

Original Research

Different ideas associated renal malformation and laminin $\alpha 5$ expression caused by maternal nicotine exposures

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Abstract: The number of smokers is increasing specially in pregnant mothers and millions of children with health problems are born from the smoker mothers. Nicotine as a toxic substance crosses from placenta and accumulates in the developing organs of fetus. In this study, the effects of maternal nicotine exposure on expression levels of kidney laminin $\alpha 5$ in newborn mice were examined. Timed pregnant mice were injected subcutaneously with nicotine at a dose of 2 mg/kg/day from day 7 of gestation to the last day of the pregnancy (Group 1) and from day 7 until the two weeks of postnatal (Group 2). Sham control groups were injected with saline. After the last injection, all the newborn mice were anesthetized; their kidneys were removed and prepared for analysis of mRNA and protein expression of laminin $\alpha 5$ using Real-Time PCR and immunohistochemical techniques, respectively. Our results showed that mRNA levels of kidney laminin $\alpha 5$ in newborn mice were increased in group 1 when compared to sham control group and also group 2. Immunohistochemical analysis demonstrated that the protein levels of laminin $\alpha 5$ in the glomerulus have significantly increased in group 1 when compared to group 2. In the proximal convoluted tubules, the parameter had a high significant increase in group 1 in comparison to control and also group 2. According to the results, it seems that maternal nicotine exposure may induce abnormal laminin $\alpha 5$ expression which may cause defects in kidney function during life time.

Key words: Laminin $\alpha 5$, proximal convoluted tubules, glomerulus.

Introduction

Investigations demonstrated that smoking is increasing world widely, especially among young women, who are in the peak of reproductive age (1). It has been reported that spontaneous abortion, fetal and neonatal death, intra uterine retardation (IUGR) and premature delivery are the pregnancy complications which are associated with maternal smoking (2, 3). Nicotine, as one of the main materials in the cigarette, is a toxic substance and easily crosses the placenta to concentrate in the fetus at a higher level than mother's body (4, 5). The kidney is a vital organ playing important roles to regulate the volume of body fluids and to excrete of waste produced metabolites (6). Studies showed that maternal smoking cause a variety of effects on neonatal kidneys such as: loss of renal weight, urinary organ malformations, renal agency, renal polycystic and nephropathy (7, 8). It also has been proven abnormal expression of collagen type IV as a part of basement membrane and extracellular matrix is caused by mother's smoking (9). Extracellular matrix composition is essential for morphogenesis and differentiation of virtually all tissues (10). Basement membranes are distinctive extracellular matrices play essential roles in tissue organization and development. Laminin, type IV collagen, entactin/nidogen, and sulfated proteoglycans are the common components to all basement membranes (11). Among the matrix molecules found, laminins (as glycoproteins) modulate adhesion and signaling through integrin binding. Additionally, they are able to adhere to other extracellular matrix molecules (12, 13). Each laminin molecule is a composed of three non-identical subunit, called α , β , and γ chains (14). At least 17 isoform

types of laminin have been identified which substantially were synthesized and expressed during fetal and adult periods (15). Different components are involved in induction of urethral bud into metanephric parenchyma such as extracellular matrix. Extracellular matrix provides progression and branching of urethra into renal parenchyma (15). Any changing in basement membrane and extracellular matrix may affect the cell differentiation of each organ. Several studies indicated that Alpha 5 ($\alpha 5$) chain of laminin (laminin $\alpha 5$) is essential for the lung development, both in embryonic and adult lung (16), natural development of smooth muscle cell types, basal membrane of blood vessels and digestive tract (14, 17). Laminin $\alpha 5$ is also essential for propagation and polarization of epithelial cells (18). Deletion of the gene encoding alpha5 chain of laminin $\alpha 5$ during fetal development in mouse lead to death (19) and imposed abnormality in kidney and digestive tract (20).

As regarded, in embryonic period, nicotine passes through the placental barrier and plays key roles in evolution of embryonic connective tissue. So, it seems that the resulting impact on neonatal kidney could be taken place by changes in connective tissue components such as laminin. On the other hand, as mentioned in the previous sentences, nicotine concentrates in mother milk and passes to newborn babies. Therefore, the aim of

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the present study was to examine the effects of nicotine intake during pregnancy and lactation on laminin $\alpha 5$ expression in different parts of renal fetal mice.

