The Effect of Cigarette Smoke on Placental Antioxidant Enzyme Expression

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Abstract:

Cigarette smoking is associated with systemic oxidative stress leading to an upregulation of antioxidant systems (superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and haeme oxygenase (HO)) in some tissues but the response in the human placenta is unknown. The aim of this study was to determine the effect of cigarette smoke exposure on placental antioxidant expression in vivo, as well as the effect on antioxidant expression in the HTR-8SVNeo trophoblast cell line. Methods: In vivo experiment: Normal term placentas were obtained following elective caesarean section. The chorionic villi (CV), anchoring villi (AV), and basal plate (BP) were dissected, and western blot analysis was carried out for HO-1, HO-2, SOD, CAT, and GPx. In vitro experiment: A cigarette smoke extract (CSE) was prepared by bubbling the smoke form 3 cigarettes through 15mL of RPMI. This 100% CSE was syringe filtered and diluted to 0.1, 0.5, 1, 2, 5, and 10% concentrations. HTR-8SVNeo cells were cultured with the CSE for 48 hours. The cells were harvested, protein was extracted, and run on SDS-PAGE gels, and western blot analysis was carried out for HO-1, HO-2, SOD, and CAT. Immunofluorescence for HTR-8SVNeo cells HO-1 was carried out following increasing concentrations of CSE. Results: In vivo experiment: HO-1 and HO-2 expression was increased in the BP of placentas from smokers compared to non-smokers. CAT, GPx, and SOD levels in all placental regions, and HO-1 and HO-2 expression in the AV and CV were unchanged. In vitro experiment: The 5%, 10%, and 20% dilutions were toxic to the cells. The 0.1% CSE solution did not significantly alter HO-1 expression. Treatment with the 0.5%, 1% and 2% CSE solutions resulted in a dose-dependent increase in HO-1 expression. None of the CSE treatments resulted in a
significant alteration in HO-2, SOD, GPx, or CAT expression. HO-1 immunoflorescence confirmed the HO-1 expression studies. **Conclusions:** Cigarette smoke exposure increases HO-1 and HO-2 expression in the placental basal plate and increases HO-1 expression in the HTR-8SVNeo cell line. Increased HO-1 and HO-2 protein expression may increase the production of the antioxidants biliverdin and bilirubin, which are products of haeme metabolism. This could function to reduce the oxidative load that is released into the maternal plasma from the pre-eclamptic (PE) placenta, and may contribute to the observed decreased incidence of PE in smokers.
**Introduction:**

Cigarette smoking during pregnancy is a major public health concern that is associated with an increased risk of premature birth, spontaneous abortion, placenta previa and intrauterine fetal growth restriction (IUGR) (Lambers and Clark. 1996). The effect of fetal growth is dose-dependent with birth weight decreasing 250g/pack/day smoked (Wang et al. 1997). Paradoxically, women who smoke during pregnancy exhibit a 32% decreased incidence in pre-eclampsia (PE) (Conde-Agudelo et al. 1999; Yang et al. 2005). In order for this effect to occur, the woman must smoke for the duration of the pregnancy (England et al. 2002) and the incidence of PE is inversely correlated with the number of cigarettes smoked per day (Conde-Agudelo et al. 1999). A recent review of the subject (Bainbridge et al. 2005) hypothesized that the effect is likely multifactorial due in part to increased circulating levels of carbon monoxide (CO) which acts as a placental vasodilator (Bainbridge et al. 2002) and decreases placental apoptosis (Bainbridge et al. 2006), as well as possible upregulation of placental antioxidant systems.

