were rapidly sacrificed by cervical dislocation and the lung of mice were removed and fixed for 24 hours at room temperature in formaldehyde 10% and used for immunohistochemistry method.

**Immunohistochemistry Study**

After being raised twice in 0.05 Tris buffer, the sections were blocked in 0.3% Triton X-100 in TB-NaCl and 5% goat serum for 1-2 hr. Then slides were incubated for 12-24 hr at 4 °C with monoclonal anti-collagen IV antibody (sigma, Germany) diluted 1: 50 in TB-NaCl with 0.3% Triton and 2% serum. Samples were washed three times with TB-NaCl and incubated in biotinylated goat anti-rabbit IgG for 2 hr. After three further rinses, for blocking endogenous peroxidase activity the slides incubated in 0.03% H2O2 in methanol for 30 min. Then, tissues were incubated for 2 hr in 1:100 avidin-biotinylated horseradish peroxidase complexes. After being raised three times in 0.05 Tris buffer, samples visualized with 0.03% solution of 3,3-diaminobenzidine for 10-15 min. Slides were washed and counterstained with hematoxylin. Tissues were immersed in glycerol and were evaluated by a microscope. Firth’s method was used for grade staining collagen.\textsuperscript{12} Different fields of the slides were captured by an Olympus (Japan). Collagen reaction in BM of alveolus and lung parenchyma was graded by two separate observers.

 Besides, the alveoli and bronchioles of the lung offspring were counted, using morphometric method.\textsuperscript{13} For this purpose, the serial sections from lungs of each group were studied with light microscope. The counting carried out by placing a scaled square (Figure 1) over the lens of microscope. Then, one field out of each four fields was studied by displacing the samples under microscope. Alveolar numbers in unit volume were obtained by counting the thickness of sections and the removed serial sections. Also, the greatest alveolar diameters were measured.

**Statistical Analysis**

Data were analyzed using the SPSS software by Kruskal-Wallis and Mann-Whitney test. The results were considered significant were \( p<0.05 \).

**RESULTS**

Tracing of collagen in different parts of the lung indicated that collagen reaction in BM of bronchioles observed light brown (Figure 2a). These reactions increased to dark brown in the alveolar basement membrane in experimental groups but did not indicate significant change in experimental groups when compared to the control groups (Figure 2b).

Morphometric studies and microscopic observations of bronchioles in experimental group 2 recognized that alveolar diameter decreased while compared with the
control group and canalization did not take place well (Figure 2b vs Figure 2a).

Mesenchyme cells have observed in some bronchioles as an undifferentiated cellular mass and while canalization completed in other areas but there were closed bronchioles in these regions (Figure 2b). Our finding indicated that collagen reaction increased in alveolar basement membrane and ECM of lung alveolar of experimental groups (Table 1) and although this reaction did not show significant change in experimental groups (experimental groups 1,2), the collagen reaction showed remarkable increased in compared to the control groups (Figure 2c-2f). Morphometric studies distinguished that the alveolar numbers of the lung increased in the control samples in contrast with the experimental samples (Table 1). Besides, the mean alveolar number in the control group increased significantly when compared to the experimental group (Table 1).

Figure 1. Section through the lung parenchyma which scaled square has been shown over it and the alveoli wall were counted which were situated into the scaled square. (measurements were calculated according to micrometer).

Figure 2. Sections through the bronchial epithelium and the alveolar lung which are incubated against collagen type IV antibody. In these images, (a) section through bronchiol and parenchymal space in control group 2 (circle). (b) Similar section in experimental group 2 that have remained some blocked regions (arrowsheads). Images the lung alveolar in control groups 1, 2 (c, d) that were observed partitioning of the primitive alveolar and alveolar formation (asterisk), while in experimental group 1,2 (e, f) were detected interruption of the alveolar walls and increasing the alveolar air volume (asterisks). (scale bar=100 µm, Haematoxylin counterstained).
DISCUSSION

Our previous studies showed that collagen type IV is one of the most abundant proteins of BM of lung epithelium and Extra Cellular Matrix. This protein plays important roles in differentiation and cell interactions during embryonic period as well as after birth. Our findings indicated that there is a significant difference in the lung collagen of the offspring that consist with other studies. On the other hand, the data indicated that maternal nicotine exposure may disturbance collagen expression in respiratory system which leads to abnormal bronchogenesis in next generation. Besides, our data showed that the mean alveolar diameter in experimental group increased in compared to the control groups. But the alveolar number per volume unit of the lung in experimental groups especially experimental group 2 decreased significantly in compared to the control groups. This data indicate that the newborns which were exposed to nicotine via the placenta and mother's milk are more susceptible to damages that remarkable its signs are alterations collagen type IV, cell necrosis and defects in the alveolar sepa and lung surface area reduction. Also, our previous investigation indicated that formation of various embryonic organs is dependent on collagen type IV and confirm the key role of this protein. It seems that any teratogenic factor such as nicotine can effect on gene expression α-chains and leads to disruption in differentiation. Hence, it’s clear that collagen type IV is vital protein of basement membrane and extra cellular matrix in various tissues as well as lung parenchyma. It seems that any factor which affects collagen regulation during lung development may put at risk the normal health of the respiratory system. The results of this study also indicated that maternal nicotine exposure may change bronchogenesis and alveolarization in the lung of the mouse offspring. It have reported that maternal exposure to nicotine during pregnancy leads to disorders in the alveolar development that have been attributed to decreased lysyl oxidase enzyme. The findings show that lysyl oxidase enzyme is necessary for cross-linking of elastic and collagen fibers. Sekhon et al reported that maternal nicotine exposure effects on health of the respiratory system of the offspring and may lead to alterations in airway structure such as increasing collagen fibers.

It concludes that nicotine effects on signaling pathway that causes differentiation of lipofibroblast to myofibroblast. Hence, this phenomenon effects on alveolarization process, which leads to defects in lung development. Considering, lipofibroblast protects pneumocyte against free radicals and also its important role in myofibroblast differentiation suggests that nicotine may disturb genes expression of the lung and as a result causes incidence of defects in the lung of the offspring.

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