Materials and Methods

Nicotine administration and tissue preparation

This study was performed on 28 virgin mice Balbc/c. After mating and specifying the zero day of pregnancy, mice were randomly divided into two experimental (Group 1 and 2) and two sham control groups (Group 3 and 4). Group 1 was received 2 mg/kg of nicotine (according to the previous investigations (4, 21-23)) from day 7 of gestation to the last day of the pregnancy and the group 2 was injected by nicotine from day 7 until the two weeks of postnatal (lactation period) (9). Thus, in the group 1, nicotine is transferred through the placenta and affected its development stages, while, in the group 2, the stages were additionally affected by nicotine in breast milk. The sham control groups received nicotine solvent (normal saline) at the same period.

Animals were investigated in a standard condition (standard pellet food and water, $23 \pm 2^\circ\text{C}$ room temperature and humidity of $55 \pm 5\%$).

Nicotine was purchased from Sigma Company (N 3876, Sigma, USA) and dissolved in saline to prepare stock solutions. Mice were injected subcutaneously (SC) with 2 mg/kg nicotine (according to the previous investigations (4, 21-23)) or saline at 10:00 a.m. and the animals were housed in a standard transparent rectangular cage. The experimental protocol was approved by the Rafsanjan University Animal Care Committee.

Finally, in an ethical approved condition, the animals were rapidly sacrificed using cervical dislocation and subsequently their kidneys were removed in postnatal days, one and fourteen, and then fixed by incubation in buffer formalin 10% for 24 hours at room temperature to use for immunohistochemistry investigation.

Immunohistochemistry method

The kidney tissues from the groups were prepared according the routine histological methods. The full method has been described in our previous investigation (24).

Real-time PCR

RNA extraction, cDNA synthesis and Real-Time PCR condition have been described in our previous study (24) except primer sequencing which are presented in the table 1.

Statistical analysis

Based on the staining intensity, sections were graded, and according to the non-continues data, non-parametric statistical Mann-Whitney test was used to analyze the differences between groups using SPSS software (version 17). In contrast with the results from immu-

Table 1. The sequences of primers used in the project.

Chin name	Design
Laminin $\alpha 5$ - F	CGTCCCACAGGAATAGGCT
Laminin $\alpha 5$ - R	TACCAACGAAGGGCTGCG
GAPDH-F	AACTCCATTCTTCCACCTTTG
GAPDH-R	CTGTAGCCATATTCATTGTCATACCAG

nohistochemical analysis, the data from mRNA expression of laminin $\alpha 5$ were continues, hence, student t-test was used for comparing the result and are presented as mean \pm SD. Differences were considered significant at $P < 0.05$.

Results

Our results showed that the relative expression of mice kidney laminin $\alpha 5$ in the group 1 was increased significantly comparing with the control group ($p = 0.008$). In contrast, in group 2 (1.62 ± 0.19), there was not a significant increase in the expression of laminin $\alpha 5$ in comparison to control group (1 ± 0.20) ($p < 0.067$). The results also demonstrated that mRNA levels of laminin $\alpha 5$ were significantly decreased in group 2 in comparison to group 1 ($p < 0.001$) (Figure 1).

Immunohistochemical reactivity of kidney tissue to evaluate protein levels of laminin $\alpha 5$ in glomerulus, proximal convoluted tubules showing positive reactivity. The locations of laminin $\alpha 5$ expression in kidney tissue were determined according to the intensity of color darkness. Immunohistochemistry data illustrated that the differences regarding intensity of laminin $\alpha 5$ in glomerulus of group 1 (1 (1, 1.5)) in comparison to its control group (group 3) (1 (0.75, 1.5)) and group 2 (1 (1, 0.75)) in comparison to the corresponded controls (group 4) (1 (1, 0.75)) were not significant ($p = 0.434$ and $p = 0.791$, respectively). The statistical analysis revealed that the intensity of laminin $\alpha 5$ in the glomerulus of group 1 were significantly increased when compared to group 2 ($p = 0.045$). Figures 2 and 3 illustrate the intensity of laminin $\alpha 5$ in the groups.