Placental oxidative stress appears to be critical for the development of PE (Madazli et al. 2002; Mutlu-Turkoglu et al. 1998; Poranen et al. 1996; Rinehart et al. 1999; Walsh and Wang. 1993). Therefore, any increase in the levels of endogenous antioxidants may prevent the development of PE. Several antioxidant systems have been identified within the placenta, including copper/zinc superoxide dismutase (Cu/Zn SOD; inactivates O$_2^-$) (Wang and Walsh. 1996), catalase (CAT; inactivates H$_2$O$_2$) (Wang and Walsh. 1996) glutathione peroxidase (GPx; inactivates H$_2$O$_2$ and lipid peroxides) (Wang and Walsh. 1996) and the haeme oxygenase enzymes HO-1 and HO-2 (McLean et al.
2000). HO enzymes act as antioxidants by degrading haeme (a pro-oxidant) into equimolar quantities of CO, biliverdin (bilirubin) and Fe^{2+} (Bainbridge and Smith. 2005); biliverdin and bilirubin are potent physiologic antioxidants. HO-1 (32 kDa) is the inducible form of the enzyme, while HO-2 (36 kDa) is constitutively active (McCoubrey et al. 1997).

A better understanding of the mechanism(s) by which cigarette smoking decreases the risk of developing PE will increase our understanding of the PE disease process and may lead to therapeutic options; delivery is currently the only known cure for PE. The objective of this study was determine \textit{in vivo} the effect of smoking on placental antioxidant enzyme expression as well as the \textit{in vitro} effect of cigarette smoke extract (CSE) treatment on trophoblast antioxidant systems. We hypothesize that exposure to cigarette smoke increases the expression of antioxidants within placental tissue and that this increase in placental antioxidant enzymes is linked to the decreased incidence of PE in smokers.

\section*{Methods:}

\subsection*{Tissue Collection}

Term placentas (n=6 smokers, n=6 non-smokers) from uncomplicated pregnancies were obtained immediately following elective caesarian section at the Kingston General Hospital and transported to the laboratory on ice. Inclusion criteria for smokers/nonsmokers was based on maternal end-tidal CO measured using a Micro CO meter (Micro direct, Lewiston, ME) (non-smokers <5 ppm CO, smokers >15ppm CO).

\subsection*{Tissue and Cell Preparation}
Placental tissues were blunt dissected and random samples from the basal plate, anchoring villi and chorionic villi were obtained. The tissue was flash frozen in liquid nitrogen and stored at –80°C for future analysis.

Cells of the immortalized human trophoblast choriocarcinoma cell line HTR-8SVneo were a gift from Dr. C.H. Graham (Queen’s University). The cells were maintained at 37°C in 75 cm² flasks containing RPMI (Sigma-Aldrich, Oakville, On) + 5% foetal bovine serum (FBS) (Sigma-Aldrich, Oakville, On) (Graham et al. 1993). Confluent monolayers were trypsinized (Sigma-Aldrich, Oakville, On) and then 5 x 10⁵ cells were plated in 60mm dishes for experimental procedures.

**Cigarette Smoke Extract (CSE) Preparation and Cell Treatment**

A vacuum-driven smoking devise was adapted from the protocol used by Lee et al. (Lee et al. 2001). Briefly, the smoke from three cigarettes was drawn through 15mL of RPMI media. This was established as 100% CSE. In order to ensure reproducibility from one batch of CSE to the next, dissolved CO₂ levels were measured using the ABL5 gas monitor (Radiometer, London, ON) The 100% CSE was then diluted using RPMI containing 5% FBS.

Previous work with pulmonary artery endothelial cells demonstrated that treatment with CSE concentrations of greater than 10% resulted in significant cell death (Lee et al. 2001). The HTR-8SVneo cells were incubated for 48 hours with 0.1%, 0.5%, 1%, 2%, 5%, 10%, and 20% dilutions of CSE or control RPMI.

**Protein Extraction**

Placental samples were thawed and washed in PBS to remove excess blood. 4 mL of protein extraction buffer (2% SDS, 10mM Tris, 0.15M NaCl, pH 7.5) was added for
every 1g of tissue, the tissue was homogenized and DNA was sheared by passing the homogenate 12 times through an 18-gauge needle. Protein was extracted from the HTR-8SVneo cells using 70µL of a protein extraction buffer (2% SDS, 10mM Tris, 0.15M NaCl, pH 7.5). The samples were then sonicated for 15s to denature nucleic acids. Tissue and cell culture homogenates were boiled for 5 minutes and then centrifuged at 14,000g at 22°C for 15 minutes to separate out cellular fragments. The supernatant was collected and stored at –80°C. The protein concentration of each sample was determined using the DC protein assay (Bio-Rad Laboratories, Hercules, CA).

