Published in final edited form as:

Am J Obstet Gynecol. 2015 March ; 212(3): 370.e1–370.e8. doi:10.1016/j.ajog.2014.12.042.

Vitamin C Supplementation Ameliorates the Adverse Effects of Nicotine on Placental Hemodynamics and Histology in Non-Human Primates

Jamie O. Lo, MD¹, Matthias C. Schabel, PhD², Victoria H.J. Roberts, PhD³, Terry K. Morgan, MD PhD⁴, Juha P. Rasanen, MD PhD¹, Christopher D. Kroenke, PhD^{2,5}, Ms. Sophie R. Shoemaker³, Eliot R. Spindel, MD PhD⁵, and Antonio E. Frias, MD^{1,3,6}

¹Department of Obstetrics and Gynecology, Oregon Health & Science University, Portland, OR

²Advanced Imaging Research Center, Oregon Health & Science University, Portland, OR

³Division of Diabetes, Obesity and Metabolism, Oregon National Primate Research Center, Portland, OR

⁴Department of Pathology, Oregon Health & Science University, Portland, OR

⁵Division of Neuroscience, Oregon National Primate Research Center, Portland, OR

⁶Division of Reproductive & Developmental Sciences, Oregon National Primate Research Center

Abstract

Objective—We previously demonstrated that prenatal nicotine exposure decreases neonatal pulmonary function in non-human primates (NHP) and maternal Vitamin C supplementation attenuates these deleterious effects. However, nicotine's effect on placental perfusion and development is not fully understood. This study utilizes non-invasive imaging techniques and histological analysis in a NHP model to test the hypothesis that prenatal nicotine exposure adversely affects placental hemodynamics and development, but is ameliorated by Vitamin C.

Study Design—Time-mated macaques (n=27) in 4 treatment groups: control (n=5), nicotine only (n=4), Vitamin C only (n=9), and nicotine plus Vitamin C (n=9). Nicotine animals received 2mg/kg/day of nicotine bitartrate (~0.7mg/kg/day free nicotine levels in pregnant human smokers) from days 26–160 (term, 168 days). Vitamin C groups received ascorbic acid at 50, 100 or 250mg/kg/day with or without nicotine. All underwent placental Dynamic Contrast-Enhanced MRI (DCE-MRI) at 135–140 days and Doppler ultrasound at 155 days to measure uterine artery

© 2014 Elsevier Inc. All rights reserved.

Corresponding Author: Jamie Lo, MD, Department of Obstetrics and Gynecology, Oregon Health & Sciences University, 3181 SW Sam Jackson Park Road, Mail Code L466, Portland, Oregon 97239, Work Phone: (503) 494-2685, Home Phone: (503) 679-2025, Fax: (503) 494-5680, loj@ohsu.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

The authors report no conflicts of interest.

This research was presented orally at both the 34th and 35th Society of Maternal Fetal Medicine Annual Meetings in New Orleans, Louisiana, February 3–8th, 2014 and San Diego, California, February 2–8th, 2015

and umbilical vein velocimetry and diameter to calculate uterine artery volume blood flow (cQuta) and placental volume blood flow (cQuv). Animals were delivered by cesarean section at 160 days. A novel DCE-MRI protocol was utilized to calculate placental perfusion from maternal spiral arteries. Placental tissue was processed for histopathology.

Results—Placental volume blood flow (cQuv) was significantly reduced in nicotine only animals compared with controls and nicotine plus Vitamin C groups ($p=0.03$). Maternal placental blood flow was not different between experimental groups by DCE-MRI ranging from 0.75–1.94 ml/ml/min ($p=0.93$). Placental histology showed increased numbers of villous cytotrophoblast cell islands ($p<0.05$) and increased syncytiotrophoblast sprouting ($p<0.001$) in nicotine only animals, which was mitigated by Vitamin C.

Conclusion—Prenatal nicotine exposure significantly decreased fetal blood supply via reduced placental volume blood flow, which corresponded with placental histologic findings previously associated with cigarette smoking. Vitamin C supplementation mitigated the harmful effects of prenatal nicotine exposure on placental hemodynamics and development suggesting its use may limit some of the adverse effects associated with smoking during pregnancy.

Keywords

Nicotine; non-human primate; non-invasive imaging; placental perfusion; Vitamin C

INTRODUCTION

Approximately 12% of women smoke during pregnancy, corresponding to more than 450,000 smoke-exposed infants born in the United States per year¹. Smoking during pregnancy poses a serious health risk to the fetus; it has been estimated to cause 5 to 10% of all fetal and neonatal deaths^{2–5}. Nicotine is the major component of cigarettes and a key mediator of the negative effects of smoking⁶. It readily crosses the placenta and accumulates in the amniotic fluid⁷. Given the addictive nature of nicotine and lack of effective treatments for smoking cessation in pregnancy, it is an issue that is not likely to be resolved in the near future. Thus, looking for methods to lessen the impact of smoking during pregnancy is important, even while maintaining a focus on smoking reduction and cessation in pregnancy.

