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Full Length Research Paper

Nicotine decreased articulation of fibronectin in infant lung parenchyma

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Smoking during pregnancy may impair pulmonary function in infants and children. Nicotine is one of the chemical substances with high level of toxicity. It crosses the placenta and accumulates in the developing organs of fetus. Previous investigations indicated that maternal nicotine exposure induces abnormal collagen IV expression and causes defects in bronchopulmonary development. In this study, the effect of maternal nicotine exposure on fibronectin expression in the lungs of newborn mice has been evaluated. Female Balb/C mice were mated and positivity in vaginal plug was designated as day 0 of pregnancy. Pregnant mice were divided into 2 experimental and 2 control groups. The experimental group 1 received 3 mg/kg intra peritoneal (IP) nicotine from day 7 of gestation to the last day of pregnancy. Also, the same amount of nicotine was injected into experimental group 2 during the same gestational days as well as day 14 after birth (lactation). However, 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 removed for Immunohistochemical method and real-time polymerase chain reaction (PCR). Our finding indicated that fibronectin mRNA expression in the lung of newborn during gestation and lactation period was decreased by 0.7-and 1-fold, respectively compared with control groups. Fibronectin immunoreactivity intensity was not similar in the different parts of the lungs including alveoli, bronchiole and small vessels, having a significant decrease in immunoreactivity of the experimental groups in contrast with the control groups. These data also indicate that maternal nicotine exposure may induce abnormal fibronectin expression which may cause defects in lung function during life time.

Key words: Fibronectin, lung, mouse, nicotine.

INTRODUCTION

Nicotine is a major component of cigarettes. It is a habit forming substance and is prescribed by health professionals to assist smokers to quit smoking (Maritz, 2008). Maternal smoking has been associated with pregnancy complications, including intrauterine retardation (IUGR), fetal and neonatal death, spontaneous abortion and premature delivery (Hafstrom et al., 2005; Wickstrom, 2007). Nicotine is the causative agent for these effects, because it is a major pharmacological constituent of tobacco that easily crosses the placenta and is concentrated in the fetus to a higher level than the mother (Chen et al., 2005).

The use of nicotine replacement therapy (NRT) is widely promoted by health practitioners as a safe way to quit smoking habit. Although, there are some idea that nicotine is not harmful, but several studies indicate the fetal lungs, heart, and central nervous system damage by

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nicotine administration (Argentin and Cicchetti, 2004; Kleinsasser et al., 2005).

The lung development has important role as a gas exchanger in survival of the breathing organism. The lungs develop in uterus and are prepared to function at birth time but similar to other mammals; final stages of its development do not complete until after birth. Disturbance in lung developmental stages may affect its maturation and resistance to diseases in future life (Sekhon et al., 2004; Wasowicz et al., 1996, 1998).

Investigation in animal models showed that maternal nicotine exposures cause a variety of effects on neonatal lungs including significant suppression of alveolarization (Maritz, 1988). Also, nicotine may cause structural changes such as decreased elastic staining of lung parenchyma and increased type I and III collagens (Pierce and Nguyen, 2002). Increase in collagen IV expression and abnormal bronchogenesis have also been reported (Jalali et al., 2010). Extracellular matrix (ECM) composition is essential for morphogenesis and differentiation of virtually all tissues (Gullberg and Ekblom, 1995). ECM contains various parts such as reticular fibers, glycoprotein, proteoglycans and glycosaminoglycans (Jalali et al., 2010). Among the matrix molecules found, fibronectin, a cell adhesive glycoprotein implicated in tissue injury, repair (Bitterman et al., 1983). It is highly expressed during morphogenesis and in tissue remodeling processes (Degen et al., 2009). Fibronectin can modulate many cellular functions ranging from cell adhesion, migration and chemo taxis to proliferation, differentiation and apoptosis (Roman, 1997; Han et al., 2004). The exact role(s) of fibronectin in tobacco-related lung disease is unknown. Several studies have found an association between tobacco abuse and the expression of the matrix glycoprotein fibronectin (Zheng et al., 2007; Roman et al., 2004). Cigarette smoke inhibits fibroblast proliferation and migration by increasing cell-cycle transit time, thereby, reducing the rate of alveolarization (Nakamura et al., 1995). Increased production of fibronectin in alveolar macrophages of smokers, produce fibronectin in lung fibroblasts by stimulating α7 nicotine acetylcholine receptor (nAChR) and contributes to airway wall remodeling (Roman et al., 2004). Fibronectin, as an important component of the fibro proliferative response, triggered in tissues after injury (Roman, 1997). Because ECM components are essential for morphogenesis of all tissues in this study, we evaluated the expression of fibronectin in lung connective tissue development of the offspring during gestational time and lactation period.

