Reduced placental size and increased apoptosis are associated with prenatal nicotine exposure in rats

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Abstract. – OBJECTIVE: Smoking during pregnancy has been linked to a variety of negative embryonic and neonatal consequences. Nicotine is the major constituent of tobacco smoke, which was associated with adverse impacts on histological and functional features of the placenta. This study aims to investigate the potential influence of nicotine exposure on the rat placenta and fetus.

MATERIALS AND METHODS: Nicotine was administrated through the drinking water of female pregnant rats. The placental size, as well as the fetal body weight and size, were measured at E20. The mRNA expression of the Bax gene (pro-apoptotic), the Bcl-2 gene (anti-apoptotic) and the angiogenic genes VEGF, Flt-1, and HIF1 were measured in placental tissue. Furthermore, Immunohistochemistry (IHC) using Bax, caspase 9 and VEGF antibodies were performed on placental sections.

RESULTS: The results of the current study showed a significant reduction in the size of the placenta along with fetal body weight in nicotine treated group compared to the control group. Apoptosis was observed to be boosted in the placenta of the nicotine-treated group. This was associated with upregulation of Bax expression combined with no change in the expression of Bcl-2 in the treated group. On the other hand, there was no difference in the expression of angiogenic factors VEGF, Flt-1, or HIF1 between tested groups.

CONCLUSIONS: In utero nicotine exposure in pregnant rats showed deleterious impacts on fetus growth and weight, as well as placental size. These were accompanied by increased apoptosis within the placenta, as revealed by Bax gene upregulation.

Key Words: Nicotine, nAChR, Placenta, Apoptosis, Bax, Bcl-2.

Introduction

Cigarette smoking during pregnancy has been considered an important risk factor for a wide variety of adverse reproductive outcomes1. Specifically, it has been associated with deleterious outcomes such as increased neonatal morbidity, premature delivery, low birth weight, and increased neonatal mortality2,3, in addition to increasing the risk of neurobehavioral and psychiatric problems4. Nicotine is considered the main pathogenic element of tobacco smoke; it exerts its biological effect via the activation of nicotinic acetylcholine receptors (nAChRs). nAChRs are mammalian ligand-gated ion channels composed of sixteen subunits of pentameric structure (α1–10, β1–4, δ, ε, and γ)5-7.

Previous human research has proven that maternal smoking has negative impacts on the structure and function of the placenta, which include: Reduced chorionic villi diameter, vasoconstriction, increased apoptosis8-11, inhibition of trophoblast migration, invasion, and differentiation12,13, as well as inhibition of amino acid transport14. While the exact composition of cigarette smoke that causes the above-mentioned downstream histological alterations is unknown, nicotine has been suggested as a major contributor.

As a component of the placental cholinergic system, which plays a key role in regulating developmental processes related to placental growth, nAChRs subunits (α1–7, α9, α10, β1–4, δ, ε, and γ) are ubiquitously expressed in all human and rat placental cell types15-19. Cigarette smoking during pregnancy may lead to chronic stimulation of the nAChR by nicotine, which can lead to imbalanced receptor activation or functional desensitization, as...
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well as smoking-related placental malfunction. The purpose of the current study was to evaluate the in vivo deleterious effects of nicotine on placental development in terms of placental size and fetal growth, as well as to evaluate the expression of major factors involved in placental apoptosis and angiogenesis.

**Materials and Methods**

**Oral Nicotine Administration**

Formal approval was granted by the Ethics Committee at Yarmouk University for use of animals in the current research (No. IACUC/2021/6). Eight to ten weeks-old female Wistar rats (with an average weight of 200 grams) were mated, pregnancy was validated by visualizing the vaginal plug. Pregnant females (n=15) assigned for nicotine treatment were singly housed and immediately given free access to drinking water containing 0.06 mg/ml nicotine and 2% saccharin as their sole water supply. Nicotine exposure was performed by using nicotine hydrogen bitartrate salt from (Sigma-Aldrich, St. Louis, MO, USA) and concentration was calculated concerning the free base. The concentration of nicotine selected in this study was used as it has been shown previously to obtain daily nicotine consumption similar to that experienced by habitual smokers20,21. The control group (n=10) was given normal tap water. Rats in both groups had free access to water and food with an average water consumption of 22-24 ml per day.

Pregnant rats (control and nicotine treated) were killed at embryonic day (E) 20. Fetal and placental size and weight were measured. The placental tissues were used in quantitative real-time reverse transcription qRT-PCR and immunohistochemical studies.

**Immunohistochemistry**

Placentas fixation was performed in paraformaldehyde (4%) followed by tissue processing and embedding in paraffin as described before22 and sectioned at 5 µm. For the assessment of the target markers by IHC, sections were incubated with the appropriate primary antibodies: anti-Bax (Cat # sc-7480), anti-Caspase 9 (Cat # sc-56076) and anti-VEGF (Cat # sc-7269) from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Immunopositive staining was identified using the biotinylated secondary antibodies and avidin-peroxidase (ABC-HRP kit, Vector Laboratories, Peterborough, UK) using diaminobenzidine (DAB) chromagen from (Vector Laboratories, CA, USA). Counterstaining was performed using H & E from (Sigma-Aldrich, St. Louis, MO, USA). The processed tissues were examined under an Optika microscope and evaluated using Fiji Image J software.

