European Review for Medical and Pharmacological Sciences 2022; 26: 1586-1593

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

A. ALZU'BI[1], M.S. AL ZOUBI[1], G.T. ABDELHADY[1,2], B. AL-TRAD[3], S. OMARI[3], M.I. ABUALARJAH[1], W. EL-HUNEIDI[4], D.R. ALZU'BI[5], J.M. BANI-ISSA[1], G.J. CLOWRY[6]

[1]Department of Basic Medical Sciences, Faculty of Medicine, Yarmouk University, Irbid, Jordan
[2]Department of Anatomy and Embryology, Faculty of Medicine, Ain Shams University, Cairo, Egypt
[3]Department of Biological Sciences, Faculty of Science, Yarmouk University, Irbid, Jordan
[4]Department of Basic Medical Sciences, College of Medicine, University of Sharjah, Sharjah, United Arab Emirates
[5]Pediatric Department, King Abdullah University Hospital, Irbid, Jordan
[6]Biosciences Institute, Newcastle University, Newcastle upon Tyne, United Kingdom

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 placentas 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 outcomes[1]. Specifically, it has been associated with deleterious outcomes such as increased neonatal morbidity, premature delivery, low birth weight, and increased neonatal mortality[2,3], in addition to increasing the risk of neurobehavioral and psychiatric problems[4]. 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 apoptosis[8-11], inhibition of trophoblast migration, invasion, and differentiation[12,13], as well as inhibition of amino acid transport[14]. 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 types[15-19]. 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

Corresponding Author: Ayman Alzu'bi, MD; e-mail: ayman.alzubi@yu.edu.jo

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 smokers[20,21]. 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 before[22] 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 I. List of primers used for qRT-PCR validation.

Gene	Primer sequences Forward primer	Reverse primer
Bax	5′-CGGCGAATTGGAGATGAACTGG-3′	5′-CTAGCAAAGTAGAAGAGGGCAACC-3′
Bcl-2	5′-TGTGGATGACTGACTACCTGAACC-3′	5′-CAGCCAGGAGAAATCAAACAGAGG-3′
VEGF	5'-GCAATGATGAAGCCCTGGAG-3'	5'-GGTGAGGTTTGATCCGCATG-3'
Flt-1	5'-AACAACAGGACCATGCAC-3'	5'-GCT TCA GTT TTC GGA TGA-3'
HIF-1α	5'-CAACTGCCACCACTGATGAA-3'	5'-TGGGTAGAAGGTGGAGATGC-3'
GAPDH	5'-ATGGTGAAGGTCGGTGTG-3'	5'- GAACTTGCCGTGGGTAGA-3'

