Journal of Cell and Animal Biology Vol. 6(15), pp. 226-232, October, 2012
Available online at http://www.academicjournals.org/JCAB
DOI: 10.5897/JCAB12.042
ISSN 1996-0867 ©2012 Academic Journals

Full Length Research Paper

Maternal nicotine exposure altered expression of laminin α 5 in lung tissue newborn mice

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Accepted 4 September, 2012

Maternal smoking has been clearly demonstrated to be associated with increased health problems in infants and children. Nicotine is a chemical substance with high level of toxicity. It crosses through the placenta and accumulates in the developing organs of fetus. Previous investigation indicated that maternal nicotine exposures induce decreased fibronectin expression in lung parenchyma. In this study, the effect of maternal nicotine exposure on laminin expression of the newborn mice lungs has been evaluated. 24 female pregnant Balb/C mice were divided randomly into four groups as follows: Experimental Group 1 (Exp D1); received 3 mg/kg nicotine intra peritoneal injection (IP) from gestational day 7 (GD7) to the last day of pregnancy, Experimental Group 2 (Exp D14); received 3 mg/kg nicotine from GD_7 to post natal day 14, Groups 3 and 4; as sham control groups (Sha-Con) received the same volume (3 mg/kg) of normal saline parallel to experimental groups. At the end of exposure times, all the newborns were anesthetized, their lungs were removed and prepared for immunohistochemical method and real-time polymerase chain reaction. Our finding indicated that laminin alpha 5(Lama5) mRNA expression in the lung of newborn in the nicotine treated Exp D1 decreased by 0.63 fold but increased in Exp D14 by 1.57 fold comparing to Sh-Con groups. Lama5 immunoreactivity was not similar in different parts of the lungs including alveoli and bronchiole, having a significant increase in the experimental groups in contrast to the Sh-Con groups. These data also indicate that maternal nicotine exposure may induce abnormal laminin expression which may cause defects in lung function during life time.

Key words: Laminin, lung, nicotine, mouse.

INTRODUCTION

Maternal smoking has been associated with pregnancy complications, including intra uterine 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 lung has important role as a gas exchanger in survival of the breathing organism. The lungs development occurs in uterus and is prepared to function at birth time but similar to other mammals, final stages of its development do not complete until birth. Disturbance in developmental stages of lung may affect its maturation and resistance to diseases in future life (Sekhon et al., 2004; Wasowicz et al., 1998; Wasowicz et al., 1996). Investigation in animal models showed that maternal nicotine exposures cause a variety of effects on neonatal lungs including; significant suppression of

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alveolarization (Maritz, 1988) and decreased elastin staining of lung parenchyma (Pierce and Nguyen, 2002). Extracellular matrix (ECM) composition are essential for morphogenesis and differentiation of virtually all tissues (Gullberg and Ekblom, 1995). Basement membranes are distinctive extracellular matrices having essential roles in tissue organization and development. Components common to all basement membranes include laminin, type IV collagen, entactin/nidogen, and sulfated proteoglycans (Kruegel and Miosge, 2010). Among the matrix molecules found, laminins are glycoproteins that modulate adhesion and signaling through integrin binding; additionally, they adhere to other ECM molecules (Aumailley and Smyth, 1998). The laminins have such important roles as; cell adhesion, migration, growth, differentiation, angiogenesis and tumor invasions (Suzuki et al., 2005). Each laminin molecule is composed of three non-identical subunit, called the α, β, and γ chains (Bolcato-Bellemin et al., 2003). At least 15 isoform types of laminin have been identified which substantially were synthesized and expressed during fetal and adult periods (Lefebvre et al., 1999; Miner et al., 1997; Nguyen et al., 2002). Several study indicated that Alpha 5 chain of laminin is essential for the lung development, both in embryonic and adult lung (Nguyen et al., 2005; Nguyen et al., 2002), natural development of smooth muscle cell types, basal membrane of blood vessels (Vainionpaa et al., 2006) and digestive tract (Bolcato-Bellemin et al., 2003). Lama 5 is also essential for propagation and polarization of epithelial cells (Fukumoto et al., 2006). Deletion of the gene encoding alpha5 chain of laminin during fetal development in mouse lead to death (Miner et al., 1998) and imposed abnormality in kidney and digestive tract (Lefebvre et al., 1999). In the lungs it would bring about a delay in its evolution along with abnormality in the growth of the surface alveolar cells and disorganization in growth and development of vessels and alveoli (Rahuel et al., 2008; Rebustini et al., 2007). Because Laminins are important BM component essential for morphogenesis of all tissues, in this study we evaluated the effect of nicotine on the expression of lama 5 in lung tissue development of the offspring during gestational time and lactation period.

