www.roavs.com EISSN: 2223-0343
RESEARCH OPINIONS IN ANIMAL & VETERINARY SCIENCES

Effects of vitamin C on expression of renal laminin-α5 in offspring of nicotine-treated mice

Hasan Pahang[1], Mehdi Jalali[2], Mohammad Reza Nikravesh[2]*, Ariane Sadr-Nabavi[3] and Alireza Ebrahimzadeh bideskan[2]

[1]Student Research Committee, Mashhad University of Medical Sciences, Mashhad, Iran; [2]Department of Anatomy and Cell Biology, Faculty of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran; [3]Department of Human Genetics, Faculty of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran

Abstract

Nicotine has been suggested to have deleterious effects on the outcome of pregancy. This study investigated the effects of maternal nicotine and vitamin C during pregnancy on the expression of laminin-α5 in kidneys of offspring. In this study, eighteen female BALB/c mice, after pregnancy confirmation, were randomly divided into three groups: experimental group 1 (T1) received 0.3 mg/kg/day nicotine and experimental group 2 (T2) received simultaneously 0.3 mg/kg/day nicotine and 9 mg/kg/day vitamin C and control group (T3) received 3 ml/kg/day normal saline parallel to other groups since the 6th day of gestation to the end of prenatal life. At birth, laminin-α5 content of the renal cortex, glomerulus and convoluted tubules were determined immunohistochemically (IHC) and real-time polymerase chain reaction (RT-PCR). Maternal nicotine reduced birth weight but increased kidney weight although not significantly ($P>0.05$). The IHC results showed that laminin-α5 decreased significantly ($P<0.05$) in the renal cortex, glomerule proximal and distal convoluted tubule in T1 compared to the T2 and T3 ($P<0.05$). The RT-PCR also confirmed this outcome. The results suggested that vitamin C consumption by the mother during the gestation period could ameliorate the nicotine effects on renal extracellular matrix of the offspring.

Keywords: Vitamin C, nicotine, laminin-α5, kidney

To cite this article: Pahang H, M Jalali, MR Nikravesh, A Sadr-Nabavi and AE bideskan, 2014. Effects of vitamin C on expression of renal laminin-α5 in offspring of nicotine-treated mice. Res. Opin. Anim. Vet. Sci., 4(7): 405-410.

Introduction

Nicotine is one of the major toxic products of cigarette smoke and has been identified as a risk factor for many diseases. Its concentration in serum, amniotic fluid and foetus body is higher than smoking mothers (Luck et al., 1985). Nicotine impairs protein synthesis and thus destroys cell communication with extracellular matrix (Snyder et al., 2002). Previous studies revealed that maternal nicotine during pregnancy and lactation stimulates collagen synthesis in lung parenchyma and also caused alveolar remodelling and abnormal bronchogenesis in the lungs of offspring in mice (Jalali et al., 2010). Also other studies have demonstrated that maternal nicotine reduces expression of laminin-α5 in newborn lung (Mahdi Shariati et al., 2012). Furthermore, significant decrease was observed in skin laminin expression with maternal hyperthyroidism (Amerion et al., 2013).

Laminin-α5, in kidney is a major epithelial and endothelial basal lamina component (Miner et al., 1995). Also mutations in laminin and collagen IV gene destructs kidney ultrafiltration (Miner, 2012). Therefore, disturbance in synthesis of laminin-α5 causes developmental defects in the structure in basal lamina of kidney of foetus (Miner et al., 1998).

***Corresponding author:** Mohammad Reza Nikravesh, Department of Anatomy and Cell Biology, Medical Faculty, Mashhad University of Medical Sciences, Pardis Complex, Azadi Square, Mashhad, 917794-8564, Iran Tel: +98 511 8002490

Laminin-α5 in this organ participates in the process of glomerular vascularization and glomerulo-genesis and ureteric bud branching (Shannon et al., 2006).

To alleviate the adverse effects of maternal nicotine, vitamin C consumption in gestation period has been recommended in some studies. For example the development of newborn rat lung parenchyma was ameliorated with co-administration of nicotine and vitamin C in the prenatal period (Maritz and Rayise, 2011). Also maternal antioxidant consumption during pregnancy and lactation period prevented β-cell apoptosis in offspring in response to nicotine exposure (Bruin et al., 2012). Some study proved that vitamin C could not protect lungs tissue from harmful effects of nicotine in the prenatal period (Maritz and van Wyk, 1997). Similar observation demonstrated that vitamin C treatment in the prenatal period did not prevent deleterious effects of maternal nicotine exposure (Gunes et al., 2008). Also vitamin C could not prevent adverse effects of nicotine exposure during prenatal and postnatal periods on neonatal rat bone development (Koklu et al., 2006). However, the effect of maternal vitamin C uptake on foetus kidney extracellular matrix structure in nicotinic mothers has not been examined. Therefore, the present study was designed to determine the simultaneous effects of maternal nicotine exposure and vitamin C consumption on laminin-α5 expression in the extra cellular matrix tissue of kidney in the off spring.

