ORIGINAL ARTICLE

Tanaffos (2011) 10(2), 32-37

Maternal Nicotine Induces Collagen Type IV Changes in the Mice Lung Parenchyma and its Vessels

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ABSTRACT

Background: *One of the undesirable effects of maternal nicotine exposure during pregnancy is pulmonary hypertension. Since nicotine binds to its receptors on pulmonary vessels the hypothesis of this research was the possible structural changes that nicotine may cause on newborn vessels.*

Materials and Methods: *Twenty-four female BALB/c mice were mated and finding vaginal plug was assumed as day zero of pregnancy. Pregnant mice were divided into 2 experimental and 2 control groups. Experimental group 1 received 3 mg/kg nicotine intraperitoneally from day 5 of gestation until the last day of pregnancy. Experimental group 2 received the same amount of nicotine during the same gestational days as well as the first 2 weeks after birth (lactation). The control groups received the same volume of normal saline during the same periods. At the end of exposure times, all the newborns (experimental and control) were anesthetized, their lungs were removed and immunohistochemical studies were carried out for tracing collagen.*

Results: *Our findings indicated that collagen reaction in the bronchial basement membrane (BBM) and extracellular matrix (ECM) of the lung parenchyma in experimental groups increased significantly compared to the control groups but these changes were not observed in BM of lung vessels in the experimental groups.*

Conclusion: *These data indicate that nicotine exposure during pregnancy does not cause a significant change in collagen type IV in BM of lung vessels. But this does not mean that other types of collagen fibers do not indicate change because the wall thickness of pulmonary vessels in experimental groups increased significantly compared to the control groups.* ***(Tanaffos2011; 10(2): 32-37)***

Key words: *Nicotine, Collagen type IV, Lung vessels, Mouse*

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Received: 16 July 2010

Accepted: 23 February 2011

INTRODUCTION

The lungs should develop in the uterus and be prepared to function at birth but similar to other mammals, final stages of its development do not complete until after birth. Studies have shown that the natural process of lung development is important due to its future role as a system of gaseous exchange. Disturbance of the lung developmental stages may affect lung maturation and resistance and lead to diseases in the future (1-4).

Along with the appearance of lung buds and bronchogenesis, angiogenesis must happen for the nutrition of the lung parenchyma and as a pathway of circulatory system (5).

Mesenchymal cells guide the cytoskeleton by growth factors during angiogenesis (6,7).

Since expansion of vascular connective tissue is necessary for tissue development, nicotine may affect lung development through vessels alterations.

Studies have shown that mechanical stretch prevents from the proliferation of fibroblasts and induces apoptosis during the canalicular stage; it also causes changes in angiogenesis (8, 9). This shows that apoptosis naturally happens during the lung development and the effect of factors on apoptosis may lead to defects in lung development as well as some changes in vessels' structure (10).

Previous studies have shown that BM of lung vessels and extracellular matrix comprise different molecules such as collagen fibers, glycoproteins, proteoglycans and glycosaminoglycans that among them, collagen especially type IV is the most abundant composition (11-15). Considering all the above, fetal nicotine exposures via placenta during embryonic period and through mother's milk after birth may have adverse effects on the development of lung connective tissue.

This study aimed to investigate the effects of nicotine exposure on basement membrane of lung parenchyma and pulmonary vessels.

MATERIALS AND METHODS

1- Nicotine administration and tissue preparation

Twenty-four virgin female BALB/c mice with 35 gram body weight were obtained from the animal house of Mashhad University of Medical Sciences and were divided randomly into 2 experimental and 2 control groups. Finding vaginal plug was designated as day zero of pregnancy. The environmental conditions were 22±1°C temperature, relative humidity of 50-55% and 12 hr light-dark cycle with free access to water and food. The experimental group1 received daily intraperitoneal injection of 3 *mg/kg* nicotine (Sigma company) from day 5 of gestation until the last day of pregnancy (16) and experimental group 2 received nicotine for two weeks postnatal. The control groups received nicotine solvent (normal saline). Then, the animals were sacrificed by cervical dislocation and the lungs of mice were removed and fixed for 24 hours in formaldehyde 10% and immunohistochemistry technique was used for tracing collagen type IV.

2-Immunohistochemistry study

The Avidin-Biotin peroxidase procedure was used for immunohistochemistry studies. All samples were fixed and placed in paraffin blocks (Merck, Germany) and sectioned serially at a thickness of 5 µm. After deparaffination and rehydration, sections of lungs were washed twice for 5 min with Tris buffer (containing 1.5% sodium chloride at PH=7). For blocking nonspecific antibody, sections were preincubated in 0.3% Triton X-100 in TB-NaCl followed by 5% goat serum (GIBCO, UK) for 1-2 hr. Then sections were reacted for 12-24 hr at 4 °C with primary antibody (anti-collagen IV) (Sigma Aldrich) and diluted 1: 50 in TB-NaCl with 0.3% Triton and 2% serum. Tissues were washed with TB-NaCl for three times, each time for10 min and incubated for 2 hr in biotinylated goat anti-rabbit IgG (1:400 in TB-NaCl). After three further rinses, each for 1 hr,