It has been previously demonstrated in both our pregnant non-human primate (NHP) model⁸ and in a rat model^{9,10} that prenatal nicotine exposure produces changes in offspring's pulmonary function (e.g. decreased expiratory flow) similar to those in offspring of human smokers. In addition, supplementation with Vitamin C is found to ameliorate the harmful effects of smoking on fetal lung development^{8–10}. As some of these adverse changes correspond with changes induced by oxidants, it can be expected that natural antioxidants, such as Vitamin C, will be protective to both mother and baby. Despite the protective benefit of Vitamin C on nicotine exposure on fetal pulmonary function, its effect on placental development and perfusion has not been studied.

The mechanism and specific vasoactive effects of nicotine on the placenta and fetal development have not been identified. Nicotine acts via nicotinic receptors, which are widely distributed not only in the fetal lung, but also in the placenta¹¹. Nicotine freely

crosses the placenta^{12,13} and binds to the α -subunit of nicotinic acetylcholine receptors, expressed in all placental cell types¹¹ along with endogenous acetylcholine, an essential placental signaling molecule. Nicotinic acetylcholine receptors are also located in placental vessels where nicotine is thought to be involved in the cholinergic regulation of placental blood flow and fluid volume¹⁴.

Understanding the impact of nicotine exposure on placental perfusion and development is critical to reduce and prevent its deleterious effects. Doppler ultrasound (D-US) is an established antenatal surveillance method routinely used in the clinical management of pregnancy^{15,16}. It has the ability to semi-quantitatively evaluate uterine blood flow for assessment of fetal well-being, but not comprehensive placental perfusion. We have recently developed a complementary non-invasive method of advanced imaging, Dynamic Contrast-Enhanced Magnetic Resonance Imaging (DCE-MRI), to quantify blood flow to the entire placental unit simultaneously¹⁷. Prior small animal studies have used DCE-MRI for evaluation of placental blood flow by acquiring T₁-weighted placental images prior to, during, and following the administration of a gadolinium-based contrast reagent^{18–20}. In rodents, previous studies have quantified placental perfusion and maternal-fetal interface permeability, including a recent study that demonstrated different perfusion regions in the mouse placenta²¹. Anatomically the primate placenta is distinct from those of small animals and is hemochorial like humans^{22–26}. Non-human primates also have a longer gestation and developmental ontogeny resembling that of humans. Given the similarity in vascular organization and development of the placenta, the NHP provides a powerful translational model for human pregnancy studies.

Our study focuses on the effects of chronic maternal nicotine exposure with and without Vitamin C supplementation in a relevant NHP model on placental perfusion and development. We hypothesized that nicotine exposure during pregnancy would decrease placental perfusion and increase placental pathology reportedly associated with cigarette smoking^{27–29}, and that these adverse effects would be ameliorated by maternal Vitamin C supplementation.

MATERIALS AND METHODS

Experimental Design

Time-mated female Rhesus macaques (n=27) were randomly allocated to control (n=5), nicotine only (n=4), Vitamin C only (n=9), or nicotine plus Vitamin C groups (n=9). On gestational day 26 of pregnancy, control animals received subcutaneous (midscapular) Alzet miniosmotic 2ML4 pumps containing bacteriostatic water (Abbott Laboratories, North Chicago, IL) and nicotine animals received subcutaneous osmotic mini-pump infusions of nicotine bitartrate (Sigma, St. Louis, MO) in bacteriostatic water to deliver 2.0 mg/kg/day nicotine bitartrate^{30,31}. Pumps were replaced every three weeks and animals received Cefazolin (150 mg/twice daily) for three days after pump insertion. The Vitamin C treated group received supplemental ascorbic acid at 50, 100, or 250mg/kg/day in the form of a children's chewable vitamin. Standard dosing of Vitamin C in human prenatal vitamins is 90 to 120 mg. Given the low toxicity of Vitamin C in pregnancy, a range of doses were chosen to assess dose response effects^{32–34}. All groups were fed Purina Mills (Richmond, IN) High-

Protein Monkey Diet, containing 0.75 mg of Vitamin C per gram of diet, with an average daily Vitamin C intake from the diet of 50 mg. In addition, all groups received multivitamins twice weekly (Zoo Chews; IVC Industries, Portland, OR) containing 60 mg Vitamin C. All animal procedures were approved by the Oregon Health and Science University Institutional Animal Care and Use Committee and conformed to all applicable regulations.

All animals underwent D-US at 155 days of gestation and placental DCE-MRI at 135–140 days gestation. Cesarean section was performed at gestational day 160 (term is 168 days) to ensure delivery of offspring prior to the onset of spontaneous labor. At the time of delivery, maternal blood, cord blood, and amniotic fluid were collected for metabolite analyses. Nicotine and cotinine (metabolite of nicotine) levels were measured by gas chromatograph, as previously described^{30,31}. Placental weights and measures were recorded.

Imaging

Doppler-US—Doppler-US data was collected by a sonographer blinded to treatment group using image-directed pulsed and color Doppler equipment (GE Voluson 730) with a 5- to 9-MHz sector probe. Prior to D-US, animals were sedated with 10mg/kg ketamine. The lowest high-pass filter level was used (100 Hz), and an angle of 15° or less between the Doppler beam was deemed acceptable. Blood flow velocity waveforms were obtained from the proximal portion of the uterine artery (Uta) as previously described^{35–37}. Doppler waveforms measurements for the Uta, umbilical artery and ductus venosus were performed using machine-specific software and the following measurements were obtained: pulsatility index (PI), velocity time integral (VTI), and fetal heart rate (HR). The diameter of the Uta was measured using power angiography as previously described^{35–37}. The cross sectional area (CSA) of the vessel was calculated as $CSA = \pi(\text{diameter}/2)^2$. cQ_{Uta} was calculated using the following formula: $cQ_{Uta} = VTI \times CSA \times HR$. For the placental volume blood flow (cQ_{UV}), the Doppler waveforms were obtained from the straight portion of the intra-abdominal umbilical vein (UV) as previously described^{35,38}. The mean velocity (V_{mean}) was calculated as 0.5 of the maximum velocity. cQ_{UV} was calculated as: $V_{\text{mean}} \times CSA \times 60$.