**Total mRNA Extraction, cDNA Synthesis and qRT-PCR**

A commercial kit (Total-RNA Extraction Kit) supplied by (Jena Bioscience, Jena, Germany) was used for the total RNA from placenta tissue, and the manufacturer’s recommendations were followed for the total mRNA extraction protocol. The RNA concentration was assessed using Quantus Fluorometer after RNA staining (Promega, Madison, WI, USA). cDNA synthesis was performed according to the supplied protocol using Revert Aid First cDNA Synthesis Kit from (Thermo Fisher Scientific, Waltham, MA, USA). All extracted and total RNA and synthesized cDNA were stored at -80°C for the next use. For gene expression assessment, the quantitative real-time PCR (qRT-PCR) was conducted using 5X EvaGreen Supermix (Soils Biodyne, Tartu, Estonia). Briefly, the final volume was 20 µl which contained 2 µl EvaGreen Supermix, 1 µl of the forward primer, 1 µl of the reverse primer, 14 µl of nuclease-free water, and 2 µl cDNA. The amplification cycling was performed according to the following amplification conditions: Activation step (95°C/3 min), 40 cycles of (95°C/5s and 60°C/30s). GAPDH gene was used as a reference gene. Gene primers sequences

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer sequence</th>
<th>Reverse primer sequence</th>
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<tbody>
<tr>
<td>Bax</td>
<td>5′-CGGCCGAGTTGGAGATGAACTGG-3′</td>
<td>5′-CTAGCAAAGTAGAAGAGGCAACC-3′</td>
</tr>
<tr>
<td>Rel-2</td>
<td>5′-TGAGTGATGACTGACTAACC-3′</td>
<td>5′-CACAGGAGGAACCAACACAGAGG-3′</td>
</tr>
<tr>
<td>VEGF</td>
<td>5′-CCAACTGAGTGACCTGGAG-3′</td>
<td>5′-GTGAGGGTCTGATCCACGATG-3′</td>
</tr>
<tr>
<td>Flt-1</td>
<td>5′-AACACAGGGACATGAC-3′</td>
<td>5′-GCT TCA GTT TTC GGA TGA-3′</td>
</tr>
<tr>
<td>HIF-1α</td>
<td>5′-CAACTGCCCACCACTGATG-3′</td>
<td>5′-TGGGTCAGGGATGGATGC-3′</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5′-ATGGTGAAGGTCGGTG-3′</td>
<td>5′-GAACCTTGCCCGTGGTAG-3′</td>
</tr>
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Table I. List of primers used for qRT-PCR validation.

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(Table I) were designed using Primer3 software. All primers were supplied by IDT (Integrated DNA Technologies Inc., Coralville, IA, USA). 2\( \Delta \Delta CT \) approach was used for the analysis and assessment of genes expression.

Statistical Analysis

SPSS V19 (SPSS Inc., IBM, Armonk NY, USA) was used for the calculation of the Student’s \( t \)-test where the \( p \)-value was considered significant when it is less than 0.05.

Results

The Effect of Nicotine on Placental Size and Fetal Growth

As illustrated in Figures 1A and B, placental size (diameter) was assessed at E20, where a substantial reduction in the diameter of nicotine-exposed groups’ placentas (1.08 ± 0.02 cm) was observed in comparison to control groups’ placentas (1.30 ± 0.04 cm).

Additionally, fetal growth was evaluated by measuring the fetal length and weight at E20 (Figure 1C). We noticed a significant reduction in fetal body length (2.6 ± 0.04 cm) and weight (1.9 ± 0.07 gram) in nicotine exposed group compared to the fetuses from the control group as shown in Figure 1 D and E (Fetal Length: 2.9 ± 0.04 cm; Fetal Weight: 2.3 ± 0.03 g).

The Effect of Nicotine on Placental Apoptosis

The apoptotic activity in the placenta was evaluated by assessing the expression of the \( Bax \) gene and \( Bcl-2 \) gene as apoptotic and anti-apoptotic genes, respectively, using qRT-PCR. As shown in figure 2A, the expression of the \( Bax \) gene was considerably upregulated in the placentas of the nicotine-exposed group compared to those of the control group (+2.7-fold overexpression; \( p < 0.05 \)). Whereas the anti-apoptotic \( Bcl-2 \) gene did not show any significant change in the nicotine-treated group compared to the control group (Figure 2B).
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In line with the qRT-PCR results, the placentas of nicotine-exposed dams had consistently elevated quantities of Bax protein in the nuclei of the trophoblast layer of villi than control group placentas (Figure 2C, D). Furthermore, increased apoptosis in nicotine-treated placentas was also demonstrated by a significantly increased amount of Caspase 9 protein in the treatment group as shown in Figures 2 E and F.