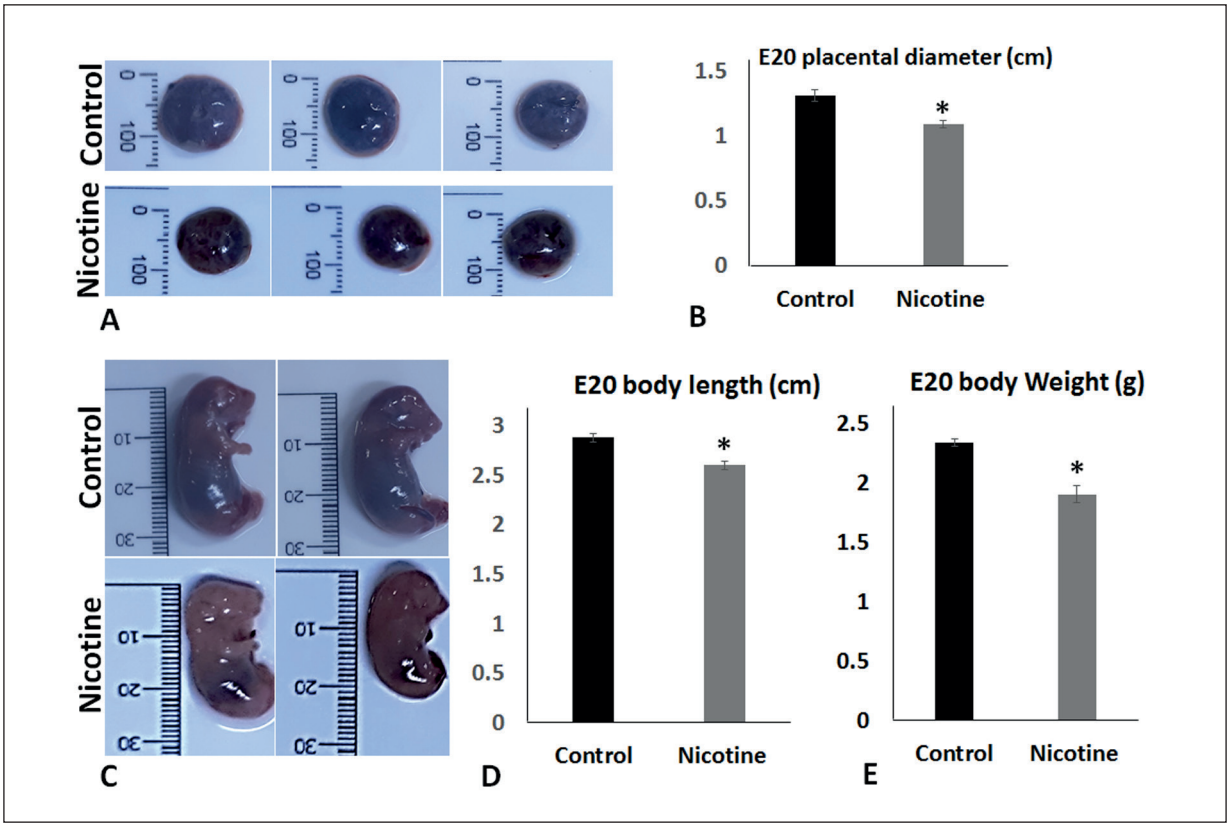

Figure 1. Reduced placental size and fetal growth in nicotine treated mothers. (**A**) Representative images of E20 placentas from nicotine-exposed and control mothers. (**B**) Diameter of E20 placentas. (**C**) Representative images of E20 fetuses from nicotine-exposed and control mothers. (**D**) Fetal body length at E20. (**E**) Fetal body weight at E20. Bar charts show the results of n = 15 placentas/fetuses randomly selected from 3 females from each group. (*= $p < 0.05$).

(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).

