Published in final edited form as:

Am J Obstet Gynecol. 2011 September ; 205(3): 246.e1–246.e7. doi:10.1016/j.ajog.2011.06.023.

MATERNAL TOBACCO USE IS ASSOCIATED WITH INCREASED MARKERS OF OXIDATIVE STRESS IN THE PLACENTA

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Abstract

Objective—We sought to extend our prior observations and histopathologically characterize key metabolic enzymes (*CYP1A1*) with markers of oxidative damage in placental sections from smokers.

Study Design—Placental specimens were collected from term singleton deliveries from smokers ($n=10$) and non-smokers ($n=10$), and subjected to detailed histopathologic examination. To quantify the extent of oxidative damage, masked score-graded (0–6) histopathology against 4-hydroxy-2-nonenal (4-HNE) and 8-hydroxydeoxyguanosine (8-OHdG) was performed. Minimal significance ($p<0.05$) was determined with Fisher's-exact and two-tailed T-test as appropriate.

Results—We observed a significant increase in the presence of syncytial knots in placentas from smokers (70% versus 10%, $p=0.02$). These gross observations were accompanied by significant aberrant placental aromatic hydrocarbon metabolism (increased CYP1A1, 4.4 vs. 2.1, $p=0.002$) alongside evidence of oxidative damage (4-HNE 3.4 vs. 1.1, $p=0.00005$; 8-OHdG 4.9 vs. 3.1, $p=0.0038$).

Conclusions—We observe a strong association between maternal tobacco use and aberrant placental metabolism, syncytial knot formation, and multiple markers of oxidative damage.

Keywords

maternal smoking; placenta; oxidative stress; IUGR; immunohistochemistry; metabolic stress

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DISCLOSURE: The authors report no conflict of interest.

Presented at the 31st Annual Meeting of the Society for Maternal Fetal Medicine, San Francisco, California, February 10, 2011.

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INTRODUCTION

Although the concerning effects of maternal tobacco smoke on fetal growth have been well reported for over three decades, it remains today one of the leading preventable causes of fetal growth restriction in developed and developing countries. (1–4) In the seminal report from Simpson it was reported that mothers who smoked 10 cigarettes or more per day delivered infants with a decrease in birth weight of approximately 200 grams compared with neonates from non-smoking mothers. (5) However, not all fetuses exposed to maternal tobacco smoke are growth restricted. (1, 2, 6, 7) Susceptibility to tobacco exposure likely involves several factors including, but not limited to, epidemiological, genetic, epigenetic and socioeconomic. (1,2)

Nicotine, a principal alkaloid of tobacco smoke, has been shown to mediate constriction of the intrauterine vessels and result in increased proliferation of placental syncytiotrophoblasts. (8) Potentially harmful DNA adducts (metabolic products of polycyclic aromatic hydrocarbons; PAH) are known to cross or collect in the placenta of smokers. (9, 10) PAH compounds, together with nitrosamines, comprise likely carcinogenic species in tobacco smoke. (11, 12) The majority of chemical carcinogens are metabolized in a sequential series of two-phase enzymatic metabolic reactions (Figure 1). (1,2) Phase I enzymes such as *CYP1A1* metabolically activate PAH compounds into oxidized derivatives, resulting in reactive oxygen intermediates capable of covalently binding DNA to form adducts. (13) In turn, these reactive electrophilic intermediates can be detoxified by phase II enzymes, such as the glutathione *S*-transferase (*GSTT1*), via conjugation with endogenous species to form hydrophilic glutathione conjugates which are then readily excreted. (13) Thus the coordinated expression of these enzymes and their relative balance may determine the extent of cellular DNA damage and related development of adverse outcomes.