Dynamic Contrast-Enhanced MRI

Dynamic contrast-enhanced MRI data were acquired on a 3T Siemens Tim Trio scanner dedicated for NHP imaging, equipped with a high resolution 15 channel extremity RF coil, as described previously¹⁷. Prior to MRI, animals were sedated with 10mg/kg ketamine, intubated and maintained on 1.5% isoflurane. Subsequent to anatomic imaging using standard T1- and T2-weighted sequences for localization of the placenta, a time series of highly T1-weighted, 3D spoiled gradient echo measurements spanning the entire uterus with 2.5 mm isotropic spatial resolution was acquired (TR=86 ms, TE=1.5 ms, flip angle=20°) with 5.5 s temporal resolution. Data was acquired for at least 8 minutes, with a standard dose of 0.1 mmol/kg of gadoteridol (Prohance®) injected as a bolus at the end of the 10th time frame.

The custom postprocessing algorithm, implemented in MATLAB (Mathworks, Natick, MD) and previously described¹⁷ was slightly modified, as described in the following, to improve robustness and performance. After manually-generating a region of interest (ROI) covering

each lobe of the placenta, curves of contrast uptake were fit via nonlinear regression to the GCTT model³⁹ to obtain parametric maps of model flow (F) and contrast bolus delay time (t_d). The F map was smoothed by 3D convolution with a gaussian of standard deviation 3 mm to minimize spatial noise. Local maxima were identified in the smoothed F map and used to initialize a region-growing segmentation algorithm based on the fast marching method⁴⁰, as implemented in MATLAB File Exchange submission #24531 by Dirk-Jan Kroon). Once individual perfusion domains were segmented, the average volume of the region growing shells within each domain was plotted against the corresponding median delay time and resulting curves fit via linear regression to obtain volumetric flow in ml/min. The total volume of each perfusion domain, determined by multiplication of the number of voxels contained within the segmented domain mask by the known MRI voxel volume, was used to compute normalized blood flow (ml/min/ml), defined as the total blood flow for the domain divided by the domain volume.

Histologic Analyses

Representative samples from each fresh placenta were collected using four quadrant sampling and fixed in 10% zinc formalin, paraffin-embedded and histological sections were stained for hematoxylin and eosin. A placental pathologist (T.K.M.) evaluated the placental histologic sections while blinded to treatment group and scored for the presence of specific pathological features previously reported to be associated with smoking; including, villous cytotrophoblast cell islands related to abnormal differentiation, villous capillary density (with and without chorangiosis), and syncytiotrophoblastic sprouting^{27–29}. Findings had to be present in multiple villi in multiple sections for each case to be considered positive.

Immunohistochemistry

Immunohistochemistry was performed as previously described^{35,42}. Primary antibodies were as follows: 4-Hydroxyalkenals, 4-Hydroxynonenal (4-HNE) mouse monoclonal (Abcam, diluted to 1:250), 8-Hydroxydeoxyguanosine (8-OHdG) goat polyclonal (Millipore, diluted to 1:250), and Ki67 mouse monoclonal (Dako, diluted to 1:500). Slides were incubated with the primary antibody overnight at 4°C, followed by 1:10,000 HRP-conjugated secondary antibody (Jackson ImmunoResearch Laboratory) for 60 minutes at room temperature prior to standard color reaction and hematoxylin counterstain procedures. Immunostained slides were examined by two blinded reviewers. Ten random high-power fields were observed per slide and graded using a 0 to 3 scale where 0 indicated the absence of positive staining, and 3 indicated intense positive staining.

Statistical Analysis

Data are expressed as mean $\pm$ SD. All animals (n=27) were analyzed, unless otherwise noted. Three different Vitamin C dosing groups were studied, but because no differences were observed between groups, they were combined for all analyses of *in vivo* imaging and *in vitro* studies. For the maternal and fetal imaging data, differences between all groups were tested by one-way ANOVA. Fisher's exact analysis was used for placental pathologic analysis and an independent sample T-test was used for immunohistochemistry analysis with significance designated at $p < 0.05$.

RESULTS

Nicotine and cotinine levels in maternal serum, amniotic fluid, and cord blood at the time of delivery are shown in Table 1. Maternal plasma levels were similar in both groups. Nicotine and cotinine were undetectable in amniotic fluid from control animals. Nicotine levels were significantly higher in the amniotic fluid of the Vitamin C plus nicotine group, while cotinine showed a less notable increase, compared to the nicotine only group.

Maternal, fetal birth and placental weights are given in Table 2. There were no significant differences in these parameters between any of the four groups. By D-US, we demonstrated a significant reduction in cQuv in the nicotine group compared with each of the other three groups, $p=0.024$ (Figure 1). There was no significant difference in cQuta as well as ductus venosus, umbilical artery, and uterine artery pulsatility indices (Table 3).