endogenous peroxidase activity was blocked by incubation in 0.03% H_2O_2 in methanol for 30 minutes. Tissues were incubated for 2 hr in 1:100 avidin-biotinylated horseradish peroxidase complex. Then they were washed three times, each time for 30 min in TB-NaCl and finally reacted with 0.03% solution of 3,3-diaminobenzidine tetrahydrochloride for 10-15 min. Tissues containing 0.03% H2O2 were washed and lightly counterstained with hematoxylin. Subsequently, they were washed and mounted in PBS glycerol (Merck, Germany) and were evaluated by a microscope. Because collagen immunoreaction is a proper index for determination of its density, Firth's method was used for grade staining (12). Images of different regions of the lungs were captured by a light microscope (Olympus BH-2) and collagen reaction in BM of alveolus and lung parenchyma was graded by two separate individuals.

Besides, the alveoli and bronchioles of the offspring lungs were counted, using morphometric method (17). For this purpose, serial sections from the lungs of each group were studied with light microscope. By putting a scaled square (Figure1) over the lens of microscope, a specific unit for measuring microscopic field was designed. Then, one field out of each four fields was studied by displacing the samples under the microscope. Alveolar numbers in unit volume were obtained by measuring the thickness of sections and the removed serial sections. Also, the greatest alveolar diameters were measured.

3-Statistical Analyses

The data were analyzed using SPSS software and Kruskal-Wallis and Mann-Whitney U tests. The results were expressed as mean ± SD and differences less than 0.05 were considered statistically significant.

RESULTS

Tracing of collagen in different parts of the offspring lung indicated that reaction of this protein was not significant in experimental groups compared to controls and there was a weak reaction in all samples (Figures 1a, 1d). The only difference observed in the experimental groups was accumulation of vessels per unit volume that remarkably increased compared to the control groups but no significant change was observed in experimental groups compared to controls (Table1). Besides, wall thickness of pulmonary vessels increased significantly in experimental groups compared to controls and these changes were related to adventitia and media (Figures 1e, 1f).

Tracing of collagen in the lungs of different groups indicated that type IV collagen reaction in the alveolar basement membrane of the control groups appears light brown. These reactions appeared dark brown in the alveolar basement membrane of experimental groups and significant changes were observed in experimental groups compared to the control groups (Figures 1a, 1b).

Table 1. Comparison between lung parenchyma parameters in the experimental and control groups (counting and measurement were done with the magnification of 20 in 100 random fields from each group). The intensity of collagen reaction was rated from weak to strong.

Variables	Control 1	Experimental 1	Control 2	Experimental 2
Vessel sections per unit volume (mm^3) (Mean ±SD)	1.01×10^2 ±11.61	3.64×10^2 ±23.24 *	1.16×10^2 ±26.34	3.71×10^2 ±31.32 *
The greatest alveolar diameter (μm) (Mean ±SD)	3.81×12.11±4.12	4.13×17.22±6.64	8.22×11.22±5.16	11.56×21.28±5.37 *
Type IV collagen reaction in BM of bronchioles	30	30	30	40
Type IV collagen reaction in BM of alveoli and matrix	20	30	20	30 *