**The Effect of Nicotine on Placental Vascularization**

The placental expression of VEGF, Flt-1, and HIF1 genes was measured using qRT-PCR to assess the influence of nicotine on placental angiogenic factors. There was no apparent difference between the groups (Figure 3). Furthermore, immunohistochemistry examination of VEGF protein expression in both treated and control groups revealed a similar expression pattern (data not shown).

**Discussion**

The current study aimed to evaluate the influence of nicotine on placental development in vivo. Our results revealed that nicotine administration
during pregnancy can significantly affect placental size and fetal growth. In addition, nicotine treatment was found to be involved in altering the rate of apoptotic activity in their placentas, with no to minimal effect on placental vascularization. To best mimic chronic placental exposure to nicotine experienced by frequent smokers, nicotine was administered to pregnant rats in the drinking water, which was one of numerous nicotine administration strategies used in the literature. By applying this mode of administration, we may, more accurately, simulate the pulsing mode of nicotine delivery by smoking while avoiding the desensitization of nAChRs that can occur with chronic continuous nicotine exposure employed in other modalities.

We demonstrated that nicotine treatment lowers placental size considerably in the model employed in this study. Furthermore, the lighter and smaller fetuses of nicotine-treated mothers compared to control mothers were noticeable. We know that the placenta promotes oxygen and nutrient transport from the mother to fetus, and that altered placental development, structure, and shape have been linked to placental insufficiency, which leads to intrauterine growth restriction. Various human studies have connected maternal tobacco use to a variety of consequences on the placental structure, as well as lower birth weight. These studies, however, have not determined which component of cigarette smoke causes this detrimental effect. Our study revealed that nicotine, one of over 4000 chemicals in tobacco smoke, can negatively affect the placental structure, resulting in lower placental size and impaired fetal growth.

Aside from the key findings in this study on the effect of nicotine on the placental structure, we found nicotine to be directly implicated in the placenta’s dysregulation of apoptotic activity, which is crucial for the placenta’s growth and aging. Several studies suggested that the rate of apoptosis increases progressively during pregnancy, which is considered a fundamental process in the normal placental development that is essential for normal pregnancy and term delivery. Abnormal apoptosis in trophoblast cells has been linked to a variety of undesirable pregnancy outcomes, including intrauterine growth restriction, preterm birth, and preeclampsia. The regulation of the apoptotic process is mediated by several genes. The pro-apoptotic gene Bax and the anti-apoptotic gene Bcl-2 are the most well-known of these genes. The ratio of the activity of these two genes has been found to determine whether apoptosis in the placenta is stimulated. In the present study, our analysis showed significant overexpression of the Bax gene in placentas of nicotine-exposed pregnant compared to those of control pregnant, while no significant differences were detected in placental Bcl-2 gene expression. These results are in agreement with previous analysis on placental samples from pregnancies with intrauterine growth restriction. It’s possible that the increased expression of Bax in nicotine-exposed dams’ placentas, while the Bcl-2 gene remains intact, may lead to an imbalance between proapoptotic and antia apoptotic activities, reducing the amount of apoptosis-inhibiting mechanisms. In addition to the overexpression of Bax, the induced apoptosis in
placentas of nicotine-exposed pregnant was also confirmed by the overexpression of Caspase 9 protein, which regulates physiological cell death as a cysteine-aspartic protease known for its role as an activator of intrinsic apoptosis. This study, on the other hand, found that nicotine does not affect placental vascularization. Nicotine treatment did not influence the expression of the main placental angiogenic factor VEGF and its receptor VEGFR1(FLT-1) in the placenta. Similarly, no effect was found on the expression of HIF1α, which regulates the expression of VEGF, FLT-1 and other angiogenic factors in a hypoxic environment. Smoking during pregnancy has been linked to a lower incidence of preeclampsia. This can be attributed to whether smoking promotes the expression of VEGF, which regulates cytotrophoblast survival and differentiation and is linked to a lower risk of preeclampsia, or suppresses the production of soluble Flt-1 in the placenta, thus increasing the VEGF/sFLT-1 ratio, which enhances placental angiogenesis. Consequently, and based on our findings that nicotine treatment did not affect the placental expression of VEGF or FLT-1, we propose that other components of cigarette smoking, rather than nicotine, may be responsible for the protective mechanism against preeclampsia. It is worth mentioning that during this study, the results were based on placentas from embryonic day (E) 20, which is a late stage in pregnancy, while placentas from earlier stages were not investigated which could be a limitation of this study.

Conclusions

The present study demonstrates that nicotine exposure in pregnant rats has a negative impact on fetus growth and weight, as well as placental size. These were associated with increases in apoptosis within the placenta. These findings could be used to perform further investigations to elucidate the potential molecular mechanism underlying the observed effect.

Ethical Approval

All procedures involving animals in this study were approved by the Ethics Committee for Use of Animals in Research at Yarmouk University (No. IACUC/2021/6).

Conflict of Interests

The authors declare there is no conflict of interest in the current study.

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