We have previously demonstrated that in a large matched cohort, deletion of fetal *GSTT1* (a phase II pathway gene, Figure 1) is associated with birth weight reduction in pregnancies exposed to maternal tobacco use. (6) We have also shown that increased placental *CYP1A1* expression was specifically and significantly associated with hypomethylation of XRE-proximal CpG dinucleotides in the *CYP1A1* promoter region in smokers compared with non-smokers. (14) An increase in Phase I enzymes without a compensatory increase in Phase II enzymes has the potential to create reactive species within the cell. These unprocessed ROSs have the unmitigated potential to lead to DNA-adduct mediated damage and lipid oxidation, perpetuating the cycle of modulated cellular and molecular physiology. (Figure 1) In this study, we hypothesized disrupted metabolic pathways converge at the cellular level to increase markers of oxidative stress in the placenta. To quantify the extent of DNA damage and oxidative damage we used two well characterized markers: 8-OHdG (a marker of DNA damage) and 4-HNE (a marker for oxidative lipid damage) as determinates of cellular oxidative stress. (15, 16) We therefore sought to extend our prior observations and histopathologically characterize key metabolic enzymes (*CYP1A1*) with markers of oxidative damage in placental sections from smokers.

MATERIALS AND METHODS

Study Population

Placental samples ($n=20$) for this study were obtained from subjects selected from a well-described cohort of 20 self-reported smokers alongside 53 non smoking controls; this has been previously validated as an accurate measure of maternal tobacco exposure. (17) The Institutional Review Board of Baylor College of Medicine and its affiliated institutions approved this study, and written informed consent was obtained from each participant at the time of enrollment. Data collected from each patient included age, ethnicity, height and

weight, past obstetrical history, gestational age at delivery, and potential maternal comorbidities. Data collected from the newborns included gender, Apgar scores, weight and length, and level of resuscitation interventions if any. Exclusion criteria included multiple gestation, known fetal anomalies, and maternal hepatic, hypertensive, or endocrine disorders. For the analysis reported herein, subjects were matched in a nested cohort design by virtue of maternal age (± 3 years), race/ethnicity, BMI, and gestational age (± 1 week). Consistent with a nested cohort design, matching was performed prior to knowledge of the primary outcomes (i.e., histopathology and immunohistochemistry) and without consideration of fetal factors (beyond gestational age) including fetal weight, length or neonatal outcome. In such a manner, an initial 20 matched subjects were analyzed with minimized potential for selection bias. This is as noted in Table 1.

Collection and standardized processing of placental samples

Placental specimens were collected immediately after delivery, systematically stored, and processed for histopathology within 12 hours. Standardized collection and section methodology included uniform triplicate 3 cm excisional blocks at a prescribed 4 cm trinary distance from the umbilical cord insertion, along with a section from the insertion point and random 3 marginal sections. All sections collected were full-thickness. The excised sections were embedded into paraffin blocks and stained with hematoxylin and eosin (H&E) for microscopic examination. In addition, unstained sections were prepared for use in immunohistochemistry.

Placental histopathology analysis

All H&E stained sections were examined by reviewers masked to maternal cohort. Pathologic changes were recorded as present or absent, and the prevalence of abnormalities observed (e.g. infarcts, inflammation, syncytial knots) was compared between the two groups and analyzed with the statistical software package SPSS v 11.5 using Fisher's exact test with a minimal p value of <0.05 denoting significance.

Immunohistochemistry

Primary antibodies employed were 4-HNE mouse monoclonal (Abcam), CYP1A1 rabbit polyclonal (Millipore), and 8-OHdG goat polyclonal (Millipore). For immunostaining, unstained paraffin sections were deparaffinized in four changes of xylene for five minutes each, and then rehydrated through a series of graded alcohols with a final rinse in distilled water. Endogenous peroxidase was quenched by soaking sections in two changes of 3% H_2O_2 in Methanol for 10 minutes at room temperature. Prior to immunohistochemistry, antigen retrieval was performed to facilitate antibody binding to antigen. Slides were incubated at $99^\circ C$ for 20 minutes in either Target Retrieval Citrate Solution pH 6.0 (Dako Corporation, Carpinteria, CA) for CYP1A1 and 4-HNE, or for 28 minutes in Diva Decloaker Retrieval Solution pH 6.2 (Biocare Medical, Concord, CA). Slides were then allowed to cool down for 10 minutes in the same solution, rinsed in three changes of distilled water, and placed in Tris Buffered Saline with Tween 20 pH 7.4 (Signet Pathology Systems, Inc., Dedham, MA) for five minutes to decrease surface tension and facilitate coating by the subsequent reagents. The PolyVue HRP/DAB non-biotin polymer detection system (Diagnostic Biosystems, Pleasanton, CA) was used in the immunostaining protocols for CYP1A1 and 8-OHdG, while the MACH 4 HRP biotin-free Universal Polymer Detection System (Biocare Medical, Concord, CA) was used for 4-HNE. Incubations occurred at room temperature unless otherwise specified, and for each step the sections were coated with 200 micro-liters of reagent. Tris buffered saline with Tween 20 pH 7.4 was used to rinse the sections between each of the immunohistochemistry steps. Background Sniper solution (Biocare Medical, Concord, CA) was used to block non-specific staining for 10 minutes at room temperature. The Primary antibody was diluted using the solution provided