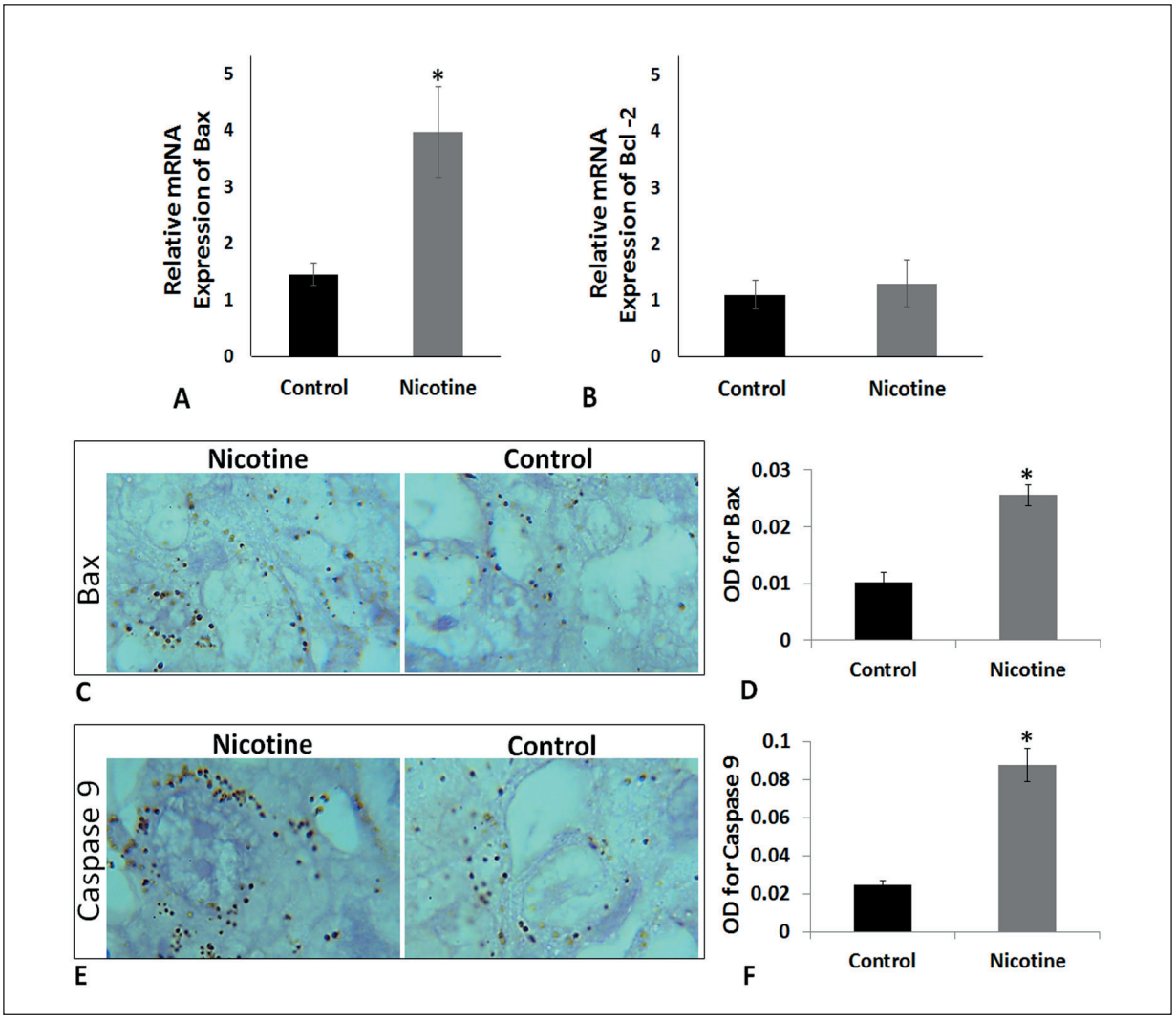

Figure 2. Increased apoptosis in placentas of nicotine-exposed mothers. (**A**) Bax mRNA expression in placentas of nicotine-exposed mothers compared to control mothers (*$p < 0.05$). (**B**) Bcl-2 mRNA expression in placentas of nicotine-exposed mothers compared to control mothers. (**C**) Immunohistochemistry of Bax in extravillous trophoblasts in placentas of nicotine-exposed mothers compared to control mothers (magnification, × 400). (**D**) Immunohistochemistry optical density score of Bax. Data represent the mean ± SEM (*=$p < 0.05$). (**E**) Immunohistochemistry of Caspase 9 in placentas of nicotine-exposed mothers compared to control mothers (magnification, × 400). (**F**) Immunohistochemistry optical density score of Caspase 9. Data represent the mean ± SEM (*=$p < 0.05$).