MATERIALS AND METHODS

Nicotine administration and tissue preparation

Twenty-four (24) female Balbc/C mice were randomly divided into 2 experimental and 2 control groups. Sperm positivity in vaginal plaque was designated as day 0 of pregnancy. The environmental conditions were at temperature of 22±1°C and 12 h of light-dark cycle with free access to water and food. The experimental group1 were allowed to receive 3 mg/kg of nicotine (N 3876, sigma.com) daily intra peritoneal (IP) from day 7 of gestation to the last day of pregnancy; experimental group 2 was allowed to receive nicotine from day 7 of gestation to 2 weeks postnatal (Jalali et al., 2010). The control groups received nicotine solvent (normal saline) at the same period. Finally, the animals were rapidly sacrificed by cervical dislocation and their lungs removed and fixed for 24 h at room temperature in formalin 10% to use for immunohistochemistry (IHC) study.

Immunohistochemistry (IHC) method

The 5 µm thickness sections were deparaffinized, rehydrated and then washed in phosphate-buffered saline (PBS) (pH 7.4) for 10 min. Antigen retrieval were carried out with heat-induction by Tries/ ethylenediaminetetraacetic acid (EDTA) buffer, pH 9.0 for 20 min. The slides were washed in PBS plus 0.025% Triton X100 for 5 min, and blocked in 10% normal serum with 1% bovine serum albumin (BSA) in PBS for 2 h at room temperature. All the sections were incubated with monoclonal anti fibronectin antibody (Abcam, 2413, USA) diluted 1: 170 in PBS with BSA 0.1% for overnight at 4°C and then washed three times with PBS. For blocking endogenous peroxides activity, the slides were incubated in 0.03% H_2O_2 dissolved in methanol for 30 min. Next, tissues were incubated for 2 h with secondary antibody (Abcam, 97051, USA) diluted 1:800 in PBS with BSA 0.1% for 2 h. After incubation, the sections were washed extensively with PBS for 3 min and treated with 3,3'-diaminobenzidine (DAB) solution (0.03 g DAB in 100 ml PBS and 200 µl H_2O^2/100 ml PBS) for 15 min at room temperature in dark. After being washed in running water, all the sections were counterstained with hematoxylin for 1 min. Finally, the sections were dehydrated in increasing graded ethanol, cleared in xylene and mounted in glass slide. Fibronectin reaction in alveolus and lung parenchyma was graded by three separate observers (Ebrahimzadeh et al., 2011).

Real-time study

RNA extraction

Total RNA was isolated by RNA plus (Cinnagen.co) according to the manufacturer's instructions. Briefly, 50 to 70 mg of lung tissue was homogenized in RNA plus using homogenizer (polytron PT 1200E, Switzerland). The homogenate was centrifuged at 12000 × g for 10 min at 4°C to remove insoluble debris, and the supernatant was transferred to a fresh micro centrifuge tube. Samples were allowed to sit at room temperature for 5 min, and 0.2 ml of chloroform was added per 1 ml of RNA plus™. The samples were vortexed for 15 s and allowed to stand for 5 min at room temperature. The mixture was centrifuged at 12000 × g for 15 min at 4°C. The aqueous phase was transferred to a fresh micro centrifuge tube and an equal amount of isopropanol was added. After 30 min incubation at -20°C, the mixture was centrifuged at 12000 × g for 15 min at 4°C. The pellet was washed with 75% ethanol, air-dried, and resuspended in 50 µl of diethylpyrocarbonate-treated water. The total RNA was examined by measuring the optical density at 260/280 nm.

cDNA synthesis

First strand cDNA was made using a cDNA synthesis kit

Table 1. Effect of nicotine treatment (3 mg/kg) from the day 7 of gestation to the day 14 postnatal in the newborn mice.

