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Original contribution

Smoking during pregnancy causes double-strand DNA break damage to the placenta

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Summary Despite the adverse effects of smoking, many pregnancies are exposed to tobacco smoke. Recent studies have investigated whether smoking damages placental DNA by measuring DNA adducts. This study investigated whether a more severe lesion, double-strand DNA breaks, was also present in the tobacco smoking–exposed placenta. Term placentae from women who smoked during their entire pregnancies (n = 52), from those who had ceased smoking for at least 4 weeks before delivery (previous smokers, n = 34), and from nonsmoking women (n = 150) were examined using the DNA double-strand break marker phosphorylated γ H2AX. The extent of DNA damage was assessed according to cell type and additional markers were applied for cell fate (apoptosis and DNA repair), and function (human chorionic gonadotropin, human placental lactogen, and glucose transporter 1), to characterize the effect of the DNA damage on placental integrity. Marked phosphorylated γ H2AX–positive cells occurred in the villous syncytiotrophoblast and syncytial knot nuclei in placentae from smokers ($P < .001$). Phosphorylated γ H2AX foci did not colocalize with the DNA repair protein 53BP1, and damaged nuclei had a marked reduction in expression of human chorionic gonadotropin, human placental lactogen, and glucose transporter 1. Minimal DNA damage, similar to nonsmokers, was present in previous smokers including those that had ceased smoking for just over 4 weeks before delivery. In summary, smoking during pregnancy was associated with marked double-strand DNA break damage to the syncytiotrophoblast. We suggest that smoking cessation is important to prevent additional DNA damage and to facilitate DNA repair.

1. Introduction

Despite the risks, 13% to 25% of pregnancies are exposed to tobacco smoke [1,2]. Tobacco smoking lowers birth weight, increases the risk of antepartum hemorrhage and abruption, and increases perinatal mortality and the risk of sudden infant death syndrome [3-6]. Long-term effects from tobacco smoke exposure in utero include an increased risk for obesity and impaired lung function [7-11].

In many cases, the smoking-exposed placenta is normal by gross and by routine microscopic analysis [12-16]. Thus, a diagnostic report for a smoking-related placenta is unlikely to document changes because these require investigation and quantification beyond the constraints of routine diagnostic

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services. In some cases, the smoking-exposed placenta does demonstrate pathologic microscopic changes of chronic uteroplacental malperfusion including infarcts, villous hypoplasia, Tenny-Parker changes, and others [17]. Smoking induces DNA damage such as DNA adducts and the more severe double-strand DNA break lesion [18]. As early as 1986, it has been known that covalent DNA adducts can occur in the smoke-exposed placenta, but it is not known if double-strand DNA breaks also occur [19-21]. Double-strand breaks are hazardous leading to an increased risk of genetic instability, and as such, this type of DNA damage has been thoroughly studied in cancer [22-24].

Double-strand DNA breaks are detected by phosphorylated γ H2AX. As a component of the DNA repair response, the histone deacetylase γ H2AX becomes phosphorylated on serine 139 and accumulates at double-strand DNA break sites [25]. Phosphorylated γ H2AX staining (γ H2AX) is approximately 100 times more sensitive than other double-strand DNA break detection methods [18,22,25] and can be applied to paraffin-embedded tissues to examine the spatial distribution of double-strand DNA breaks [22], and γ H2AX foci form de novo upon DNA damage and disappear once the DNA break is repaired [18].

The premise for this study was that double-strand DNA breaks may contribute to the complications associated with smoking during pregnancy. This study addressed whether double-strand DNA breaks are present in the placentae from maternal smokers. Placentae from current smokers and nonsmokers were accessed using γ H2AX staining [18,22]. Because of the severity of the double-strand break lesion, we also tested whether the DNA damage was associated with impaired cell function. The study was extended to test if smoking cessation before delivery could ameliorate DNA damage, by using placentae from women who ceased smoking up to 4 weeks before delivery.

2. Materials and methods

Placentae were obtained from the Otago Placenta Study, collected from 2009 to 2011 in New Zealand. Smoking history included the number of cigarettes smoked per day and the number of weeks since the last cigarette. Most women smoked factory-made cigarettes (85%) with 5 prominent brands: 30% Holiday, and 10% to 15% for Benson & Hedges, Horizon, Pall Mall, and Winfield. All of the term placentae were from women who delivered at between 38 to 42 weeks of gestation and were all from singleton births. Placentae that were associated with pregestational or gestational diabetes, hypertension, preeclampsia, or known causes of intrauterine growth restrictions were excluded.

A total of 236 singleton term placentae were collected from women who were smoking during pregnancy and up to delivery (smoking, n = 52, smoking an average of 8 cigarettes per day), women who did not smoke during pregnancy (nonsmokers, n = 150), and women who had smoked during pregnancy but had quit smoking at least 4 weeks before delivery (previous smokers, n = 34). In the previous smoker group, 24 placentae came from women who had ceased smoking in the third trimester, 4 to 12 weeks before delivery. The demographic, clinical, and fetoplacental characteristics of the smoking, nonsmoking, and previous smoking groups are given in Table 1.

A trained examiner, blinded to the clinical history, assessed fresh or refrigerated placentae (within 24 hours of delivery) by standard pathologic means. Eight standard sections were dissected per placenta and consisted of representative transmural placental sections within 50 mm of the umbilical insertion. Histologic and immunohistologic sections were examined and graded by 2 investigators including a perinatal pathologist who was blinded to the smoking status and clinical history. Additional tissue samples were taken from 10 placentae to investigate the effect of delayed fixation and prolonged fixation in 10% neutral-buffered formalin before processing to paraffin wax.

2.1. Immunohistochemistry

All antibodies were used at dilutions recommended by the manufacturers. Single-protein immunohistochemistry (IHC) analyses (γ H2AX, ab2893 and ab22551; Abcam, Cambridge, UK) used the Envision Dual Link detection system (Dako, Glostrup, Denmark) and DAB. Cells were imaged using light microscopy (DM 2000 microscope, DFC 295 camera, and Application Suite software, version 3.5.0; Leica, Solms, Germany).

γ H2AX staining was examined in a transmural placental section. Since a geographic variation in staining intensity was observed; the most intense staining being within 5 mm of the chorion, this subchorionic area was chosen for γ H2AX grading, and positive nuclei in the villous syncytium and syncytial knots were assessed. γ H2AX staining was graded from 0 to 3, and the grading confirmed with the second γ H2AX antibody. Grade 3 had greater than 10% positive nuclei, and grade 0 had less than 0.1% positive nuclei. In the syncytium, the number of γ H2AX–positive nuclei per 500 nuclei was counted. In the syncytial knots, knots were considered positive if 2 or more of the clustered nuclei were positive. At least 500 knots were assessed as syncytial knots per terminal villous or areas.

Apoptotic nuclei were detected in placental sections using the Klenow FragEL DNA Fragmentation Kit according to the manufacturer's instructions (Merck, Darmstadt, Germany). All apoptotic cells were counted per section using light microscopy.

2.2. Immunofluorescence

Detection of caspase 3 cleaved at Asp 175 (MAB835; R and D Systems, Minneapolis, MN), 53BP1 (ab36823; Abcam), E-cadherin (Abcam), human chorionic gonadotropin (hCG)