**Western Blot Analysis**

The protein extraction samples (30-60µg) were separated by electrophoresis on 12% SDS-PAGE gels at 165V for 2.5hrs. The separated proteins were then transferred onto PVDF membranes (Millipore, Billerica, MA) at 110V for one hour. The membranes were blocked overnight in PBST with 5% skim milk and 1% bovine serum albumin to prevent non-specific binding. Membranes were then incubated for one hour with primary antibody (HO-1 – RCH Antibodies, Sydenham, On; HO-2, and GPx – Stressgen, Victoria, B.C.; SOD – Santa Cruz Biotechnology, Santa Cruz, CA; and CAT – Sigma-Aldrich, Oakville, On). Following incubation with the primary antibody, the membranes were washed five times for five minutes in PBST and then incubated in the appropriate secondary antibody (1:15,000 goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA) for HO-1, HO-2, and SOD, 1:25,000 goat anti-mouse IgG (Bio-Rad Laboratories, Hercules, CA) for CAT and GPx)) for one hour, and washed as after the primary antibody. Following the second set of washes, secondary antibodies were detected by
enhanced chemiluminescence (PerkinElmer Life Sciences, Boston, MA) and exposed onto X-OMAT Blue XB-1 film (Kodak, Rochester, NY).

As an internal control all membranes were stripped of bound antibody (100mM 2-mercaptoethanol, 2% SDS, 62.5mM Tris, pH 6.7) at 50°C for 30 minutes and reprobed for the housekeeping protein β-actin (1:8,000, Sigma-Aldrich, Oakville, On). The membranes were processed as above.

**Immunofluorescence Staining of CSE treated HTR8 SVneo cells**

HTR8 SVneo cells were seeded on glass coverslips in 35mm cell culture dishes and allowed to adhere overnight in 37c incubator at 5% CO2 in complete RPMI (RPMI +10% FBS)(Sigma-Aldrich Oakville, ON.). Media was replaced with complete RPMI, RPMI + 0.1% or RPMI + 0.5% CSE and cells incubated for a further 24 hours. Cells were fixed in 4% PFA on ice for 15 minutes, washed twice in cold PBS and permeabilized in 0.5% Triton X 100 PBS for 15 minutes on ice. Cells were washed twice in PBS 01% Triton X 100 and incubated in 200ug/ml RNAse A(Sigma- Aldrich, Oakville, ON.) at 37c for one hour. Cells were then blocked in 5% normal goat serun PBST for 1 hour. Cell were then incubated with anti H0-1 chicken antibody(RCH Antibodies Sydenham, ON.) for one hour followed by incubation for one hour in anti chicken Alexa 488 secondary antibody(Molecular Probes Eugene OR.). Cells were washed and incubated in Propidium Iodide 2ug/ml.(Sigma-Aldrich). The cells were mounted on slides and photographed on a Leica TCS SP2 Confocal Microscope.

Data Analysis
The band densities were determined using the AlphaEase software (Alpha Innotech Corp. San Leandro, CA). The data are expressed as the ratio of the protein density of each lane to the β-actin density value of the same lane.

**Statistical Analysis**

Results were expressed as the mean ± standard error of the mean. The human placenta data was analyzed using a two-tailed paired students t-test and results were considered statistically significant when \( p < 0.05 \). The HTR-8SVneo data was analyzed using a one-way ANOVA and the data was subjected to a Tukey post-test.

**Results:**

**Placental Data**

The smoking group exhibited significantly higher exhaled CO than the non-smokers \((19.7 ± 2.2 \text{ vs } 3 ± 0.45 \text{ p=0.001})\). Exhaled CO is correlated with number of cigarettes smoked [26].