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

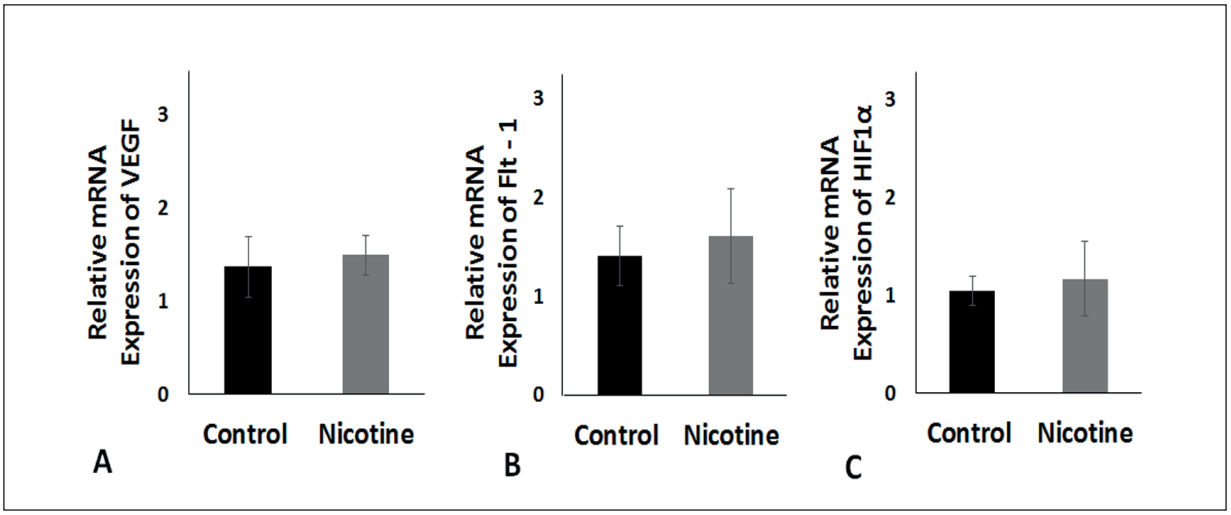

Figure 3. The mRNA expression of angiogenic factors (**A**) VEGF, (**B**) Flt-1 , and (**C**) HIF1α in placentas of nicotine-exposed mothers compared to control mothers.

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[23]. 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[21].

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[24-26]. Various human studies have connected maternal tobacco use to a variety of consequences on the placental structure[27,28] as well as lower birth weight[9,29,30]. 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[31], 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[32,33]. 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[32]. Abnormal apoptosis in trophoblast cells has been linked to a variety of undesirable pregnancy outcomes, including intrauterine growth restriction, preterm birth, and preeclampsia[34-38]. The regulation of the apoptotic process is mediated by several genes. The pro-apoptotic gene *Bax* and the antiapoptotic 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[39-41]. 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[42,43]. 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 antiapoptotic activities, reducing the amount of apoptosis-inhibiting mechanisms[43]. 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[44].

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[45,46]. Smoking during pregnancy has been linked to a lower incidence of preeclampsia[47]. 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[48], or suppresses the production of soluble Flt-1 in the placenta, thus increasing the VEGF/sFLT-1 ratio, which enhances placental angiogenesis[48]. 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[49,50]. It is worth mentioning that during this study, the results were based on palectas from embryonic day (E) 20 , which is a late stage in pregneancy, 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.

Research Funding

This research was funded by the Deanship of Scientific Research and Graduate Studies at Yarmouk University (Grant # 17/2021).