Maternal perfusion of the placental intervillous space was evaluated using DCE-MRI. Secondary to incomplete imaging acquisition, data was available for analysis in 22 animals: control ($n=3$), Vitamin C only ($n=8$), nicotine only ($n=3$), and nicotine with Vitamin C ($n=8$). Average placental blood flow (ml/min) and normalized placental blood flow (ml/min/ml), which adjusts for placental volume, were not significantly different between any of the groups and all fell within the range of 59.7 to 109.2 ml/min and 0.75 to 1.94 ml/min/ml respectively, $p=0.927$ (Table 4).

A subset of 24 animals was available for placental histologic analysis: controls ($n=5$), nicotine only ($n=4$), Vitamin C only ($n=7$), and nicotine plus Vitamin C ($n=8$). Placentas exposed to nicotine showed conspicuous villous cytotrophoblast cell islands and syncytiotrophoblast sprouting, which was less common in controls and largely absent in animals treated with Vitamin C (Figure 2). Specifically, villous cytotrophoblast cell islands were seen in all nicotine exposed animals compared with only 40% in the control group and 13% in the nicotine plus Vitamin C group, $p<0.05$ (Table 5). Syncytiotrophoblast sprouting was observed in all nicotine only animals and in 29% of Vitamin C only animals, but was absent in control and nicotine plus Vitamin C animals, $p<0.001$ (Table 5). Villous capillary density was not significantly different between groups (e.g. all sections were negative for chorangiosis). Placental weights ($p=0.170$) and volumes ($p=0.768$) were not different amongst different treatment groups (Table 2 and 4).

Expression of 4-HNE and 8-OHdG were examined as markers of oxidative stress. There was a slight, non-significant trend towards increased expression with 4-HNE and 8-OHdG in nicotine only animals with a mean grade of 2.17–2.2 compared with the Vitamin C (1.86–2) only and nicotine with Vitamin C groups (1.9–2), but this difference was not statistically significant. In addition, there was a slight trend towards decreased placental villous staining with Ki67, a marker of trophoblastic proliferation²⁸, observed in placentas exposed to nicotine only (2.17) compared with Vitamin C only (2.29) and nicotine with Vitamin C groups (2.3). Staining was observed to be diffusely localized throughout the villous (Figure 3).

COMMENT

We found that prenatal nicotine exposure significantly reduced overall fetal blood supply, which corresponded with significant histologic findings characteristic of placental hypoxia. These effects were ameliorated in nicotine exposed animals that received maternal Vitamin C supplementation. The presence of nicotine, however, did not significantly alter maternal perfusion of the placenta. Prenatal nicotine exposure has been associated with fetal hypoxia *in utero*⁴³. Our finding of decreased cQuv is suggestive of decreased oxygen delivery to the fetus, which is supported by our histologic observations. Specifically, the placental histology was notable for significantly increased villous cytotrophoblast cell island formation and conspicuous syncytiotrophoblastic sprouting, which are features previously described in placentas of smokers^{27–29}. Although chronic prenatal nicotine exposure resulted in significant histologic changes, we did not observe significant differences in proliferation index (Ki-67), which suggests the late gestation sampling may have revealed hyperplastic islands that formed earlier in gestation²⁸. We did not observe increased chorangiosis (fetoplacental angiogenesis) or expression of placental oxidative stress markers (4-HNE and 8-OHdG).

Our study revealed that chronic nicotine exposure resulted in decreased fetal blood supply without significant alterations in maternal placental hemodynamics on D-US assessment of uterine artery Doppler waveforms. Consistent with our D-US assessment, DCE-MRI results showed no significant maternal perfusion differences amongst all treatment groups. The most likely explanation for the absence of altered maternal placental blood flow from nicotine exposure in our study is because nicotine was administered from gestational day 26 and in the macaque, maternal blood vessel penetration by the trophoblasts occurs by gestational day 14 to 16⁴⁴. This suggests that adequate spiral artery remodeling occurred and thus maternal perfusion of the intervillous space in our model was less susceptible to the effects of nicotine exposure because the placental vasculature was well established. However, previously published studies in humans also reported no significant difference in uterine artery Doppler measurements in smokers compared with non-smokers^{45,46}. This was also observed in a prior sheep study that demonstrated no effect from chronic nicotine exposure on uterine artery flow⁴⁷.

Although chronic prenatal nicotine exposure resulted in decreased cQuv and histologic changes characteristic of hypoxia, we found that these were mitigated with the addition of Vitamin C. The protective effects of maternal Vitamin C supplementation have also been shown in fetal lung function in addition to some, but not most, aspects of the developing brain^{8,48}. However, the mechanism of action remains unclear, but Vitamin C has been shown to affect coronary blood flow and arterial stiffness in smokers, suggesting potential involvement of elastin and collagen, with other evidence indicating that Vitamin C alters cytokine production^{8,49,50}. This suggests that the actions of nicotine may provide a target for Vitamin C to block some of the deleterious effects of smoking during pregnancy on fetal development. These findings support the consideration of Vitamin C supplementation in pregnancy as a safe and inexpensive treatment that may help lessen the morbidity and mortality of infants born to women who cannot quit smoking during pregnancy.