The results also showed that protein levels of laminin $\alpha 5$ in proximal convoluted tubules of group 1 (2.5

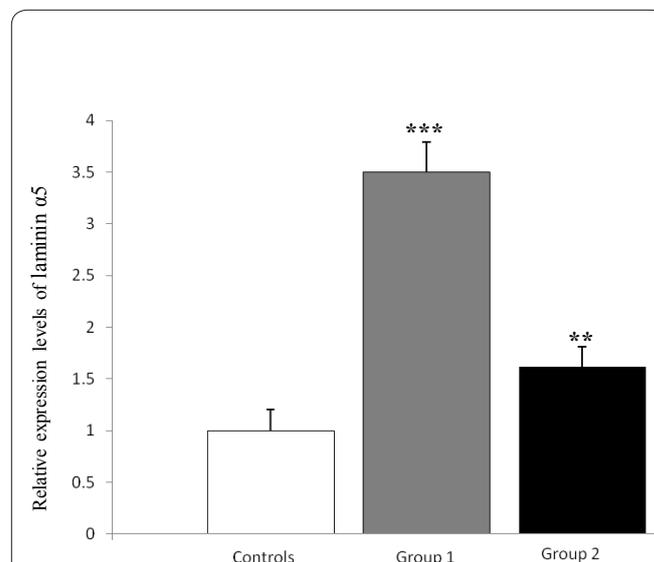


Figure 1. Effect of maternal nicotine exposure on the relative transcription level of laminin $\alpha 5$ in mice kidney. Pregnant animals were treated with nicotine (2 mg/kg) on different days (days 1 and 14 of newborn), and then, kidneys of newborn mice were sampled and studied using real-time PCR.

Each bar represents the mean \pm SD.

*** Significant differences between control and experimental group 1 ($p = 0.00001$).

** Significant differences between experimental group 1 and 2 ($p = 0.008$).

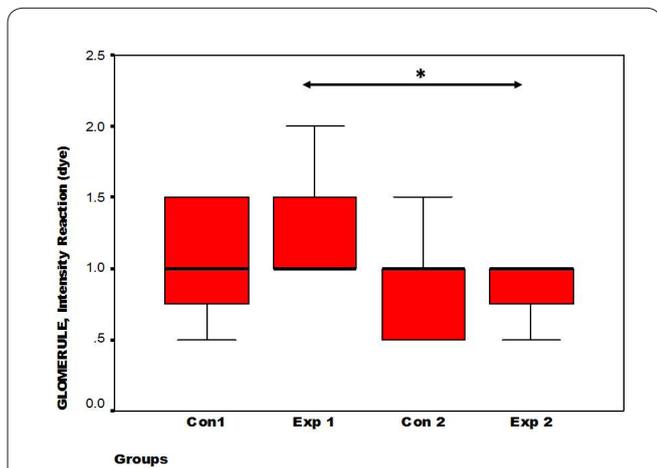


Figure 2. Box plots showing the effect of treatment of maternal nicotine exposure on the protein level of laminin in glomerulus of mice kidney. Pregnant animals were treated with nicotine (2 mg/kg) on different days (days 1 and 14 of newborn), and then, kidneys of newborn mice were sampled and studied using immunohistochemistry. The figure demonstrated that expression levels of laminin were decreased in glomerulus of mice kidney in group 2 in comparison to group 1.
* Significant differences between experimental group day1 and day 14 ($p= 0.045$).

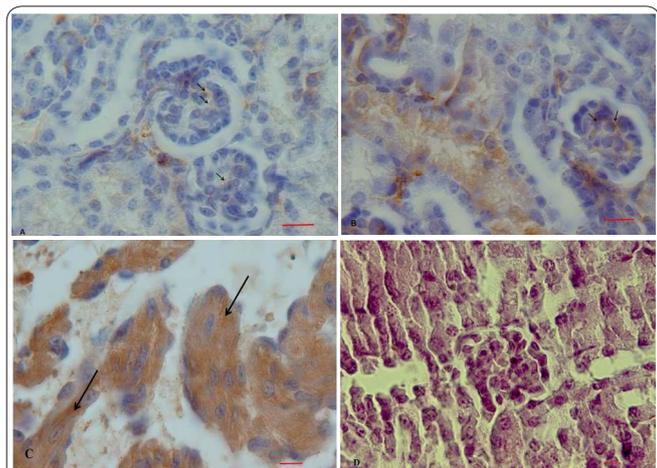


Figure 3. Sections of mice kidney incubated with laminin $\alpha 5$ antibody in the glomerulus. Left Upper panel (A): experimental group day 14, Right Upper panel (B): experimental group day 1, Left Lower panel (C): Control Positive, Right Lower panel (D): Control negative. The figure demonstrated that the reactivity was higher in experimental group day 1 (dark brown). Scale bar=10 μ m.