Group / variable	Control groups (Day 1)	Experimental groups (Day 1)	Control groups (Day 14)	Experimental groups (Day 14)
B wt (g)	1.55± 0.05	1.43 ± 0.04*	5.84 ± 0.33	5.28 ± 0.39*
L wt (g)	0.029± 0.001	0.025 ± 0.004*	0.11 ± 0.006	0.09 ± 0.007*
L wt/B wt (g)	0.018 ± 0.0009	0.017 ± 0.0019	0.018 ± 0.001	0.018 ± 0.003

The body weight, lung weight and ratio of lung weight/body weight (L wt/B wt) index in new born mice at different days are compared to control groups. Values are means ± SD. B wt, body weight; L wt, lung weight; *, P<0.05.

(fermentas) according to the manufacturer's instructions. RNA (3 µl) was mixed with 1 µl of DNase and incubated for 30 min at 37°C, and then 1 µl of 100 pmole/µl *Oligo* (*dT*) and 8 µl of H_2O were added to each and incubated 10 min at 70°C. After the stated step, 2 µl of 10 mM deoxynucleosides triphosphates (dNTP) mix with 4 µl of 5x reaction buffers, 1 µl of Ribolck and 1 µl of reverse transcriptase were added to each sample tube. The tubes were sequentially incubated at 42°C for 60 min and 70°C for 5 min, and stored at -20°C.

Primers and real-time polymerase chain reaction (PCR)

Real-time PCR was performed using the Stratagene Max3000p (USA) in a total volume of 20 µl per well, containing 10 µl SYBR Green® PCR Master Mix(Pars tous, Iran), 1 µl of cDNA, 1 µl of primer forward, 1 µl of reverse and 7 µl of H_2O. The designed primers are as follows:

Fibronectin forward primer: (TAGGAGAACAGTGGCAGAAAG)
Reverse primer (CCATCGGGACTGGGTTCA)
GAPDH forward primer (ACTCCCATTCTTCCACCTTTG)
Reverse primer (CTGTAGCCATATTCATTGTCATACCAG)

The cDNA was denatured for 10 min at 95°C and the 35 cycle of 95°C for 30 s, 58°C for 20 s, and 72°C for 20 s. After final cycle, the temperature was 95°C to construct a melting curve. The cDNA content of each specimen was determined using a comparative threshold cycle (Ct) method. The results were presented as relative expression of a specific gene normalized to the GAPDH gene. Signals from control lung were considered a relative value of 1.0.

Statistical analysis

Data were analyzed using the SPSS software by student t-test and Mann-Whitney test. The results were considered significant at P<0.05.

RESULTS

Our results showed that decrease in lung weight and body weight of mice offspring born to the mothers with nicotine exposure was significant compared to control groups (P<0.01). However, the ratio of lung weight to body weight index in experimental group compared with that of the control groups was not significant (Table 1). In immunohistochemical method, the locations of fibronectin expression in lung tissue were determined according to the intensity of color darkness. Immunohistochemical reactivity of lung tissue using rabbit polyclonal antibody against mice was specific for fibronectin in alveolar septum, bronchioles and small vessel, showing positive reactivity. Therefore, intensity of Immunohistochemical reactivity increased significantly in experimental alveolar septum especially in marginal part of lung tissue (++ to +++ in central and marginal areas of the tissue respectively).