with the detection kit, using a dilution of 1:200 for CYP1A1 and 8-OHdG, or 1:50 for 4-HNE. Slides were incubated with the primary antibody solution for 30 minutes at room temperature (CYP1A1), 60 minutes at room temperature (4-HNE), or overnight at 4°C (8-OHdG). Sections were then incubated in the universal secondary antibody provided with the kit for 15 minutes, followed by the HRP label reagent. Afterwards, Stable DAB Plus (Diagnostic Biosystems, Pleasanton, CA) was applied for 5 minutes as chromagen. The slides were rinsed in distilled water and manually counterstained with Harris Hematoxylin (Fisher Scientific,) for 15–30 seconds, and then rinsed in distilled water. Coverslips were then applied to each slide, using synthetic glass and permount mounting media. Negative controls and non-specific antibodies were included in each immunostaining procedure.

IHC analysis

Immunostained slides were examined by two independent reviewers masked to whether the case was a smoker or non-smoker. For each slide examined, ten random high-power fields were graded using a 0 to 6 scale where 0 indicated the absence of positive staining, and 6 indicated intense and diffuse positive staining. The location of positive staining areas was also recorded. The average of all grades was calculated for each slide, and IHC grades of smokers were compared with those of non-smokers using the independent sample T-test, after equal variance test was performed, using the statistical software package SPSS v 11.5 with minimal significance designated at $p < 0.05$.

RESULTS

Study Subjects

Our nested cohort design of singleton gestations at term (>37 weeks gestation) yielded matched cohorts which were designated to differ by virtue of maternal smoking, but manifest a significant decrease in infant birth weight in smokers ($3159\text{g} \pm 144$ versus $3619\text{g} \pm 128$, $p=0.028$; Table 1). By design, gestational age as well as maternal age, BMI, race/ethnicity, maternal comorbidities did not differ significantly in the two groups (Table 1). There was no observed difference among infant length or neonatal outcome among cohorts (Table 1).

Histopathology

No significant differences in gross pathologic abnormalities (i.e., placental abruption, subchorionic hematoma, nor umbilical cord abnormalities) were observed among the cohorts; a single case of chorioamnionitis was observed in our smoking cohort. Meticulous standardized examination of 6 to 8 H&E-stained placental sections from subject triplicate samples was undertaken. In 7 of 10 smokers, all sections of villous parenchyma were remarkable for prominent syncytiotrophoblastic knots (clusters of syncytial nuclei that form on the surface of a terminal villus characterized by a display of highly condensed chromatin); conversely, this feature was observed only in one of 10 non-smokers, and was statistically significant ($p=0.020$; Figure 2A and B).

The umbilical cord was sectioned and examined for histopathologic abnormalities. As none were observed, sections were not further included in subsequent immunohistochemistry staining studies.

Immunohistochemistry

Table 2 presents a quantitative summary with noted significance of the immunohistochemistry grades for placental CYP1A1, 4-HNE, and 8-OHdG staining depicted in Figure 3, and in association with our syncytial knot formation (Figure 2). The interobserver variability of immunohistochemistry scores was negligible. We observed a

significant overall enhancement of CYP1A1 placental immunostaining among smokers, manifest primarily as positive staining of large decidual cells and extravillous trophoblast (Table 2, Figure 3A). In the villous parenchyma, the intervillous fibrinoid occasionally stained positive, and often syncytiotrophoblast also showed positive staining (Figure 3A). Amniotic epithelial cells stained positive in most fields examined (Figure 3A). Conversely, in controls, very faint staining was occasionally observed in the basal plate, albeit primarily within decidual cells with rare focal staining observed in the villous parenchyma (Table 2; Figure 3A).