(2, 2.75)) were significantly increased in comparison to control (1.5 (1, 2)), while, the intensity of laminin $\alpha 5$ in proximal convoluted tubules of group 2 (1.5 (1.5, 2)) did not change significantly comparing with the corresponded control group (2 (1.5, 2.5)) ($p= 0.437$). Statistical analysis showed that the expression of laminin $\alpha 5$ in proximal convoluted tubules significantly decreased in group 2 compared to group 1 ($p<0.001$) (Figures 4 and 5).

Discussion

Basement membrane and its components as one the most important element of the parenchyma play vital role in development, function, and structural stability of kidney parenchyma (9, 10). Accordingly, each

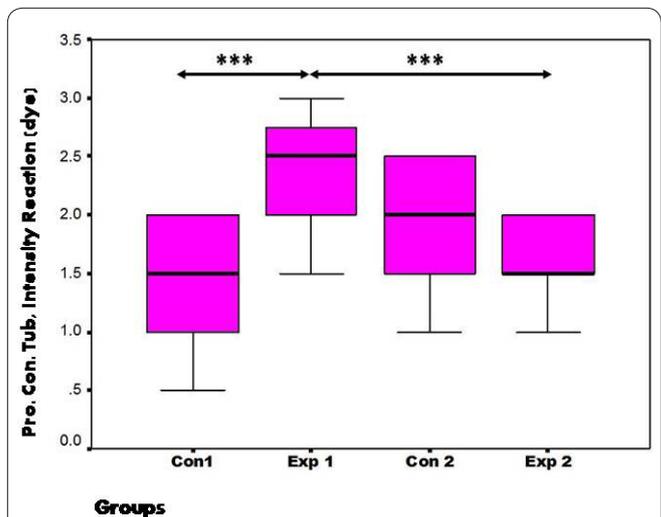


Figure 4. Box plots showing the effect of treatment of maternal nicotine exposure on the protein level of laminin in proximal convoluted tubules of mice kidney. Pregnant animals were treated with nicotine (2 mg/kg) on different days (days 1 and 14 of newborn), and then, kidneys of newborn mice were sampled and studied using immunohistochemistry technique. Figure demonstrated that protein levels of laminin were increased in group 1 in comparison to its corresponded controls and decreased in group 2 in comparison to group 1.
*** Significant differences between control and experimental group day1 ($p< 0.001$).
*** Significant differences between experimental day1 and experimental day14 group ($p< 0.001$).

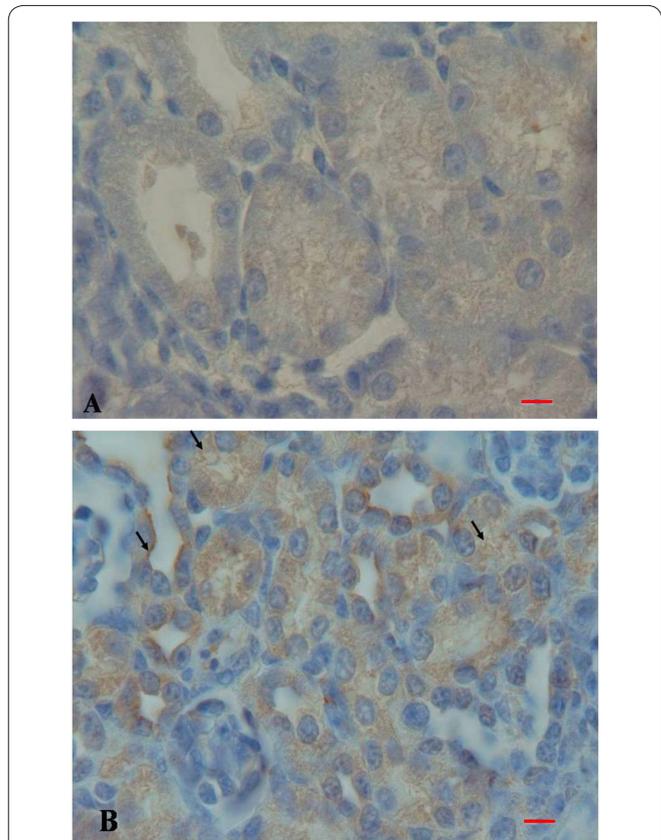


Figure 5. Sections of mice kidney incubated with laminin $\alpha 5$ antibody in the proximal convoluted tubules. Upper panel (a): control group day1, Lower panel (b): experimental group day1. The figure demonstrated that the reactivity was higher in experimental group day 1 (dark brown) in comparison to its corresponded control group. Scale bar=10 μ m.