References

1) Jauniaux E, Burton GJ. Morphological and biological effects of maternal exposure to tobacco smoke on the feto-placental unit. Early Hum Dev 2007; 83: 699-706.
2) Cliver SP, Goldenberg RL, Cutter GR, Hoffman HJ, Davis RO, Nelson KG. The effect of cigarette smoking on neonatal anthropometric measurements. Obstet Gynecol 1995; 85: 625-630.
3) Kramer MS. Intrauterine growth and gestational duration determinants. Pediatrics 1987; 80: 502-511.
4) Pauly JR, Slotkin TA. Maternal tobacco smoking, nicotine replacement and neurobehavioural development. Acta Paediatr 2008; 97: 1331-1337.
5) Albuquerque EX, Pereira EF, Alkondon M, Rogers SW. Mammalian nicotinic acetylcholine receptors: from structure to function. Physiol Rev 2009; 89: 73-120.
6) Alzu'bi A, Middleham W, Shoaib M, Clowry GJ. Selective expression of nicotinic receptor sub-unit mRNA in early human fetal forebrain. Front Mol Neurosci 2020; 13: 72.
7) Kulbatskii D, Bychkov M, Lyukmanova EJRJ. Human nicotinic acetylcholine receptors: part I—structure, function, and role in neuromuscular transmission and CNS functioning. Russian Journal of Bioorganic Chemistry 2018; 44: 595-607.
8) Chhabra D, Sharma S, Kho AT, Gaedigk R, Vyhlidal CA, Leeder JS, Morrow J, Carey VJ, Weiss ST, Tantisira KG, DeMeo DL. Fetal lung and placental methylation is associated with in utero nicotine exposure. Epigenetics 2014; 9: 1473-1484.
9) Garrabou G, Hernandez AS, Catalan Garcia M, Moren C, Tobias E, Cordoba S, Lopez M, Figueras F, Grau JM, Cardellach F. Molecular basis of reduced birth weight in smoking pregnant women: mitochondrial dysfunction and apoptosis. Addict Biol 2016; 21: 159-170.
10) Rath G, Dhuria R, Salhan S, Jain AK. Morphology and morphometric analysis of stromal capillaries in full term human placental villi of smoking mothers: an electron microscopic study. Clin Ter 2011; 162: 301-305.
11) Romani F, Lanzone A, Tropea A, Tiberi F, Catino S, Apa R. Nicotine and cotinine affect the release of vasoactive factors by trophoblast cells and human umbilical vein endothelial cells. Placenta 2011; 32: 153-160.
12) Genbacev O, Bass KE, Joslin RJ, Fisher SJ. Maternal smoking inhibits early human cytotrophoblast differentiation. Reprod Toxicol 1995; 9: 245-255.
13) Holloway AC, Salomon A, Soares MJ, Garnier V, Raha S, Sergent F, Nicholson CJ, Feige JJ, Benharouga M, Alfaidy N. Characterization of the