Similarly, 4-HNE immunohistochemistry demonstrated more diffuse and intense membranous and cytoplasmic staining in placental sections from smokers (Table 2, Figure 3B). This again manifests as intense diffuse staining of the amniotic epithelium, syncytiotrophoblast, vascular endothelium, and rarely of large Hofbauer cells (Figure 3B). On the maternal interface, similar intensively positive extravillous cytotrophoblast and decidual staining was consistently observed among the entire smoking cohort (Figure 3B). In contrast, staining was focal, less intense, and mostly noticeable on the basal plate, within decidual cells and extravillous cytotrophoblast among non-smokers (Figure 3B).

Finally, 8-OHdG placental immunostaining significantly differed both quantitatively and qualitatively between smokers and non-smokers (Table 2, Figure 3C). Overall and consistent with our HNE observations, positive staining of large decidual cells and extravillous trophoblast was observed (Figure 3C). Interestingly, within villi there was intense staining of the syncytiotrophoblast and to a lesser extent the cytotrophoblast; positive staining of Hofbauer cells was common (Figure 3C). This was both quantitatively and qualitatively distinct when comparing the two cohorts (Table 2, Figure 3C).

COMMENT

The presence of smoking-associated cellular damage in the placenta has been shown in several investigations, although the specificity and uniformity of these findings are variable. (18, 19) We have attempted to circumvent these issues by logically extending our prior findings to the cellular level in a well-matched, nested cohort design of systematically gathered and processed samples, with a focus on both histopathology and well-validated analyses tools for measuring cellular oxidative damage.

We have observed a number of likely clinically-relevant findings in our systematic examinations. Placental sections from gravidae who smoke demonstrated a marked (70%) rate of syncytiotrophoblastic knot formation compared to sections collected from controls (10%, $p=0.02$). Syncytiotrophoblastic knots (also called syncytial knots) are clusters of syncytial nuclei that form on the surface of a terminal villus characterized by highly condensed chromatin. Although often present in normal placenta, syncytial knots are more frequent in mature (term) than in premature placenta, and have historically been used to assess villous maturation. (18, 20) It is felt that increased syncytial knotting is a response of the villi to hypoxia, where villi attempt to increase their surface area to facilitate oxygen exchange with maternal blood. (21–23) Consistent with this notion, an increase in the number of syncytial knots observed is often associated with uteroplacental hypoperfusion and oxidative damage, and thus with conditions such as preeclampsia. (19–23) Our observation that syncytial knots were observed more frequently in placentas of smokers, confirms the observations of Demir et al. (8) and suggests that malperfusion and oxidative damage are increased in smoking mothers compared to controls. We have extended these findings herein to demonstrate their presence in placental sections from gravidae who smoke, in the noted absence of maternal comorbidities.

Building on our prior molecular observations, (6, 7, 14) we now demonstrate that the tobacco-mediated metabolic gene pathway perturbations manifest with significant placental accumulation of both 4-HNE and 8-OHdG (Figure 3). In our investigation, both 4-HNE and 8-OHdG showed increased levels in the placenta of smokers compared to controls. The staining pattern in the two groups was similar, involving large decidual cells, syncytiotrophoblast, and vascular endothelium. The marker 4-HNE was predominantly localized to the syncytiotrophoblast and to the vascular endothelium; cytoplasmic staining was also rarely observed in large Hofbauer cells. Semi-quantitative analysis showed a statistically significant difference in grades between placentas of smokers and controls (3.4 vs 1.1, $p=0.000095$). Similar observations were made for 8-OHdG, however in this case the staining appeared less prominent in the syncytiotrophoblast, and frequent in Hofbauer cells, possibly suggesting phagocytosis of cellular material subsequent to nuclear DNA damage. The difference in IHC grades for 8-OHdG between smokers and controls was also statistically significant (4.9 vs 3.1, $p=0.0038$).