Rhesus monkeys were treated with 2 mg/kg/ day nicotine bitartrate, which is equivalent to 0.7 mg/kg free nicotine per day and is similar to that of a moderate to heavy smoker^{30,31}. This dosing regimen yielded average concentrations of nicotine and cotinine in monkey amniotic fluid and maternal blood that were similar to pregnant human smokers¹³. Interestingly, we observed that levels of nicotine and cotinine in the amniotic fluid were increased with Vitamin C supplementation compared to nicotine only and control animals. Although the mechanism behind this unexpected finding is unknown, we can postulate that Vitamin C does not alter maternal distribution, metabolism or clearance of nicotine or cotinine, but may increase fetal metabolism and excretion.

Despite the variation in nicotine and cotinine levels in the amniotic fluid of different treatment groups, we did not observe a significant difference in neonatal birth weight in the nicotine treated animals compared to control and Vitamin C only animals. Smoking cigarettes in pregnancy has been associated with decreased neonatal birth weight in humans^{51,52}, but animal models studying the effects of prenatal nicotine exposure on offspring development do not recapitulate the effects on birth weight. Prior rat models of maternal nicotine exposure have also demonstrated no effect on offspring growth^{53,54}. This inconsistent effect on birth weight between animal and human studies may be because most animal models have chosen to study the adverse outcomes of nicotine rather than cigarettes. Indeed, there is current evidence showing that the physiological effect of smoking on fetal growth may be secondary to other components of cigarettes or the nicotine delivery method. There are several large human studies demonstrating that the use of smokeless tobacco⁵⁵ or nicotine replacement therapy prenatally does not significantly affect birthweight, which supports our NHP findings^{56–58}.

In conclusion, our study demonstrates that prenatal nicotine exposure in a NHP model, at maternal nicotine levels commensurate with human smoking during pregnancy, adversely affects fetal blood supply and placental histology, which is ameliorated by maternal Vitamin C supplementation. Although the priority must be for smoking cessation in pregnancy, Vitamin C is a simple, safe, and inexpensive supplementation that may have potential clinical application in limiting the harmful effects of prenatal nicotine exposure on placental hemodynamic function and development.

Acknowledgments

Funding: This study was supported by NIH grant R01 HL087710, R21 HD076265 and P51 OD011092-53.

REFERENCES

1. Martin JA, Hamilton BE, Sutton PD, Ventura SJ, Menacker F, Munson ML. Births: final data for 2002. *Natl Vital Stat Rep.* 2003; 52:1–113. [PubMed: 14717305]
2. Stocks J, Dezateux C. The effect of parental smoking on lung function and development during infancy. *Respirology.* 2003; 8:266–285. [PubMed: 14528876]
3. Hoo AF, Henschen M, Dezateux C, Costeloe K, Stocks J. Respiratory function among preterm infants whose mothers smoked during pregnancy. *Am J Respir Crit Care Med.* 1998; 158:700–705. [PubMed: 9730993]