MATERIALS AND METHODS

Nicotine administration and tissue preparation

24 female Balbc/c mice were randomly divided into 2 experimental and 2 control groups (n = 6). Sperm positivity in vaginal plaque was designated as day zero of pregnancy. The animals were maintained at the animal house under controlled conditions (12 h light and dark cycle, 21°C and 50% relative humidity) with laboratory chow and water provided *ad libitum*. The experimental Group1 (Exp D1) was received 3 mg/kg of nicotine (N 3876, sigma .com) daily intra peritoneally (IP) from day 7 of gestation to the last day of pregnancy and experimental Group 2 (Exp D14) was received nicotine from day 7 of gestation to two weeks postnatal (lactation period) (Jalali et al., 2010). The sham control groups (Sh-Con) were received nicotine solvent (Normal saline) at the same period. Finally, the animals were rapidly sacrificed by cervical dislocation and their lungs were removed in postnatal days one (PD1) and fourteen (PD14), then fixed for 24 h at room temperature in formalin 10% to use for immunohistochemistry (IHC) study. Finally the tissues were dehydrated in increasing graded ethanol, cleared in xylene and embedded in paraffin.

Immunohistochemistry method

The 5 µm thickness sections were deparaffinized, rehydrated and then washed in PBS (pH 7.4) for 10 min, Antigen retrieval were carried out with Heat-induction by Tries/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 (goat, Sigma, USA) with 1% bovine serum albumin (BSA) (Sigma, USA) in phosphate-buffered saline (PBS) for 2 h at room temperature. All the sections were incubated with monoclonal anti laminin antibody (Abcam, 75344, USA) diluted 1: 150 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 (Merk, Ggermany) dissolved in methanol (Bidestsn, Iran) 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 DAB (Sigma, USA) solution (0.03 grDAB 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. Laminin reaction in alveoli and lung parenchyma were graded blind by three separate observers (Table 1), then percentile median intensity reactivity was calculated and presented in the form of 50% (25%, 75%) (Kranenburg et al., 2006).

Real time study

RNA extraction

Total RNA was isolated by RNA plus (Cinnagen, Iran) 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 (Eppendorf, Germany). 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 (Velp, Italy) 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 (Merk, Germany) 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 (fermentas, Lithuania) according to the manufacturer's instructions. RNA (3 µl) was mixed with 1 µl of DNase and incubated for 30 min at 37 °C, (Incubator, Memmert, Germany) and then *1* µl of 100 pmole/µl *Oligo* (*dT*) and 8 µl of H_2O were added to each incubated

Table 1. Grade of immunoreactivity intensity reaction to antibody laminin α5.

Grade	Reaction
(-)	negative
(±**)**	very weak
(+)	weak
(++)	moderate
(+++)	strong
(++++)	very strong

Table 2. Effect of nicotine treatment (3 mg/kg) from the 7th day of gestation to the 14th day postnatal in the new born mice. The body weight and Lung weight index in new born mice at different days are compared to control groups.

Groups / Variable	Control Group PD1	Experimental Group PD1	Control Group PD14	Experimental Group PD14
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*

B wt, body weight; L wt, Lung weight; Values is means ± Sd, *: P < 0.05

10 min at 70°C (Thermal cycler, Bioeer, China). After the above step, 2 µl of 10 mM dNTP Mix 4 µl (fermentas, Lithuania) of 5x reaction buffer, 1 µl of Ribolck (fermentas, Lithuania) and 1 µl of reverse transcriptase (fermentas, Lithuania) were added to each sample tube. The tubes were sequentially incubated at 42°C for 60 min *and.* 70°C for 5 min (Thermal cycler, Bioeer, China), and stored at -20°C (Freezer, Sikat, Iran).