Increased activation of these two markers of DNA oxidation was often co-localized with areas of increased expression of aryl-hydrocarbon hydroxylase, suggesting that oxidative damage within the cellular compartments is associated with the metabolism of smoking by-products into their reactive species; this is consistent with our prior observations. (6, 7, 14) Specifically, we have projected these findings to the cellular level and observed a significant cellular uptake in CYP1A1 staining in smokers compared to controls (4.4 VS 2.1, $p=0.0023$). Taken together, our data suggest that smoking is associated with significant dysregulation of the xenobiotic metabolic pathway leading to increased oxidative damage. (Table 2)

Both 4-HNE and 8-OHdG have been previously investigated in severe pregnancy complications associated with oxidative damage, such as preeclampsia, but with mixed findings. Both Hnat et al. and Noris et al. observed increased 4-HNE levels in vascular endothelial cells in placenta of preeclamptic gravidae compared to normal pregnancies (24, 25); conversely, Takagi et al. showed no significant difference in placental 4-HNE levels between cases of preeclampsia, IUGR, and normal pregnancies. (26) In the latter report, 8-OHdG showed increased levels in pre-eclampsia and IUGR compared to controls, (26) which appeared in disagreement with the observation of Wiktor et al. showing lack of a statistically significant difference in the level of 8-OHdG between preeclamptic, growth restricted, and normal pregnancies. (27)

Other investigators have sought to examine placental sections from smokers and non-smokers for evidence of oxidative damage, and have again reported mixed findings. Rossner et al (28) and Topinka et al (29) reported a trend toward higher levels of respective markers in groups exposed to tobacco smoke (*i.e.* 8-oxodG levels in placenta, 15-F2t-IsoP in newborns, protein carbonyls in mothers). These differences were not significant, probably reflecting the small number of mothers exposed to tobacco smoke in their study (12 subjects, most of whom had low levels of plasma cotinine; 27). In further support of this supposition, the authors noted that the levels of both protein carbonyls and 15-F(2t)-IsoP in cord blood significantly correlated with those in maternal plasma ($p<0.001$). Moreover, because 8-oxodG levels positively correlated with both plasma carbonyls in cord plasma, cotinine levels in maternal plasma and bulky DNA adducts in lymphocyte DNA of newborns and mothers and with PAH-DNA adducts in the placenta (29) it is indeed probable that 8-OHdG associations would have reached significance (28) had they employed a nested cohort design with heavy smokers such as ours as reported herein. With these observations of others in mind, and because no prior observations in regard to 4-HNE and 8-OHdG were made in smokers without concomitant comorbidities, our contributions are further noteworthy. Moreover, they likely speak to the importance of carefully matching cohorts when querying

the effect of maternal exposures on the in utero environment. In addition, our findings may point to significant differences among individuals comprising study cohorts which are deserved of future investigation.

Our cellular findings in this study are also supported by previous molecular characterizations (1–4; 6–15). In sum, mechanisms leading to growth restriction following *in utero* tobacco exposure are poorly understood, but have often been attributed to chronic fetal hypoxia. Of note and with respect to both our current and prior work, all of these factors converge on a limited number metabolic pathways which convert the vast majority of over 4000 compounds found in tobacco smoke to reactive, potentially harmful, and (in some instances) excretable intermediates (1, 2). For these reasons, we have previously investigated and reported on genomic, epigenomic, and population-based maternal and fetal factors associated with maternal smoking and susceptibility to adverse fetal growth.(6, 7, 14) In a population-based, retrospective analysis of term singleton pregnancies, we reported that self-identified tobacco use increases the risk of an SGA infant in gravidae across all BMI strata and with respect to significant maternal comorbidities.(7) We further extended these analyses and demonstrated that in a large matched cohort, deletion of fetal *GSTT1* (a phase II pathway gene, Figure 1) is associated with a mean birth weight reduction of 262 grams specifically and significantly in pregnancies exposed to maternal tobacco use.(6) These observations were gene-environment specific, as significant birth weight ratio variance was *not* observed unless there concomitantly existed both the fetal (but not maternal) *GSTT1* deletion and maternal smoking: fetuses with the deletion but not exposed to tobacco did not demonstrate a variance in their birth weight ratio. (6)

With respect to the phase I pathways (Figure 1), we have demonstrated that increased placental *CYP1A1* expression was specifically and significantly associated with hypomethylation of XRE-proximal CpG dinucleotides in the *CYP1A1* promoter region in smokers compared with non-smokers. (14) Taken together, our findings suggest that among women who smoke, the placental phase I pathway is epigenetically upregulated to generate an accumulation of reactive intermediates (Figure 1). (1, 14) In the absence of *GSTT1* (a functional deletion which is present in >20% of the population), (6) the fetus cannot excrete these intermediates (Figure 1).