4. Salihu HM, Aliyu MH, Pierre-Louis BJ, Alexander GR. Levels of excess infant deaths attributable to maternal smoking during pregnancy in the United States. *Matern Child Health J.* 2003; 7:219–227. [PubMed: 14682499]
5. Wisborg K, Kesmodel U, Henricksen TB, Olsen SF, Secher NM. Exposure to tobacco smoke in utero and the risk of stillbirth and death in the first year of life. *Am J Epidemiol.* 2001; 154:322–327. [PubMed: 11495855]
6. US Department of Health and Human Services. The Health Consequences of Smoking: Nicotine Addiction A Report of the Surgeon General. Atlanta: US Department of Health and Human Services, Public Health Service, Centers for Disease Control, National Center for Chronic Disease Prevention and Health Promotion, Office on Smoking and Health; 1988. DHHS Publication No. (CDC) 88-8406.
7. Koren G. Fetal toxicology of environmental tobacco smoke. *Curr Opin Pediatr.* 1995; 7:128–131. [PubMed: 7787925]
8. Proskocil BJ, Sekhon HS, Clark JA, et al. Vitamin C Prevents the Effects of Prenatal Nicotine on Pulmonary Function in Newborn Monkeys. *Am J Respir Crit Care Med.* 2005; 171:1032–1039. [PubMed: 15709053]
9. Maritz GS. The influence of maternal nicotine exposure on neonatal lung metabolism: protective effect of ascorbic acid. *Cell Biol Int.* 1993; 17:579–585. [PubMed: 8348117]
10. Maritz GS, van Wyk G. Influence of maternal nicotine exposure on neonatal rat lung structure: protective effect of ascorbic acid. *Comp Biochem Physiol C Pharmacol Toxicol Endocrinol.* 1997; 117:159–165. [PubMed: 9214716]
11. Lips KS, Bruggmann D, Pfeil U, Vollerthun R, Grando SA, Kummer W. Nicotinic acetylcholine receptors in rat and human placenta. *Placenta.* 2005; 25(10):735–746. [PubMed: 16226123]
12. Luck W, Nau H. Exposure of the fetus, neonate, and nursed infant to nicotine and cotinine from maternal smoking [letter]. *N Engl J Med.* 1984; 311:672. [PubMed: 6472348]
13. Luck W, Nau H, Hansen R, Steldinger R. Extent of nicotine and cotinine transfer to the human fetus, placenta and amniotic fluid of smoking mothers. *Dev Pharmacol Ther.* 1985; 8:384–395. [PubMed: 4075937]
14. Sastry BVR. Human placental cholinergic system. *Biochem Pharmacol.* 1997; 53:1577–1586. [PubMed: 9264309]
15. Trudinger BJ, Cook CM, Giles WB, Connelly A, Thompson RS. Umbilical artery flow velocity waveforms in high-risk pregnancy. Randomised controlled trial. *Lancet.* 1987; 1(8526):188–190. [PubMed: 2880017]
16. Baschatt AA, Gembruch U, Reiss I, Gortner L, Weiner CP, Harman CR. Relationship between arterial and venous Doppler and perinatal outcome in fetal growth restriction. *Ultrasound Obstet Gynecol.* 2000; 16(5):407–413. [PubMed: 11169323]
17. Frias AE, Schabel MC, Roberts V, et al. Using dynamic contrast enhanced MRI to quantitatively characterize maternal vascular organization in the primate placenta. *Magn Reson Med.* 2014 (Epub ahead of print).
18. Taillieu F, Salomon LJ, Siauve N, Clement O, Faye N, Balvay D, Vayssettes C, Frija G, Ville Y, Cuenod CA. Placental perfusion and permeability: simultaneous assessment with dual-echo contrast-enhanced MR imaging in mice. *Radiology.* 2006; 241:737–745. [PubMed: 17065560]
19. Tomlinson TM, Garbow JR, Anderson JR, Engelbach JA, Nelson DM, Sadovsky Y. Magnetic resonance imaging of hypoxic injury to the murine placenta. *Am J Physiol Regul Integr Comp Physiol.* 2010; 298:R312–R319. [PubMed: 19923363]
20. Alison M, Quibel T, Balvay D, et al. Measurement of placental perfusion by dynamic contrast-enhanced MRI at 4.7 T. *Invest Radiol.* 2013; 48(7):535–542. [PubMed: 23462675]
21. Remus CC, Sedlacik J, Wedegaertner U, Arck P, Hecher K, Adam G, Forkert ND. Application of the steepest slopemodel reveals different perfusion territories within the mouse placenta. *Placenta.* 2013; 34:899–906. [PubMed: 23849931]
22. Kaufmann, P.; Burton, G. Anatomy and genesis of the placenta. In: Knobil, E.; Neill, JD., editors. *The physiology of reproduction.* 2nd ed.. New York: Raven Press; 1994. p. 441-483.
23. Enders AC, Blankenship TN. Comparative placental structure. *Adv Drug Deliv Rev.* 1999; 38:3–15. [PubMed: 10837743]

24. Ragavendra N, Tarantal AF. Intervillous blood flow in the third trimester gravid rhesus monkey (*Macaca mulatta*): use of sonographic contrast agent and harmonic imaging. *Placenta*. 2001; 22:200–205. [PubMed: 11170824]
25. Benirschke K. Placentation. *J Exp Zool*. 1983; 228:385–389. [PubMed: 6363611]
26. Roberts VHJ, Rasanen JP, Novy MJ, et al. Restriction of placental vasculature in a non-human primate: a unique model to study placental plasticity. *Placenta*. 2012; 33:73–76. [PubMed: 22030304]
27. van der Veen F, Fox H. The effects of cigarette smoking on the human placenta: a light and electron microscopic study. *Placenta*. 1982; 3(3):243–256. [PubMed: 7134194]
28. Shiverick K, Salafia C. Cigarette smoking and pregnancy I: Ovarian, uterine, and placental effects. *Placenta*. 1999; 20:265–272. [PubMed: 10329346]
29. Zdravkovic T, Genbacev O, McMaster M, Fisher S. The adverse effects of maternal smoking on the human placenta: A review. *Placenta*. 2005; 26:S81–S86. [PubMed: 15837073]
30. Benowitz NL, Kuyt F, Jacob P. Circadian blood nicotine concentrations during cigarette smoking. *Clin Pharmacol Ther*. 1982; 32:758–764. [PubMed: 7140139]
31. Benowitz NL, Jacob P III. Daily intake of nicotine during cigarette smoking. *Clin Pharmacol Ther*. 1984; 35:499–504. [PubMed: 6705448]
32. Cohen-Kerem R, Koren G. Antioxidants and fetal protection against ethanol teratogenicity. I. Review of the experimental data and implications to humans. *Neurotoxicol Teratol*. 2003; 25:1–9. [PubMed: 12633732]
33. Colomina MT, Gomez M, Domingo JL, Corbella J. Lack of maternal and developmental toxicity in mice given high doses of aluminum hydroxide and ascorbic acid during gestation. *Pharmacol Toxicol*. 1994; 74:236–239. [PubMed: 8090692]
34. Chappell LC, Seed PT, Kelly FJ, et al. Vitamin C and E supplementation in women at risk of preeclampsia is associated with changes in indices of oxidative stress and placental function. *Am J Obstet Gynecol*. 2002; 187:777–784. [PubMed: 12237663]
35. Frias AE, Morgan TK, Evans AE, et al. Maternal high-fat diet disturbs uteroplacental hemodynamics and increases the frequency of stillbirth in a nonhuman primate model of excess nutrition. *Endocrinology*. 2011; 152(6):2456–2464. [PubMed: 21447636]
36. Konje JC, Kaufmann P, Bell SC, Taylor DJ. A longitudinal study of quantitative uterine blood flow with the use of color power angiography in appropriate for gestational age pregnancies. *Am J Obstet Gynecol*. 2001; 185(3):608–613. [PubMed: 11568786]
37. Acharya G, Sitras V, Erkinaro T, et al. Experimental validation of uterine artery volume blood flow measurements by Doppler ultrasonography in pregnant sheep. *Ultrasound Obstet Gynecol*. 2007; 29(4):401–406. [PubMed: 17390334]
38. Acharya G, Wilsgaard T, Berntsen GK, Maltau JM, Kiserud T. Doppler-derived umbilical artery absolute velocities and their relationship to fetoplacental volume blood flow: a longitudinal study. *Ultrasound Obstet Gynecol*. 2005; 25(5):444–453. [PubMed: 15816007]
39. Schabel MC. A unified impulse response model for DCE-MRI. *Magn Reson Med*. 2012; 68(5):1632–1646. [PubMed: 22294448]
40. Hassouna MS, Farag AA. Multistencils fast marching methods: A highly accurate solution to the eikonal equation on cartesian domains. *IEEE Trans Pattern Anal Mach Intell*. 2007; 29(9):1563–1574. [PubMed: 17627044]
41. Fox, H.; Sebire, N. Pathology of the Placenta. 3rd Ed.. Philadelphia: Elsevier; 2007. Nicotine and placental villous histology; p. 221–222.
42. Ma Y, Krikun G, Abrahams VM, Mor G, Guller S. Cell type-specific expression and function of toll-like receptors 2 and 4 in human placenta: implications of fetal infection. *Placenta*. 2007; 28:1024–1031. [PubMed: 17588655]
43. Guan J, Mao C, Xu F, et al. Low doses of nicotine-induced fetal cardiovascular responses, hypoxia, and brain cellular activation in ovine fetuses. *Neurotoxicology*. 2009; 30(2):290–297. [PubMed: 19459226]
44. De Rijk E, Van Esch E. The macaque placenta-A mini-review. *Toxicol Pathol*. 2008; 36:108–118.