The HO-1 (Fig.1a-c) and HO-2 (Fig. 2a-c) expression was increased \((p<0.05)\) in the smoking group compared to the non-smoking group in the basal plate but not \((p>0.05)\) in either the anchoring or chorionic villi. Placental HO-1 protein expression was lower than that of HO-2. 50ug/ul of protein was required to visualize the 32kDa band corresponding to HO-1, while 30ug/ul of protein was sufficient for the visualization of HO-2, CAT, SOD, and GPx.

In all regions of the placenta, catalase, GPx, and SOD expression in smokers was not different \((p>0.05)\) compared to non-smokers (Data not shown).

**Cell Culture Data**
A reproducible CO$_2$ concentration of 80-100ppm was used to demonstrate consistency and accepted for use in CSE preparations. A “fresh” pack of cigarettes was used for each experiment as using “stale” cigarettes resulted in elevated CO2 concentrations that were not within this 80-100ppm working range. While it is not known which component(s) of cigarette smoke are captured in this CSE, CO levels are undetectable as measured by gas chromatography (data not shown).

All concentrations of CSE above 2% were associated with 100% HTR-8SVneo human cytотrophoblast cell death. The 2% dilution was also associated with cell death, though sufficient protein could still be collected to allow for western blot analysis. Concentrations less than 2% did not alter the rate of cell growth and division, nor were they associated with visible morphological changes in the HTR-8SVneo cell line.

HO-1 levels were increased in a dose-dependent manner following treatment with increasing concentrations of CSE (0.1%, 0.5%, 1.0% and 2.0%) (Fig. 3). HO-2, CAT, and SOD levels were unaffected by the CSE treatment (Data not shown). Immunostaining for HO-1 confirmed the dose-dependent effect of increasing CSE treatment (representative data Figure 4)

**Discussion:**

The present study characterized the effect of cigarette smoking *in vivo* during pregnancy on placental antioxidant systems and *in vitro* using CSE on the HTR-8SVneo antioxidant profile. Smoking throughout pregnancy resulted in elevated expression of the haeme oxygenase enzymes HO-1 and HO-2 in the placental basal plate region. Smoking did not alter the expression of CAT, SOD, or GPx, in any of the placental regions studied.
HTR-8SVneo cell line exhibited a dose-dependent increase in HO-1 expression and immunofluorescence following treatment with CSE. Cigarette smoke extract treatment did not alter the expression of HO-2, SOD, or CAT in this cell line.

Smoking constitutes a systemic oxidative stress, as each inhalation of cigarette smoke contains $10^{14}$ oxidants in the tar phase and $10^{15}$ oxidants in the gas phase (Hung et al. 2001). While acute oxidative stress (i.e. ischemia/reperfusion (IR) injury) is associated with tissue damage, chronic oxidative stresses such as that experienced by smokers may lead to an upregulation of the antioxidant enzymes. HO-1 is the inducible isoform of HO, and its expression is known to be increased in response to oxidative stress in the heart, kidneys, and liver (Katori et al. 2002).

While HO-1 is known to be induced by cigarette smoke in other tissues, the increased expression of HO-2 in the basal plate of smokers was unexpected as this isoform is constitutively active. However, HO-2 has recently been shown to be upregulated in nervous tissue following hyperthermic injury (Gordh et al. 2000; Sharma and Westman. 2003; Sharma et al. 2000) which can be attenuated by antioxidant pretreatment (Sharma et al. 2003) suggesting that this upregulation is at least in part due to oxidative stress.

The observed dose-dependent increase in HO-1 expression and immunoflourescence following CSE treatment in the HTR-8SVneo cells mimics the increased HO-1 expression seen in the placental basal plate of smoking women. However, the *in vivo* experiments did not examine a possible dose-dependent effect, but compared HO expression in the placentas from non-smokers with that from moderate-to-heavy smokers.
The *in vivo* effect of cigarette smoke on HO-1 and HO-2 expression was limited to the basal plate. The cells of the basal plate are mainly extravillous cytotrophoblast, while the anchoring and chorionic villi are composed of syncytiotrophoblast with scattered cytotrophoblast cells (Langerhans’ cells) found only in 20% of the villous surface at term. The different structural and functional properties of the subdivisions of trophoblast could result in different responses to the stress of maternal cigarette smoking. The HTR-8SVneo cell line employed in this study is believed to be extravillous cytotrophoblast-like in that it possesses numerous characteristics that are limited to this trophoblast cell population, including a highly invasive phenotype (Graham et al. 1993) and an inability to fuse to form a syncytium.