Primers and real-time polymerase chain reaction (RT-PCR)

Real-time PCR was performed using the Max3000p (Stratagene, 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 H2O. The designed primers were as allows: Lama α 5- F, CGTCCCACAGGAATAGGCT,Lama α 5- R, TACCAACGAAGGGCTGCG, GAPDHF, AACTCCCATTCTTCCACCTTTG, GAPDH-R, 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. At the end of the runs, melting curves were obtained to make sure there were no primer–dimer artifacts.

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was used as an internal control to measure the relative expression quantity of the target genes. Constructing a standard curve with serial dilutions of known template concentration for each target gene is not feasible. Therefore, dilutions (1:1, 1:10, 1:100, 1:1,000, and 1:10,000) of cDNA from the high quality sample were used to construct a relative standard curve for the target genes. Fold change in laminin gene expression was calculated by$2^{-\Delta(\Delta CT),}$ where ΔCt = Ct (target gene) - Ct (GAPDH), and Δ (ΔCt) = ΔCt (sample) - ΔCt (standard) (Pfaffl, 2001; Wong and Medrano, 2005)

Statistical analysis

On the basis of staining intensity, sections were graded and Mann-Whitney non-parametric statistical test was used to compare differences between samples, and student t test used for real time PCR. P-values <0.05 were considered statistically significant.

RESULTS AND DISCUSSION

Our results showed that decrease in lung and body weight of mice offspring born from mothers exposed to nicotine was significant compared to control groups (P < 0.01) (Table 2). Analysis of laminin α5 mRNA expression in lung tissue showed that mRNA expression decreased by 0.63 fold in Exp D1 and increased by 1.57 fold in Exp D14 comparing with the Sh Con groups. Statistical analysis indicated that the laminin α5 expression increased significantly from PD1 to PD14 in experimental group (P < 0.05) (Figure 1). Immunohistochemical reactivity of lung tissue using rabbit monoclonal antibody against mice was specific for laminin α5 in alveolar septum and bronchioles, showing positive reactivity in immunohistochemical method. The locations of laminin expression in lung tissue were determined according to the intensity of color darkness. Immunohistochemistry data showed that intensity of immunoreactivity of laminin α5 in infant lung alveoli of Sh-Con D1 group reacted moderate with median of 2 (2, 3) while at the same position in infants lung born from mothers affected under nicotine Exp D1 was in average reactivity with median of 3 (3, 4). Statistical analysis showed that this increase was significant (P = 0.01). In addition median of reaction intensity of alveoli in Exp D14 was very intensitive 4 (3, 4) compared with Sh Con D14 (p = 0.001). Also expression of laminin α5 between Exp D1 and Exp D14 increased significantly (p = 0.01) (Figures 2 and 4). Study of immunoreactivity of laminin α-5 in bronchioles of Exp

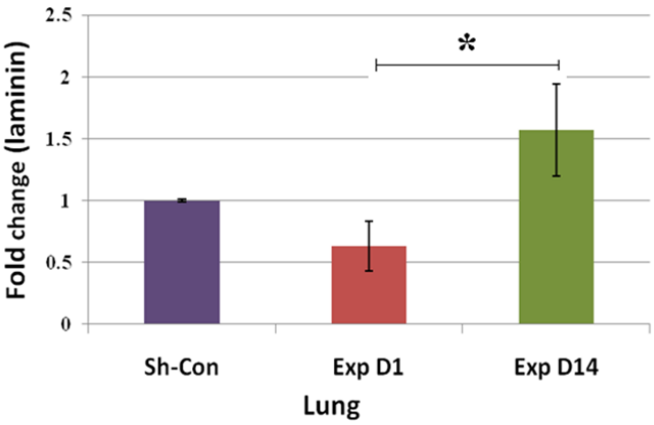

Figure 1. The graph represents the relative transcription level of laminin mRNA expression in lung tissue of the experimental groups under treatment with nicotine (3 mg/kg) on different days (days 1 and 14 of newborn) using Real-time PCR. Sh-Con (Sham control), Exp D1 (postnatal day one) and Exp D14 (postnatal day fourteen). Values represent the means ± SE (n = 6).