Also, the fibronectin reactivity of alveolar septum increased significantly in experimental group1 compared to the control group, but the amount of fibronectin in the bronchioles and small vessels decreased significantly (P<0.05) (Table 2 and Figure 1). Analysis of fibronectin mRNA expression in lung tissue showed that mRNA expression decreased by 0.7 logarithmic fold in the experimental group compared to the control group (Figure 2). Tracing of fibronectin in experimental group 2 (day 14) showed that fibronectin reaction did not change in alveolar septum of lungs but the immunoreactivity decreased significantly in bronchioles and small vessels compared to the control group (Table 2 and Figure 1). Evaluation of mRNA fibronectin expression in experimental group 2 indicated that mRNA expression decreased by 1.0 logarithmic fold compared to the control group (Figure 2).

DISCUSSION

The results of this study show that nicotine administration during gestation and lactation could change expression of fibronectin, one of the most important proteins of basement membrane. Fibronectin is a main glycoprotein of ECM involving in important process such as, proliferation, migration and differentiation (Samuel et al., 1994). The IHC results indicated that maternal nicotine exposure causes an increase in fibronectin reaction in the alveolar septum and a decrease in fibronectin reaction in both the bronchiole and lung capillaries. Therefore, the pattern of fibronectin expression is not similar in different parts of lungs. Using real-time PCR, although a little difference in fibronectin expression was seen in experimental groups but it was not significant. The same results were observed between neonates during lactation (2 weeks after birth) and gastation periods. Roman et al.

Table 2. Comparison of fibronectin immunoreactivity in different parts of lung on different days between experimental and control groups.

Group	Control group[a]			Experimental group[a]*			Control group[b]			Experimental group[b]*		
Structure	Alv	Bro	Ves	Alv	Bro	Ves	Alv	Bro	Ves	Alv	Bron	Ves
Fibronectin reaction	++	+++	+++	+++	++	++	+++	+++	+++	+++	++	++

++++**,** Very strong expression; +++, strong expression; ++, moderate expression; +, weak expression; -, negative; Alv, Alveoli; Bro, bronchiole; Ves, vessel; a, day 1; b, day 14; *, P<0.05.