Decreased HO expression has been observed in several pathological conditions in pregnancy, including spontaneous abortion, hydatiform mole, choriocarcinoma, and PE (Yallampalli and Garfield. 1993; Zenclussen et al. 2003). The decreased expression of HO in PE placentas may be the result of tissue damage as HO-2 levels are decreased in infarcted and peri-infarcted regions of the placenta (Lash et al. 2003), and the placentas from women with PE exhibit more areas of infarction. This decreased HO activity could contribute to the widespread oxidative stress that is present in PE.

Increased HO expression would result in increased biliverdin and bilirubin production locally. At physiologic oxygen tensions bilirubin has been found to be significantly more potent than vitamin E at protecting against lipid peroxidation (Stocker et al. 1987); as little as 10nM bilirubin is capable of protecting against a 10000 fold higher concentration of H$_2$O$_2$ (Baranano et al. 2002). Farrera *et al.* (Farrera et al. 1994) compared antioxidants and found that bilirubin was the most potent superoxide and
peroxyl radical scavenger. Bilirubin is also capable of preventing peroxynitrite-induced protein oxidation (Minetti et al. 1998). Therefore, any upregulation in HO-1 expression in the basal plate of smokers could potentially lead to the generation of more bilirubin, which could then function at the maternal-foetal interface to decrease oxidative stress.

In summary, smoking throughout pregnancy is associated with an increase in placental basal plate HO activity which is supported by the in vitro CSE-mediated dose-dependent increase in HO-1 in the placental cell line HTR-8SVneo. It is speculated that this upregulation of HO may explain, in part, the decreased incidence of PE in smokers by facilitating trophoblast invasion of the spiral arteries and decreasing placental oxidative damage, especially as the relationship between smoking and the development of PE is also dose-dependent. The “protective” effect of smoking is likely multifactorial representing a combination of the anti-apoptotic and vasodilatory effects of CO as well as the antioxidant actions of HO-1. Investigations into how cigarette smoking alters the HO enzyme system and the effects this has on endogenous oxidant/antioxidant balance and the development of PE could lead to the therapeutic use of inducers of this system in the prevention and/or treatment of PE.

1. Bainbridge SA, Belkacemi L, Dickinson M, Graham CH, Smith GN


15. **Lee SD, Lee DS, Chun YG, Shim TS, Lim CM, Koh Y, Kim WS, Kim DS and Kim WD.** Cigarette smoke extract induces endothelin-1 via protein kinase C in


Figure legends:

Figure 1. The effect of cigarette smoking on placental HO-1 enzyme expression density. (A) anchoring villi, (B) chorionic villi, (C) basal plate. n=6 smokers, n=6 non-smokers. The asterisk (*) indicates a statistically significant difference (p<0.05).

Figure 2. The effect of cigarette smoking on placental HO-2 enzyme expression density. (A) anchoring villi, (B) chorionic villi, (C) basal plate. n=6 smokers, n=6 non-smokers. The asterisk (*) indicates a statistically significant difference (p<0.05).

Figure 3. The effect of a 48 hour CSE exposure on HTR-8SVNeo antioxidant expression (A) HO-1, (B) HO-2, (C) CAT, (D) SOD. n = 5 for each group. The asterisk (*) indicates a statistically significant difference (p<0.05).

Figure 4. Representative images of the effect of increasing CSE exposure on HO-1 immunofluorescence in HTR-8SVNeo cells.
Dose-Response Effect of CSE on HO-1 Expression in HTR-8 Cells

HO-1 Expression Density (fold difference)

- Control RPMI
- 0.1% CSE
- 0.5% CSE
- 1% CSE
- 2% CSE

The graph shows the dose-response effect of CSE on HO-1 expression in HTR-8 cells. The expression density (fold difference) increases with higher concentrations of CSE, reaching a peak at 2% CSE.