45. Albuquerque CA, Smith KR, Johnson C, Chao R, Harding R. Influence of maternal tobacco smoking during pregnancy on uterine, umbilical and fetal cerebral artery blood flows. *Early Hum Dev.* 2004; 80(1):31–42. [PubMed: 15363837]
46. Bruner JP, Forouzan I. Smoking and buccally administered nicotine. Acute effect on uterine and umbilical artery doppler flow velocity waveforms. *J Reprod Med.* 1991; 36(6):435–440. [PubMed: 1865399]
47. Arbielle P, Bosc M, Vaillant MC, Tranquart F. Nicotine-induced changes in the cerebral circulation in ovine fetuses. *Am J Perinatol.* 1992; 9:270–274. [PubMed: 1627218]
48. Slotkin TA, Seidler FJ, Qiao D, et al. Effects of prenatal nicotine exposure on primate brain development and attempted amelioration with supplemental choline or vitamin C: neurotransmitter receptors, cell signaling and cell development biomarkers in fetal brain regions of rhesus monkeys. *Neuropsychopharmacology.* 2005; 30(1):129–144. [PubMed: 15316571]
49. Katayama Y, Shige H, Yamamoto A, Hirata F, Yasuda H. Oral vitamin C ameliorates smoking-induced arterial wall stiffness in healthy volunteers. *J Atheroscler Thromb.* 2004; 11(6):354–357. [PubMed: 15644590]
50. Teramoto K, Daimon M, Hasegawa R, et al. Acute effect of oral vitamin C on coronary circulation in young healthy smokers. *Am Heart J.* 2004; 148(2):300–305. [PubMed: 15309000]
51. Horta BL, Victora CG, Menezes AM, Halpern R, Barros FC. Low birthweight, preterm births, and intrauterine growth retardation in relation to maternal smoking. *Paediatr Perinat Epidemiol.* 1997; 11(2):140–151. [PubMed: 9131707]
52. Adriaanse HP, Kottner JA, Delgado LR, Cox HH, Essed GG. Smoking in Dutch pregnant women and birth weight. *Patient Educ Couns.* 1996; 28(1):25–30. [PubMed: 8852204]
53. Franke RM, Park M, Belluzzi JD, Leslie FM. Prenatal nicotine exposure changes natural and drug-induced reinforcement in adolescent male rats. *Eur J Neurosci.* 2008; 27:2952–2961. [PubMed: 18588535]
54. Navarro HA, Seidler FJ, Schwartz RD, Baker FE, Dobbins SS, Slotkin TA. Prenatal exposure to nicotine impairs nervous system development at a dose which does not affect viability or growth. *Brain Res Bull.* 1989; 23:187–192. [PubMed: 2819477]
55. England LJ, Kim SY, Shapiro-Mendoza CK. Maternal smokeless tobacco use in Alaska Native women and singleton infant birth size. *Acta Obstet Gynecol Scand.* 2012; 91(1):93–103. [PubMed: 21902677]
56. Lassen TH, Madsen M, Skovgaard LT, Strandberg-Larsen K, Olsen J, Andersen AM. Maternal use of nicotine replacement therapy during pregnancy and offspring birthweight: a study within the Danish National Birth Cohort. *Paediatr Perinat Epidemiol.* 2010; 24(3):272–281. [PubMed: 20415757]
57. Forinash AB, Pitlick JM, Clark K, Alstat V. Nicotine replacement therapy effect on pregnancy outcomes. *Ann Pharmacother.* 2010; 44(11):1817–1821. [PubMed: 20978216]
58. Wisborg K, Henriksen TB, Jørgensen LB, Secher NJ. Nicotine patches for pregnant smokers: a randomized controlled study. *Obstet Gynecol.* 2000; 96(6):967–971. [PubMed: 11084187]

Figure 1. Doppler ultrasound of placental volume blood flow (cQuv) as measured at 155 day gestation demonstrating significantly decreased cQuv of nicotine only exposed animals *p=0.024.