Materials and Methods

This research was performed in accordance with the rules of the Ethics Committee on Animal Experimentation, Mashhad University of Medical Sciences in Iran. For this study, 18 female BALB/c mice were selected and coupled with male BALB/c mice. Pregnancy was confirmed with vaginal plaque observation and then allowed them to habituate until embryonic day 6. The 18 pregnant BALB/c mice were randomly divided into three groups as follow: One group received 0.3 mg/kg/day nicotine (Sigma-Aldrich, Saint Louis, USA) via IP injection from the 6th day of gestation to the last day of pregnancy (T1). Second group received simultaneously 0.3 mg/kg/day nicotine and 9 mg/kg/day vitamin C (Applichem, Darmstadt, Germany) from the 6th day of gestation to the last day of pregnancy (T2). The control group received 3 ml/kg/day normal saline via IP injection for the same period (T3). On the first day of delivery, all newborns were anesthetized by chloroform inhalation. After abdomen incision, their kidneys were detached and transferred to 10% buffered formalin for IHC technique and to RNA stabilization reagent (Qiagen, Hilden, Germany) for RT-PCR technique.

Immunohistochemical studies

The right newborns kidneys were removed and transferred to 10% buffered formalin for 24 h. The IHC studies for laminin-α5 were performed on formalin-rigid sections by an indirect immune peroxidase procedure. Briefly, after the kidney tissues were sectioned at 5 µm thicknesses, deparaffinized and rehydrated then antigen retrieval was performed in a water bath at 100°C. Sections were blocked with 3% H_2O_2 to inhibited endogenous peroxides activity and transferred to 10% goat serum in phosphate-buffered saline (PBS). Now they were incubated with specific anti- laminin-α5 primary antibody (Abcam, Cambridge, UK) at 4°C overnight, followed by staining with horse radish peroxidase-conjugated secondary antibodies. When slides were exposed to diaminobenzidine (DAB), brown colour appeared. Counterstaining with hematoxylin was performed to clear the cell nuclei. The sections were dehydrated, stabilized with mounting medium and stained. Sections were examined under a light microscope (Olympus BX51, Japan). The intensity of brown colour showed the level of laminin-α5 reaction in the renal sections. Images analysis was performed by Quantitative Scoring Methods according to the following score (Dixon et al., 1980).

Intensity of Staining	without staining	weak staining	moderate staining	strong staining	very strong staining
Score	0	1	2	3	4

Real-time polymerase chain reaction

The left newborns' kidneys were removed and gene expressions were measured by RT-PCR. The kidneys were homogenized by a laboratory homogenizer (Polytron PT 1200E, Switzerland). Total RNA was extracted from the renal fragments of each newborn by RNX-plus (ParsTous, Tehran, Iran) according to the manufacturer's protocol. The purity of RNA was determined by electrophoresis on an agarose gel. Reverse transcription was performed on 3 µg of RNA using a cDNA synthesis kit (ParsTous, Tehran, Iran). The RT-PCR was performed on an ABI PRISM® 48-well optical reaction plate (Applied Biosystems Step One, Foster City, USA). The RT-PCR mixture contained 1µl of template (cDNA), 0.2 µ M forward primer, 0.2 µ M reverse primer, 3.6 µl sterilized water and 5 µl SYBR green real-time PCR master mix (ParsTous, Tehran, Iran). The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as endogenous gene control. A relative quantification method was used to compare mRNA expression (Livak and Schmittgen, 2001). Fold changes in mRNA expression were calculated using the $2^{-\Delta\Delta ct}$ equation, where ΔΔCT is the difference between laminin-α5 and GAPDH genes expression. Each test was performed in triplicate and the expression level was calculated three

times. Amplifications for both genes were performed by an optimized protocol (10 min at 95°C, 40 repeated cycles of two steps at 95°C for 15 s, 58°C for 30 s, 72°C for 30 s, 95°C for 15 s and 55°C for 1h).

Oligonucleotide primers

The sequences of oligonucleotide primers used in RT-PCR method were designed by Beacon software as follows:
Laminin-α5, forward primer 5′- CGTCCCACAGGAAT AGGCT; Laminin-α5, reverseprimer5′-ACCAACGAA GGGCTGCG; Gapdh, forward primer 5′- AACTCCCA TTCTTCCACCTTTG; Gapdh, reverse primer 5′- TGT AGCCATATTCATTGTCATACCAG. The primers were synthesized by oligo macrogen (Seoul, Korea).