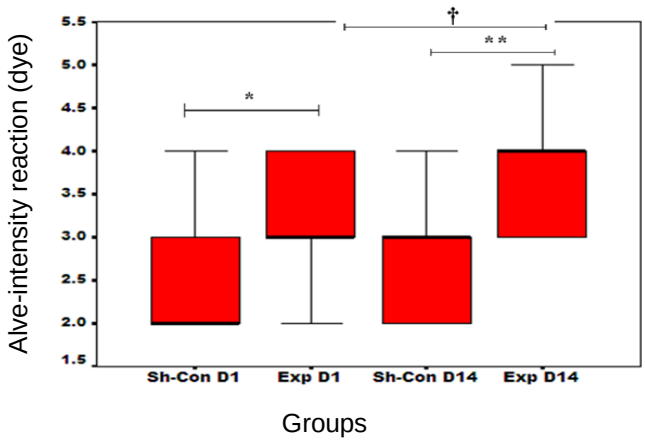

Figure 2. Boxplot shows the effect of maternal nicotine exposure in intensity reaction of laminin α5 in lung alveoli of 1 and 14 days mouse infant. Median is presented in the form of 50% (25%, 75%). Sh-Con D1: (Sham control group of day one), Exp D1: (postnatal day one), Sh-Con D14: (Sham control group of day) 14, Exp D14: (postnatal day fourteen).*p= 0.01, **p=0.001, †p = 0.01(n = 17).

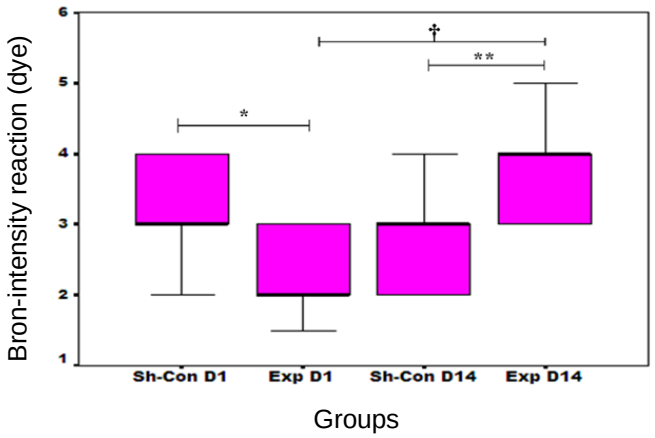

Figure 3. Boxplot shows the effect of maternal nicotine exposure in intensity reaction of laminin α5 in lung bronchiole of 1 and 14 days mouse infants. Median is presented in the form of 50% (25%, 75%). Sh-Con D1: (Sham control group of day one), Exp D1: (postnatal day one), Sh-Con D14: (Sham control group of day) 14, Exp D14: (postnatal day fourteen) 14.*p = 0.002, **p = 0.001, †p = 0.0001 (n = 17).

D14 and Exp D1 showed a decrease reactivity in Exp D1with median 2 (2, 3) comparing with sh-con 3 (2, 4) (p = 0.002). However, intensity reactivity in bronchioles Exp D14 with median 4 (3, 4) increased significantly comparing with sh-con 3(2, 3) (p = 0.001). Statistical analysis showed that the expression of laminin α-5 in bronchioles increased significantly between ExpD1 and ExpD14(p = 0.001) (Figures 3 and 4).