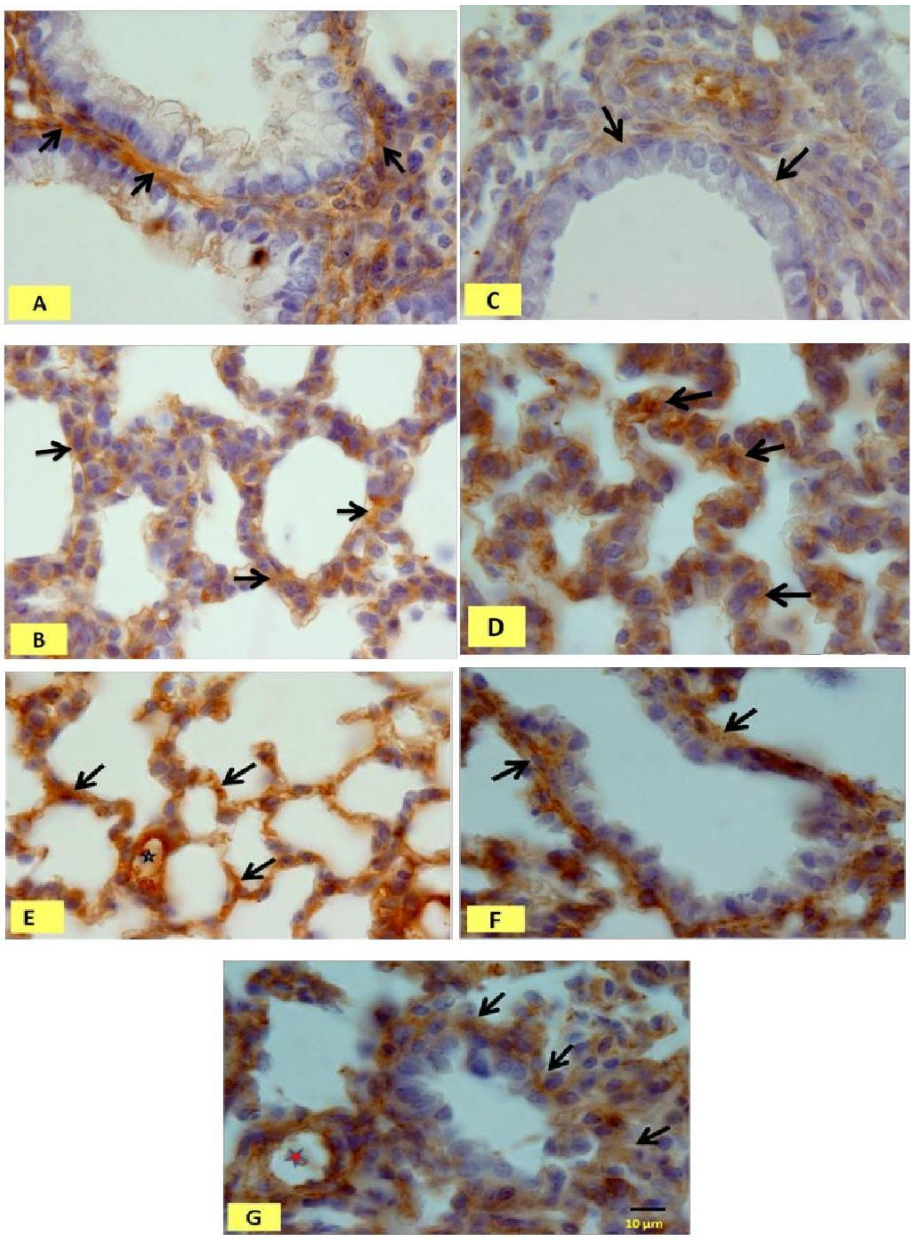

Figure 1. Photomicrographs show epithelium of bronchiole and lung alveoli which are incubated with fibronectin antibody. Photos show alveoli and bronchiole parenchyma in control groups 1 (A, B), and similar section in experimental groups (C, D). Photos (E, F) show bronchiole and lung alveoli in control groups 2 and similar section in experimental (G) that were observed shows decreasing reactivity (arrows). Haematoxylin counterstained. (Scale bar=10 µm).