Statistical analysis

Data analysis was performed using SPSS software (SPSS Inc., Chicago, IL, USA). All data were expressed as mean±SEM. The data of immunohistochemical study were analyzed by non-parametric statistical Kruskal-Wallis test. Weight data was analyzed by using one-way analysis of variance (ANOVA). P values less than 0.05 were considered to be statistically significant.

Results

The result of offspring weight, kidney weight and laminin-α5 expression (relative) are given in Table 1. The results indicated that no significant change was observed in birth and kidney weight between the control and treated groups. However, laminin-α5 expression (relative) decreased significantly in T2 group compared to control.

To confirm the results of this study, laminin-α5 gene expression was evaluated by RT-PCR. The data analysis of RT-PCR showed laminin-α5 gene expression down-regulation in T2 offspring kidney compare to T1 and T3. The IHC data analysis showed that reaction of laminin-α5 decreased significantly in the cortex, glomerular and proximal convoluted tubules of kidney of the offspring of mice that received nicotine in the gestation period contrast to control (Table 2). However laminin-α5 reaction significantly increased in the kidney cortex, proximal and distal convoluted tubules of mice offspring kidney that received simultaneously nicotine and vitamin C compared to nicotine group. Although maternal nicotine decreased laminin-α5 reaction in the glomerulus in the kidney of offspring but it increased considerably in the nicotine and vitamin C (Table 2). Data also indicated that laminin-α5 reaction decreased in the Henle loop of the mice offspring kidney that received nicotine (Table 2).

Morphological finding showed proximal convoluted tubules epithelial cells were greatly degenerated in the nicotinic mice offspring's kidney. Treatment with vitamin C maintained the cell appearance and laminin-α5 levels in the extracellular matrix suppressed by nicotine (Fig. 1). Also microscopic observation of kidneys sections in the nicotine+vitamin C group showed that urinary space size in glomerulus increased compared to the nicotine offered group (not shown).

Discussion

Our results proved that maternal nicotine in the gestation period reduced birth weight of offspring and increased kidney weight but also reduced laminin-α5 expression in the kidney of new born. There is some evidences which suggest that the main mechanism of nicotine toxicity is changing cellular oxidant-antioxidant and lipid peroxidation system (Gallo et al., 2010). Once the nicotine crosses the plasma membrane, it is transported to the endoplasmic reticulum which is the first place of nicotine metabolism (Nakayama et al., 1993).

Maternal nicotine induces oxidative stress damage through over production of reactive oxygen species (ROS) and inhibition of SOD activity in offspring's organs vascular wall (Xiao et al., 2011) leading to decrease the body growth. Additionally, the kidney glomerular mesangial cells and tubular epithelial cells were susceptible to ligands for nicotinic acetylcholine receptor (Mitsui et al., 2001). Therefore, tubular epithelial cells are more susceptible to cytokines and stimulate growth factors (Biju et al., 2005). These cells in response to the cytokines and glomerular ultrafiltrates such as nicotine stimulate myofibroblasts cells and also immune cells to proliferate and produce extracellular matrix (Wynn, 2008). In the current study,

Table 1: Effects of vitamin C on body birth weight, kidney weight and expression of renal laminin-α5 in offspring of nicotine-treated mice

Groups	Birth weight (g)	Kidney weight (g)	Laminin-α5 expression (relative ratio)
Control (T1)	1.65±0.128	0.0038±0.00024	$1±0.00^{a}$
Nicotinic (T2)	1.16±0.098	0.0046±0.00041	$0.61±0.07^{c}$
Nicotine + Vitamin C (T3)	1.48±0.11	0.0041±0.00035	$0.89±0.59^{b}$

Values are means ± SEM (n = 6) and significantly showed with alphabet superscripts in a column (P<0.05). Pregnant mice in Nicotine group received 0.03 mg/kg/day nicotine and animals in Nicotine + Vitamin C group received 0.03mg/kg/day nicotine and 9 mg/kg/day vitamin C.