The results of this study show that nicotine administration during gestation and lactation could change expression of laminin α5 as one of the most important proteins of basement membrane. According to IHC analysis, maternal nicotine exposure causes laminin reaction intensity increase in the alveolar septum and derease in bronchiole during gastation period. Therefore, the pattern of laminin expression could be different in various parts of the newborn lungs. Also decrease in expression of laminin would only occur in bronchioles of experimental Group 1 while in experimental Group 2 increased in both alveoli and bronchioles. Although a little difference in lama5 expression was observed in experimental groups using Real time PCR but it was not significant. Changes found for protein levels followed the same time course as those described for mRNA expression. Data obtained from real time PCR and IHC analyses indicated that lama5 expression increased in experimental Groups 2 follow the same developmental pattern. Thus, we concluded that nicotine could have an inhibitory impact on laminin transcription during the gestation period and an stimulatory effect on expression of laminin in laction period.

Luck et al showed that nicotine in maternal smoking results in milk concentrations between 1.5 and 3 times the simultaneous maternal plasma concentration. The nicotine in breast milk is rapidly absorbed through the infant's gut, and accumulates in some tissues (Luck and Nau, 1984). Accordingly, a reason for increase in lama5 expression in this study might be because of presence of high nicotine concentration in mother breast milk.

Several studies indicated that nicotine acts on nAChRs, which are ligand-gated ion channels controlling influx of calcium and sodium into cells (Akaike et al., 2010). Neuronal nicotinic receptors are present in bronchial epithelium and vascular endothelial cells as well as in the