change in the basement membrane may affect the cell differentiation of renal tissue (9, 10). Exogenous chemical materials which receive to the fetus via placenta could disrupt normal nephrogenous process or normal structure of kidney blood vessels (6). The same might happen with materials in the mother milk. It has been proven that nicotine as a toxic exogenous chemical material could affect fetus or newborn children from two mentioned ways (7, 8). Previous studies showed that the nicotine could affect the collagen type IV as a basement membrane component (9). We hypothesized that it may change the laminin $\alpha 5$ as another component of basement membrane.

The results of this study showed that nicotine administration during gestation and lactation could change expression of laminin $\alpha 5$ as one of the most important proteins of basement membrane.

Our results revealed that mRNA levels of laminin $\alpha 5$ were significantly increased in group 1 in comparison to controls and also decreased in group 2 in comparison to group 1. According to the results it seems that maternal nicotine exposure causes increased expression of laminin $\alpha 5$ in the kidney and exposure of nicotine via breast milk leads to decreased expression of laminin $\alpha 5$. Interestingly, the data from immunohistochemistry analysis showed that protein levels of laminin $\alpha 5$ were significantly increased in group 1 in the proximal convoluted tubules, but not in glomerulus. Overall, based on the results it may be concluded that maternal nicotine exposure leads to increased expression of laminin $\alpha 5$ in proximal convoluted tubules and increased mRNA of laminin $\alpha 5$ is also related to this region. Additionally, decreased expression of laminin $\alpha 5$ in both location (glomerulus and proximal convoluted tubules) demonstrated that exposure to nicotine during lactation leads to decreased expression of the molecule. Luck *et al.*, showed that nicotine exposure among mothers results in milk concentrations between 1.5 and 3 times more than the simultaneous maternal plasma concentration (25). The nicotine in breast milk is rapidly absorbed through the infant's gut, and accumulates in some tissues (26). Accordingly a reason for decreased expression of laminin $\alpha 5$ in group 2 might be because of presence of high nicotine concentration in mother breast milk.

Apart from concentration, the administration way of nicotine may cause the increase and decrease expression of laminin $\alpha 5$ in two different parts of our experiments. However, the changes in the expression of laminin $\alpha 5$ may lead to failure the normal kidney development. Perhaps some cases of reported failure kidney such as kidney malformations (7), wider and thicker kidney, reduction of corpuscles and kidney weight (8) may be related to change of laminin $\alpha 5$ expression by maternal nicotine exposure.

Previous studies indirectly showed that the receptor for nicotine is expressed in kidney (27) so, the change in laminin $\alpha 5$ expression in present study, may result from the direct effects of nicotine on the nicotine receptors.

Apart from nicotine, changes in the structure of basement membrane and its components have been reported (9). For example, Funabiki and his colleagues showed that the basement membrane thickness and collagen density were changed in diabetes and hyper activity of kidney (28). Similar changes such as, loss of laminin,

an increased in the expression of laminin beta2 and alpha1 chains and a reduction in laminin $\alpha 5$ expression have been reported in cases of the long term activity of kidney, aging, malnutrition, cancer, asthma and allergies (29). Common results of these studies emphasize the preservation of basement membrane structure to develop practically and structurally normal kidney, therefore, it seems that nicotine with change in laminin $\alpha 5$ expression in embryonic and lactation period disrupts basement membrane structure and reported renal abnormalities may be related to the changes in the laminin $\alpha 5$ expression.

Based on the results presented here, it may be concluded that in the gestation and lactation periods, the laminin $\alpha 5$ expression in the renal basement membrane of fetus and offspring was altered by maternal nicotine intake and perhaps the adverse effects of nicotine were induced through change of the expression of laminin $\alpha 5$.

Acknowledgments

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