adverse effects of nicotine on placental development: in vivo and in vitro studies. Am J Physiol Endocrinol Metab 2014; 306: E443-456.
14) Pastrakuljic A, Derewlany LO, Knie B, Koren G. The effects of cocaine and nicotine on amino acid transport across the human placental cotyledon perfused in vitro. J Pharmacol Exp Ther 2000; 294: 141-146.
15) King RG, Gude NM, Krishna BR, Chen S, Brennecke SP, Boura AL, Rook TJ. Human placental acetylcholine. Reprod Fertil Dev 1991; 3: 405-411.
16) Lips KS, Bruggmann D, Pfeil U, Vollerthun R, Grando SA, Kummer W. Nicotinic acetylcholine receptors in rat and human placenta. Placenta 2005; 26: 735-746.
17) Machaalani R, Ghazavi E, Hinton T, Makris A, Hennessy A. Immunohistochemical expression of the nicotinic acetylcholine receptor (nAChR) subunits in the human placenta, and effects of cigarette smoking and preeclampsia. Placenta 2018; 71: 16-23.
18) Machaalani R, Ghazavi E, Hinton T, Waters KA, Hennessy A. Cigarette smoking during pregnancy regulates the expression of specific nicotinic acetylcholine receptor (nAChR) subunits in the human placenta. Toxicol Appl Pharmacol 2014; 276: 204-212.
19) Sastry BV. Human placental cholinergic system. Biochem Pharmacol 1997; 53: 1577-1586.
20) Benowitz NL, Jacob P, 3rd. Individual differences in nicotine kinetics and metabolism in humans. NIDA Res Monogr 1997; 173: 48-64.
21) Schneider T, Bizarro L, Asherson PJ, Stolerman IP. Gestational exposure to nicotine in drinking water: teratogenic effects and methodological issues. Behav Pharmacol 2010; 21: 206-216.
22) Al-Trad B, Abo-Alrob O, Jaradat Y, Al Zoubi M, Alkarki AK, Aljabali AA, Qar J, Omari S, Shehab M, Kanan B. Effect of estrogen and progesterone hormones on the expression of angiotensin II receptors in the heart and aorta of male rats. Endocr Metab Immune Disord Drug Targets 2021; 21: 1504-1511.
23) Matta SG, Balfour DJ, Benowitz NL, Boyd RT, Buccafusco JJ, Caggiula AR, Craig CR, Collins AC, Damaj MI, Donny EC, Gardiner PS, Grady SR, Heberlein U, Leonard SS, Levin ED, Lukas RJ, Markou A, Marks MJ, McCallum SE, Parameswaran N, Perkins KA, Picciotto MR, Quik M, Rose JE, Rothenfluh A, Schafer WR, Stolerman IP, Tyndale RF, Wehner JM, Zirger JM. Guidelines on nicotine dose selection for in vivo research. Psychopharmacology (Berl) 2007; 190: 269-319.
24) Baschat AA, Hecher K. Fetal growth restriction due to placental disease. Semin Perinatol 2004; 28: 67-80.
25) Gaccioli F, Lager S. Placental nutrient transport and intrauterine growth restriction. Front Physiol 2016; 7: 40.
26) Lager S, Powell TL. Regulation of nutrient transport across the placenta. J Pregnancy 2012; 2012: 179827.
27) Bouhours-Nouet N, May-Panloup P, Coutant R, de Casson FB, Descamps P, Douay O, Reynier P, Ritz P, Malthiery Y, Simard G. Maternal smoking is associated with mitochondrial DNA depletion and respiratory chain complex III deficiency in placenta. Am J Physiol Endocrinol Metab 2005; 288: E171-177.
28) Slatter TL, Park L, Anderson K, Lailai-Tasmania V, Herbison P, Clow W, Royds JA, Devenish C, Hung NA. Smoking during pregnancy causes double-strand DNA break damage to the placenta. Hum Pathol 2014; 45: 17-26.
29) Larsen S, Haavaldsen C, Bjelland EK, Dypvik J, Jukic AM, Eskild A. Placental weight and birthweight: the relations with number of daily cigarettes and smoking cessation in pregnancy. A population study. Int J Epidemiol 2018; 47: 1141-1150.
30) Wang N, Tikellis G, Sun C, Pezic A, Wang L, Wells JC, Cochrane J, Ponsonby AL, Dwyer T. The effect of maternal prenatal smoking and alcohol consumption on the placenta-to-birth weight ratio. Placenta 2014; 35: 437-441.
31) Behnke M, Smith VC, Committee on Substance A, Committee on F, Newborn. Prenatal substance abuse: short- and long-term effects on the exposed fetus. Pediatrics 2013; 131: e1009-1024.
32) Sgarbosa F, Barbisan LF, Brasil MA, Costa E, Calderon IM, Goncalves CR, Bevilacqua E, Rudge MV. Changes in apoptosis and Bcl-2 expression in human hyperglycemic, term placental trophoblast. Diabetes Res Clin Pract 2006; 73: 143-149.
33) Smith SC, Baker PN, Symonds EM. Placental apoptosis in normal human pregnancy. Am J Obstet Gynecol 1997; 177: 57-65.
34) Allaire AD, Ballenger KA, Wells SR, McMahon MJ, Lessey BA. Placental apoptosis in preeclampsia. Obstet Gynecol 2000; 96: 271-276.
35) Diplas AI, Lambertini L, Lee MJ, Sperling R, Lee YL, Wetmur J, Chen J. Differential expression of imprinted genes in normal and IUGR human placentas. Epigenetics 2009; 4: 235-240.
36) Endo H, Okamoto A, Yamada K, Nikaido T, Tanaka T. Frequent apoptosis in placental villi from pregnancies complicated with intrauterine growth restriction and without maternal symptoms. Int J Mol Med 2005; 16: 79-84.
37) Hu R, Zhou S, Li X. Altered Bcl-2 and Bax expression is associated with cultured first trimester human cytotrophoblasts apoptosis induced by hypoxia. Life Sci 2006; 79: 351-355.
38) Hung TH, Chen SF, Liou JD, Hsu JJ, Li MJ, Yeh YL, Hsieh TT. Bax, Bak and mitochondrial oxidants are involved in hypoxia-reoxygenation-induced apoptosis in human placenta. Placenta 2008; 29: 565-583.
39) Daher S, Guimaraes AJ, Mattar R, Ishigai MM, Barreiro EG, Bevilacqua E. Bcl-2 and Bax expressions in pre-term, term and post-term placentas. Am J Reprod Immunol 2008; 60: 172-178.
40) De Falco M, De Luca L, Acanfora F, Cavallotti I, Cottone G, Laforgia V, De Luca B, Baldi A, De Luca AJTHJ. Alteration of the Bcl-2: Bax ratio in the placenta as pregnancy proceeds. Histochem J 2001; 33: 421-425.
41) Ratts VS, Tao XJ, Webster CB, Swanson PE, Smith SD, Brownbill P, Krajewski S, Reed JC, Tilly JL, Nelson DM. Expression of BCL-2, BAX and BAK in the trophoblast layer of the term human