* $p<0.05$

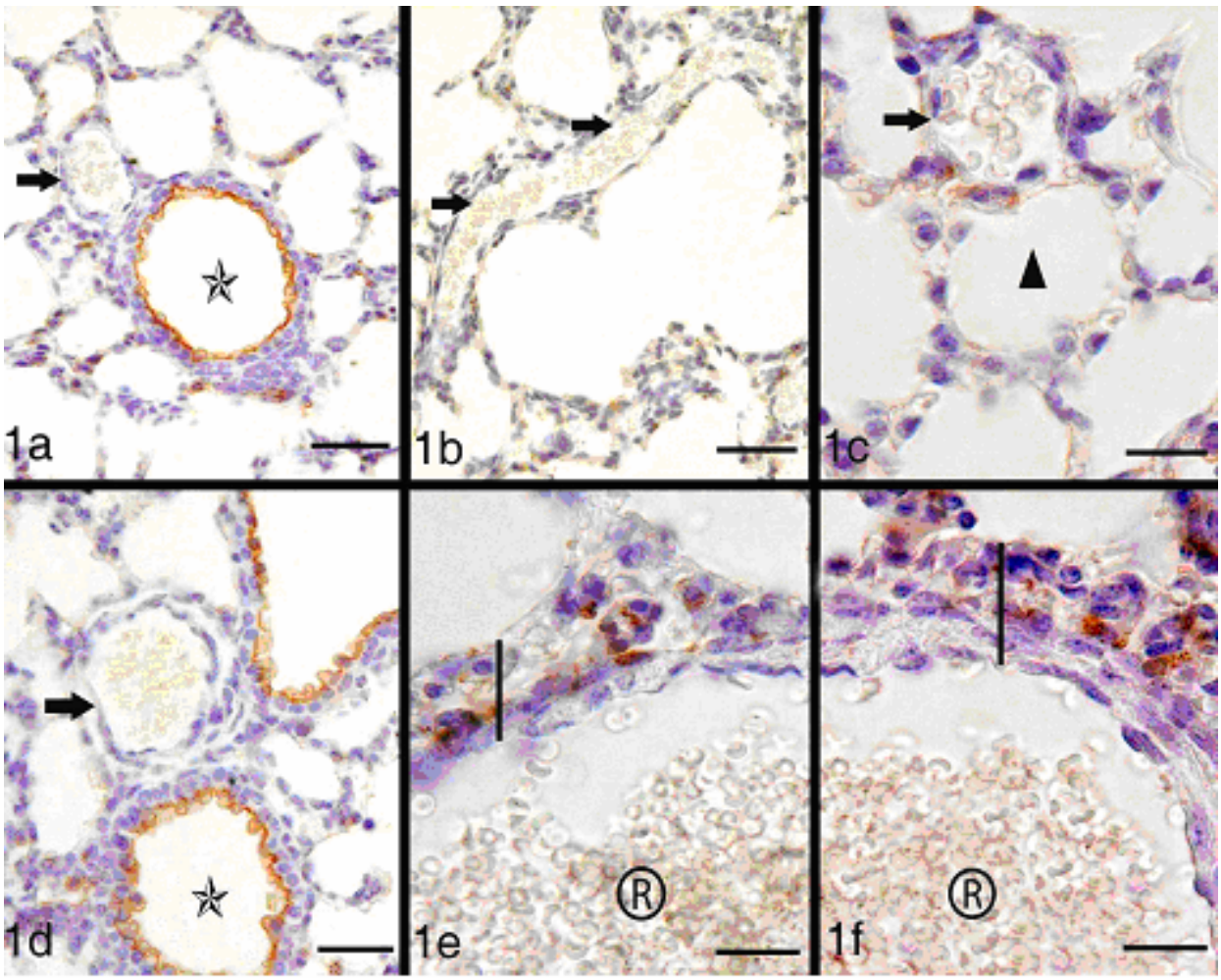

Figure1. Microscopic comparison of the lung parenchyma of the experimental and control groups incubated with antibodies against collagen type IV. These figures indicate vascular sections (arrow heads), bronchioles (asterisks) and alveolar sections (triangle) in which type IV collagen reacted in various regions from weak (light yellow) to strong (dark brown). Images 1a,1b show sections through the mice lungs of the experimental group1, Figures1c,1d experimental group2 and Figures 1e,1f are vascular sections of the experimental and control groups with a higher magnification that show globular accumulation ®) and wall thickness (vertical line on wall). (Haematoxylin counterstained, scale bar=Figure1c 200 µm, Figure 1e, 1f 400 µm and other Figures 100 µm).

DISCUSSION

This study aimed to determine the effects of nicotine on pulmonary vessels of mice offspring with maternal nicotine exposure and evaluate the changes it may cause during gestation and lactation. The mice were divided into experimental groups 1 and 2 based on the fact that weather the offspring was exposed to nicotine indirectly via placenta barrier during the embryonic period or was exposed to nicotine via mother's milk during lactation. In other words, we evaluated the effect of nicotine on lung structural changes during critical period of lung differentiation. Previous studies indicated that collagen reaction in the bronchial basement membrane (BBM) and extra cellular matrix (ECM) of lung parenchyma in experimental groups increased significantly in

comparison with controls (18).

Our findings also showed alveolar remodeling and abnormal bronchogenesis in the offspring lungs of experimental groups especially experimental group 2 (19).

Based on the morphometric results although no remarkable difference was observed between the experimental groups, accumulation of vessels increased in experimental groups in comparison with controls. In addition, adventitia and media thickness of pulmonary vessels in experimental groups increased remarkably compared to controls. Measuring the diameter of large pulmonary vessels indicated that although internal diameter of vessels decreased, wall thickness of vessels increased.

Similarly, Liu et al. reported increased thickness of adventitia and intima layers of vessels (but not media) in adult rats who were exposed to nicotine (20).

Also, they demonstrated that fibroblast cells of adventitia increased but smooth muscles of media decreased. In agreement with this report, Heeschen and coworkers showed that nicotine results in proliferation of endothelial cells and impacts the connective tissue of pulmonary vessels (21).

In Elliot et al, study, thick pulmonary wall was observed in offspring with sudden death syndrome whose mothers mostly smoked cigarette during pregnancy (22).

Besides, fibroblasts synthesize collagen fibers parallel to the nicotine receptors expression. Hence, there is an association between nicotine receptors on fibroblasts and increased collagen in the adventitia layer. Considering our results, nicotine may cross the placenta and be placed on nicotine receptors altering connective tissue proteins. Although type IV collagen in the BM did not show a significant reaction, the increased wall thickness of pulmonary vessels may be related to type I and type III collagen expression.

Acknowledgment

This study was a collaborative research project of Mashhad University of Medical Sciences and Zabol University of Medical Sciences and was funded by Zabol University Research Deputy. Also, we are grateful to Ms. Motajadded from the histology laboratory of the medical school for her technical assistance.

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