Figure 2. Placental histopathology associated with nicotine and ameliorated by Vitamin C
(A) Nicotine exposed animals showed conspicuous villous cytotrophoblastic islands (arrow) and syncytiotrophoblastic sprouting (arrowheads) compared with Vitamin C treated and control animals (B) H&E stained sections. Scale bar is 100 µm.

Figure 3. Immunohistochemistry staining

A. No staining. B. Ki67, C. 4HNE, D. 8OH-dG staining diffusely localized in the villous cytotrophoblast and syncytiotrophoblast. Scale bar is 50 μ m.

Table 1
Nicotine and cotinine levels in maternal serum, amniotic fluid, and cord blood at time of cesarean section

Group	Maternal Plasma		Amniotic Fluid		Cord Plasma	
	Nicotine	Cotinine	Nicotine	Cotinine	Nicotine	Cotinine
Nicotine	29.8 ± 8.4	101.1 ± 18.1	13.1 ± 1.0	74.6 ± 11.9	10.7 ± 1.9	92.7 ± 12.9
Nic + Vit C	28.8 ± 6.0	126.7 ± 7.9	24.8 ± 3.8 *	97.2 ± 2.6	15.3 ± 3.2	120 ± 11.8

Definition of abbreviations: Nic = nicotine; Vit C = Vitamin C
Nicotine levels (ng/ml) in the fluids shown. Data are means ± SEM. Nicotine was undetectable in control group samples.
* p < 0.05 by Tukey-Kramer test after multivariate-ANOVA compared with nicotine alone.

Table 2

Birth weight and placental weight

Parameter	Control (n=3)	Vit C (n=8)	Nicotine (n=3)	Nic + Vit C (n=8)
Maternal weight (kg)	8 ± 0.26	8.7 ± 1.1	8.5 ± 0.57	8.1 ± 0.63
Fetal birth weight (g)	469 ± 67	524 ± 53	504 ± 38	482 ± 24
Placental weight (g)	153 ± 36	147 ± 22	150 ± 19	122 ± 20

Definition of abbreviations: Nic = nicotine; Vit C = Vitamin C

1-way ANOVA test used. Data are means ± SD. There was no significant weight difference between all four treatment groups.

Table 3

Doppler ultrasound measurements of fetal and maternal hemodynamics

Parameter	Control (n=5)	Vit C (n=9)	Nicotine (n=4)	Nic + Vit C (n=9)
Ductus venosus PI	0.28 ± 0.12	0.31 ± 0.1	0.27 ± 0.08	0.34 ± 0.13
Umbilical artery PI	0.96 ± 0.06	1.1 ± 0.14	1.05 ± 0.26	1.1 ± 0.16
Uterine artery PI	0.88 ± 0.31	0.84 ± 0.15	0.75 ± 0.11	0.82 ± 0.14
cQuta (ml/min/kg)	1.35 ± 0.41	1.34 ± 0.59	1.35 ± 0.4	1.35 ± 0.29

Definition abbreviations: PI = pulsatility index, cQuta = calculated uterine artery blood flow volume corrected for maternal weight

1-way ANOVA test used. Data are means ± SD. There was no significant difference between all four treatment groups.

Table 4

Dynamic contrast-enhanced MRI measurements of placental perfusion

Parameter	Control (n=3)	Vit C (n=8)	Nicotine (n=3)	Nic + Vit C (n=8)
Placental Volume (mL)	61.7 ± 6.2	62.1 ± 5.7	74.5 ± 5.6	67.5 ± 27.4
Placental Blood Flow (mL/min)	96.0 ± 26.8	59.7 ± 25.7	109.2 ± 24.5	86.1 ± 29.1
Normalized Placental Blood Flow (mL/min/mL)	1.6 ± 0.4	1.0 ± 0.4	1.50 ± 0.5	1.4 ± 0.4

Definition of abbreviations: Nic = nicotine; Vit C = Vitamin C

1-way ANOVA test used. Data are means ± SD. There was no significant difference between all groups.

Table 5

Placental villous tissue histology. Number of animals demonstrating positive histologic findings with (%) representing the percentage per cohort.

Histology	Control (n=5)	Vit C (n=7)	Nicotine (n=4)	Nic + Vit C (n=8)
Villous CTB islands *	2 (40%)	3 (43%)	4 (100%)	1 (13%)
STB sprouting **	0 (0%)	2 (29%)	4 (100%)	0 (0%)

Definition of abbreviations: Nic = nicotine; Vit C = Vitamin C, CTB = cytotrophoblast, STB = syncytiotrophoblast

*
p<0.05 and

**
p<0.0001 by Fisher's exact analysis

There was no significant difference between all Vitamin C dosing groups.