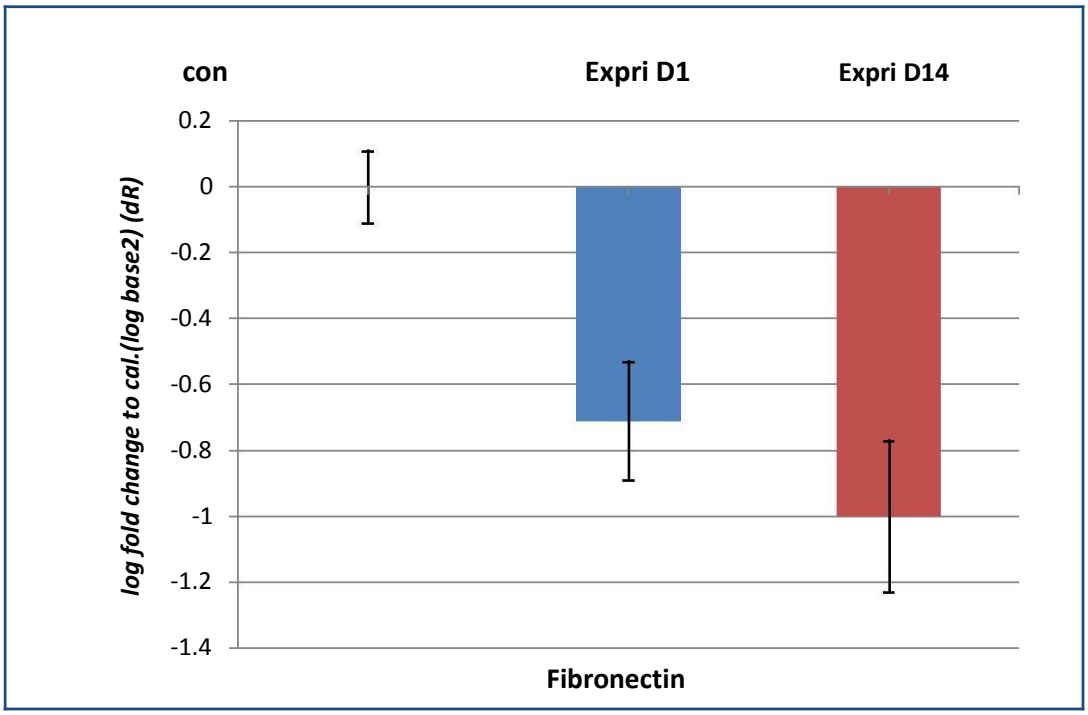

Figure 2. Comparative analysis of logarithmic fold change, (means ± SD), expression of fibronectin in lung tissue of the experimental groups under treatment with (3 mg/kg) on different days (days 1 and 14 of newborn) using real-time comparative PCR.Con, Control; Expri d1, experiment group on day 1; Expri d14, experiment group on day 14.

(2004) showed that nicotine can increase expression of fibronectin in lung fibroblast *in vitro* using real-time PCR and IHC method. IHC results in our study showed that increase in expression of fibronectin *in vivo* would only occur in alveolar septum of experimental group 1. Interestingly, data obtained from real time PCR and IHC analyses followed the same developmental pattern. Also, changes found for protein levels followed the same time course as those described for mRNA expression. Thus, we concluded that nicotine could have an inhibitory impact on fibronectin transcription during the gestation and first 2 weeks of postnatal. Moreover, this effect was similar in 2 weeks of postnatal than gestational period. Based on results of other researchers, lung fibronectin can be expressed by endothelial cells, smooth muscles of vessels, chondrocytes, fibroblasts (Astrof and Hynes, 2009) and alveolar macrophages (Samuel et al., 1994). Several studies indicated that nicotine acts on nAChRs (Akaike et al., 2010) and promotes cell proliferation upon its interaction with nAChRs on the surface of rodent bronchial epithelium (Minna, 2003). The study of Maritz (2009) showed that nicotine increase the production of free radicals parallel with a decrease in the lung antioxidant capacity. Furthermore, suppression of glycolysis and an increase in cAMP results in changes in lung growth (Maritz, 2008). Therefore, we propose that the activation or suppression of intracellular signals by nicotine could lead to increased or decreased fibronectin gene transcription. In addition, nicotine exposure during gestation and lactation may result in remarkable low pups birth weight. This finding was consistent with the results of other researchers (Sekhon et al., 2004; Ozokutan et al., 2005). A decrease in fibronectin expression caused lung disease and developmental defects, and fetal death resulted after knocking out of fibronectin gene (Astrof et al., 2007). Meanwhile, down-regulation of fibronectin expression has been studied in many diseases. Kicic et al. (2010) showed that fibronectin is an essential protein for wound healing. Also, fibronectin expression decreased in the respiratory epithelium of children with asthma and this decrease was an important factor in defects of airway epithelium *in vitro*.

Fibronectin expression reduces in children lungs with asthma, and this decrease can result in congenital abnormalities in bronchial epithelium (Lieberman, 2010). A reduction in fibronectin expression was reported in striae distensa disease (Lee et al., 1994), patients with hepatocyte carcinoma (Sell and Ruoslahti, 1982), mice and human tumoral cell lines (Taylor et al., 1998) and uterus cancer (Futyma et al., 2009). Therefore, decrease in fibronectin expression in emberyonic period may cause functional defects especially asthma during either childhood or puberty period.

Conclusions

In this study, we found that maternal nicotine exposure

during pregnancy and postnatal produce variable changes in fibronectin gene expression at different stages of lung development. This implies that intrauterine nicotine exposure might change the future development of lung dysfunction.

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