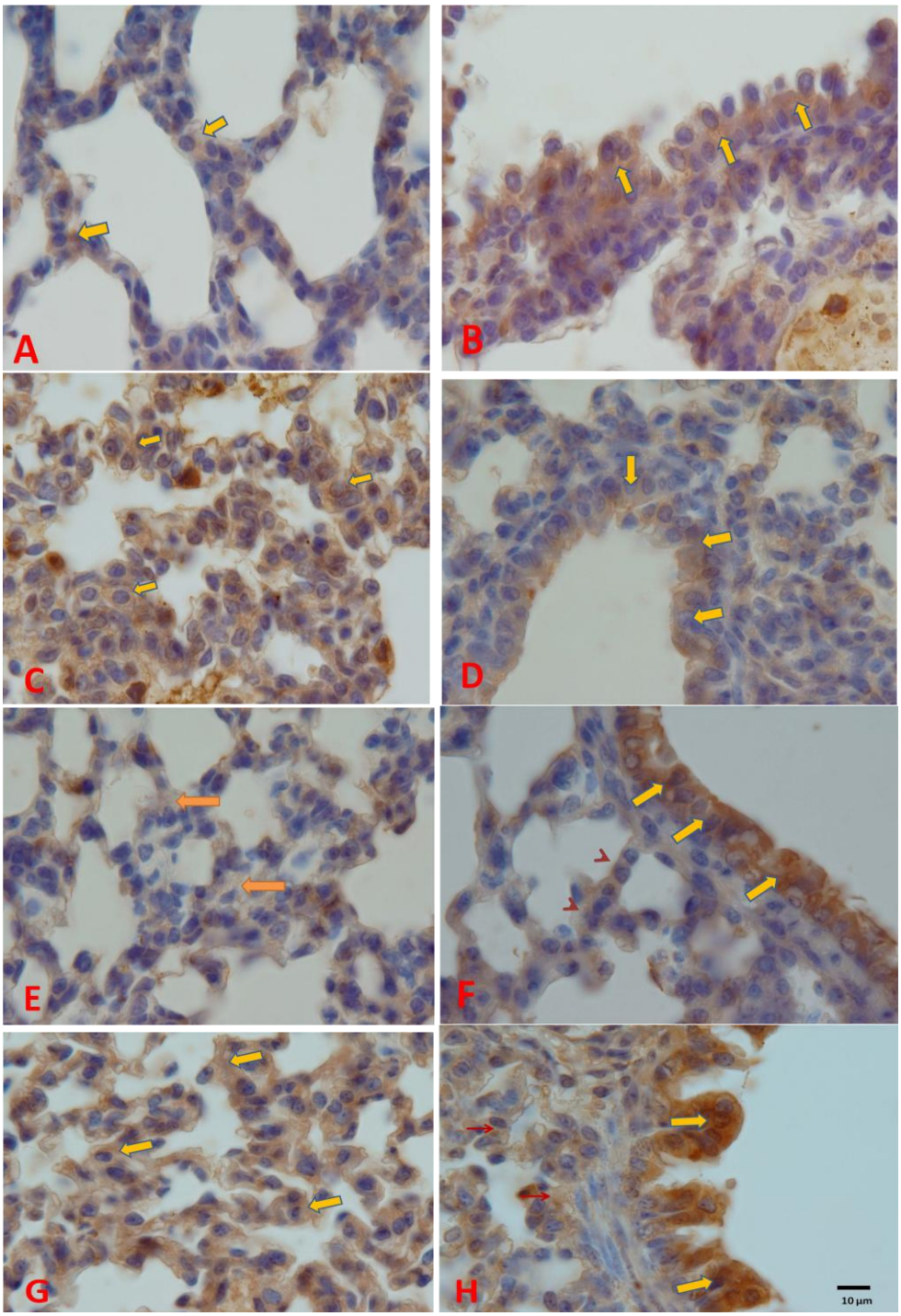

Figure 4. Photomicrographs show epithelium of bronchiole and lung alveoli incubated with laminin α5 antibody (A to H). A, B: alveoli and bronchiole parenchyma in control Group 1; C, D: similar section in experimental groups; E, F: alveoli and bronchiole parenchyma in control Group 2; G, H: similar section in experimental shows increasing reactivity (arrows). Hematoxylin counterstained. (Scale bar =10 µm).

cholinergic nerves innervating the bronchial smooth muscle (Sekhon et al., 1999; Sekhon et al., 2002). Sekhon et al. (2002) showed that nicotine administration to pregnant rhesus monkeys caused increase in expression of a 7 nicotinic cholinergic receptors within the lungs, which was accompanied by increases in collagen deposition in the airway wall in the lung. The study of Maritz (2009) showed that nicotine increase the

production of free radicals parallel with a decrease in the lung antioxidant capacity (Maritz, 2009). 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 and change in expression of a7 nicotinic cholinergic receptors could lead to increased or decreased laminin 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 (Ozokutan et al., 2005; Sekhon et al., 2004).

Based on results of other researchers, lung laminin alpha5 can be expressed by endothelial cells, smooth muscles of vessels (Bolcato-Bellemin et al., 2003) and airways epithelial (Nguyen et al., 2005). Decrease in lama5 expression observed in this study may also resulted from malfunctioning of another lung components such as smooth muscles and endothelial vessles.

Several studies showed that laminins affect lung development at multiple stages and in different cellular compartments (Schuger et al., 1990; Willem et al., 2002). Laminin α2 has been shown to be important for bronchial smooth muscle cell differentiation (Relan et al., 1999). Mice lacking laminin γ2, or α3 die 1 to 3 days after birth from malnutrition (Meng et al., 2003). A reduction in laminin α5 expression was reported in breast cancers (Martin et al., 1998), Prostate (Calaluce et al., 2001), lung (Akashi et al., 2001; Manda et al., 2000) and colon (Sordat et al., 1998). An increase in expression of laminin beta2 and alpha1 chains have been reported in airway of asthma patients, allergic airway remodeling and chronic obstructive pulmonary disease (COPD). Therefore, change in laminin expression in emberyonic period may cause functional defects especially asthma during either childhood or puberty period. Although above studies highlight the important of laminin in cancer studies but there have not found any record about the effect of nicotine in laminin gene expression.

Conclusions

In this study, we found that maternal nicotine exposure during pregnancy and postnatal produce variable changes in Laminin α5 gene expression at different stages of lung development. This implies that maternal nicotine exposure might change the future development of lung dysfunction.

ACKNOWLEDGEMENTS

The provided data in this article are from PhD student thesis supported by the grant (89566) from vice chancellor for research, Mashad University of Medical Sciences, Iran. In addition, the authors would like to thank Mrs. F. Motejaded for her technical assistance.

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