placenta: a unique model of apoptosis within a syncytium. Placenta 2000; 21: 361-366.
42) Agata KB, Anita S, Urszula KK, Agnieszka NK, Grzegorz B. Expression of caspase-3, Bax nad Bcl-2 in placentas from pregnancies complicated by treated and non-treated fetal growth restriction. Ginekol Pol 2009; 80: 652-656.
43) Barrio E, Calvo MT, Romo A, Alvarez R, Gutierrez JI, Naval J, Ferrandez Longas A. Intrauterine growth retardation: study of placental apoptosis. J Pediatr Endocrinol Metab 2004; 17 Suppl 3: 451-456.
44) Avrutsky MI, Troy CM. Caspase-9: a multimodal therapeutic target with diverse cellular expression in human disease. Front Pharmacol 2021; 12: 701301.
45) Forsythe JA, Jiang BH, Iyer NV, Agani F, Leung SW, Koos RD, Semenza GL. Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor 1. Mol Cell Biol 1996; 16: 4604-4613.
46) Manalo DJ, Rowan A, Lavoie T, Natarajan L, Kelly BD, Ye SQ, Garcia JG, Semenza GL. Transcriptional regulation of vascular endothelial cell responses to hypoxia by HIF-1. Blood. 2005; 105: 659-669.
47) Zhang J, Klebanoff MA, Levine RJ, Puri M, Moyer P. The puzzling association between smoking and hypertension during pregnancy. Am J Obstet Gynecol 1999; 181: 1407-1413.
48) Wang H, Yu M, Ochani M, Amella CA, Tanovic M, Susarla S, Li JH, Wang H, Yang H, Ulloa LJN. Nicotinic acetylcholine receptor α7 subunit is an essential regulator of inflammation. Nature 2003; 421: 384-388.
49) Venditti CC, Casselman R, Murphy MS, Adamson SL, Sled JG, Smith GN. Chronic carbon monoxide inhalation during pregnancy augments uterine artery blood flow and uteroplacental vascular growth in mice. Am J Physiol Regul Integr Comp Physiol 2013; 305: R939-948.
50) Wikström A-K, Stephansson O, Cnattingius SJH. Tobacco use during pregnancy and preeclampsia risk: effects of cigarette smoking and snuff. Hypertension 2010; 55: 1254-1259.