While these prior publications provided the initial characterization of the gene signature pathways modulated by maternal smoking, they did not address the cellular physiology *per se*. In the analysis reported herein, we have now extended our prior molecular observations to the level of cellular physiology. In total, we demonstrate that maternal smoking is significantly and specifically associated with gene and CpG-dinucleotide specific epigenomic modulations in key metabolic pathways, (6, 7, 14) which culminate at the cellular level in the form of measured alterations in oxidative stress. Ultimately, these observations allow us to understand perinatal gene-environment interactions at the molecular and cellular level. Future development will include the investigation of additional markers of oxidative damage in a much larger cohort of gravidae, and thereby allow for follow up to determine the impact of these findings on maternal and infant outcome and development.

Acknowledgments

This work was supported by the NIH Director New Innovator Award (DP2120OD001500-01 K.A.T.), NICHD/NIDDK #R01DK080558-01 (K.A.T), and the NIH REACH IRACDA K12 GM084897 (M.S.).

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Figure 1. Processing of xenobiotics in the placenta

(A) Polycyclic aromatic hydrocarbons are processed in a two step process. An increase in the Phase I enzymes is reported in the placenta in mother's who smoke compared with non-smoking controls. An increase in Phase I enzymes metabolizes PAHs into reactive oxygen species (ROS) which can lead to oxidative DNA damage, such as 8-OHdG. (B) Processing of xenobiotics by the Phase I enzyme CYP2E1 creates ROS which can lead to oxidative lipid damage such as 4-HNE.

Figure 2. Increased syncytiotrophoblastic knots in placentas of smokers

H&E staining of placental sections from smokers (A) compared to non-smokers (B) shows an increased level of syncytial knots. Original magnification (A, B): 4x.

Figure 3. Increased CYP1A1, 4-HNE and 8-OHdG in placentas of smokers

(A) Immunostaining for CYP1A1 in smokers (left) and non-smokers (right). Original magnifications, left to right: 10x, 40x, 10x, 10x.

(B) Immunostaining for 4-HNE in smokers (left) and non-smokers (right). Original magnifications, left to right: 20x, 40x, 20x, 20x.

(C) Immunostaining for 8-OHdG in smokers (left) and non-smokers (right). Original magnifications, left to right: 20x, 40x, 20x, 20x.

Positive staining appears in brown color (DAB chromagen).

Table 1
Characteristics of the study population

In our nested cohort design, after matching for maternal characteristics and gestational age, we observed a statistically significant association between infant birth weight and maternal smoking.

	Non-smokers (<i>n</i> 10)	Smokers (<i>n</i> 10)	<i>p</i> value
Maternal age (years)	29.7 ± 1.8	27.8 ± 2.1	0.504
Maternal BMI (kg/m ²)	26.5 ± 0.9	22.7 ± 3.1	0.251
Gestational age (weeks)	39.3 ± 0.7	39.0 ± 0.4	0.719
Infant weight (grams)	3619 ± 128	3159 ± 144	0.029*
Infant length (cm)	49.8 ± 0.7	48.2 ± 0.9	0.226

Table 2
Immunohistochemistry Score (Grade, $\pm$ SD)

Semi-quantitative immunohistochemistry comparing gravid smokers and nonsmokers. We observed a statistically significant difference in placental accumulation of 4-HNE, 8-OHdG, and CYP1A1. Representative photomicrographs are found in Figure 3.

	Non-smokers (n 10)	Smokers (n 10)	p value
CYP1A1	2.1 $\pm$ 1.6	4.4 $\pm$ 1.3	0.002300*
4-HNE	1.1 $\pm$ 0.7	3.4 $\pm$ 1.2	0.000095*
8-OHdG	3.1 $\pm$ 0.7	4.9 $\pm$ 1.4	0.003800*