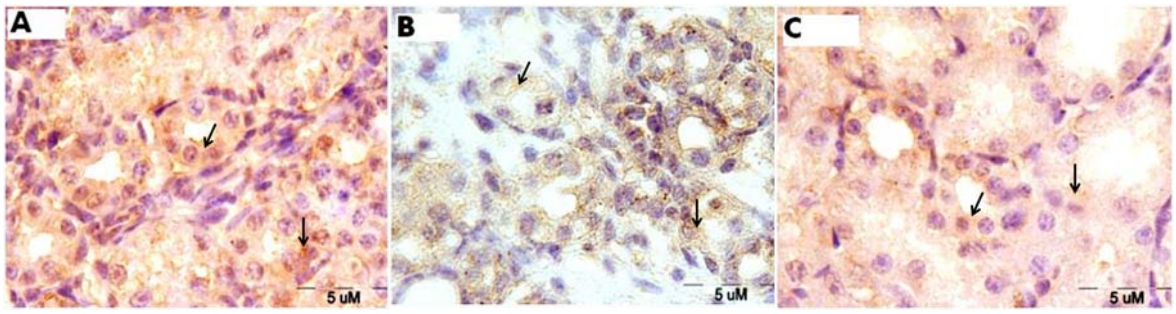

Fig. 1: Cross sectional of mice offspring kidneys in control (A), nicotine (B) and nicotine-vitamin C group (C). A: Arrows indicated laminin-α5 strong staining at the apical pole of convoluted tubules epithelium as well as extracellular matrix (ECM) in the control group. B: weak staining observed in these epithelial cells and ECM after maternal nicotine receiving in the gestation period (arrows). C: Laminin-α5 staining increased moderately in maternal nicotine + vitamin C group (arrows)

Table 2: Effects of vitamin C on expression of laminin-α5 in different regions of kidney in offspring of nicotine-treated mice

Kidney location	Control (T1)	Nicotinic (T2)	Nicotine+vitamin C (T3)
Cortex	3.5±0.16^{a}	2±0.26^{b}	3.0±0.12^{a}
Glomerulus	2.5±0.17^{a}	1±0.11^{b}	1.5±0.09^{ab}
Proximal tubule	3.0±0.21^{a}	0.5±0.15^{c}	2.0±0.25^{b}
Distal tubule	1.5±0.19^{a}	0.75±0.29^{b}	1.0±0.12^{ab}
Henle loop	0.75±0.18^{a}	0.5±0.15^{a}	0.75±0.08^{a}

Values are means ± SEM (n = 6) Different alphabets in a row differ significantly (P<0.05). T1: Control, T2: Pregnant mice received 0.03 mg/kg/day nicotine; T2: Animals in nicotine + vitamin C group received 0.3 mg/kg/day nicotine and 9 mg/kg/day vitamin C

it is suggested that accumulation of extracellular matrix in kidney of the offspring increased kidney weight. During kidney morphogenesis, laminin-α5 expressions are not similar in all epithelial cell types and proximal convoluted tubules are more susceptible to nicotine so laminin-α5 reaction intensity is higher in these tubules (Durbeej et al., 1996). Nicotine also stimulates Ca^{2+} transport across the epithelial cell membranes through non-neuronal-mediated receptors and increases intracellular Ca^{2+} concentration that controls genes expression (van Haasteren et al., 1999; Klimek et al., 2000).

In the present study, we have demonstrated that the addition of vitamin C to the diet of nicotinic mice maintained their newborn birth weight loss. Also simultaneous protective effect of maternal vitamin C consumption in nicotinic mice on the offspring renal extracellular matrix laminin-α5 synthesis was proved by the results of the present study. Briefly vitamin C scavenges free radicals in blood circulatory system and cell cytoplasm and protects cell membranes from injury (Chan, 1993). Since mitochondrial DNA is not shielded by structural proteins, it is susceptible to ROS and free radicals induced by nicotine (Wei et al., 1998). Vitamin C by activation of manganese superoxide dismutase (SOD) and glutathione peroxidase (GPx) enzymes reduces ROS generation that influence mitochondrial function (Valdecantos et al., 2010). Also vitamin C suppresses TGF-β activities leading to decrease levels of lipid and protein peroxidation products (Abhilash et al., 2012). Morphological observation in this study indicated that vitamin C reduced epithelial cell degeneration. Also other observations have shown that antioxidant such as glutathione reductase prevents nicotine toxicity by modulating of lipid peroxidation and supporting antioxidant defence system (Dey and Roy, 2010). Previous studies have demonstrated that kidney tubular epithelium cells stimulate laminin-α5 synthesis during epithelium regeneration after ischemic damage (Cheng et al., 1997).

In conclusion, the findings revealed that maternal vitamin C during pregnancy has protective effect on laminin-α5 of kidney in nicotine treated mother.

Acknowledgements

The results described in this article are parts of PhD thesis supported by Research Grant Number 900629 from Office of Vice-chancellor for Research Affairs of Mashhad Medical Sciences University. Authors appreciate Mrs Motejadded, Laboratory Assistant of Histology for her help in immunohistochemical technique. Also authors wish to thank to Medical Faculty Central Laboratory for professional consultation in PCR techniques.

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