Cyclooxygenase-1 and -2 in human placenta and placental bed after normal and pre-eclamptic pregnancies

B.Wetzka1,2,4, R.Nüsing3, D.S.Charnock-Jones2, W.Schäfer1, H.P.Zahradnik1 and S.K.Smith2

1Endocrinology and Reproductive Medicine, Universitätsfrauenklinik Freiburg, Hugstetter Str. 55, D-79106 Freiburg, Germany, 2Reproductive Molecular Research Group, Department of Obstetrics and Gynaecology, University of Cambridge Clinical School, Robinson Way, Cambridge CB2 2SW, UK and 3Medical Center of Pediatrics, University of Marburg, Deutschhausstr. 12, D-35037 Marburg, Germany

4To whom correspondence should be addressed at: Endocrinology and Reproduction Medicine, Universitätsfrauenklinik Freiburg, Hugstetter Str. 55, D-79106 Freiburg, Germany

Introduction

Pregnancy-induced hypertension (PIH) and pre-eclampsia are complications unique to human gestation which occur in the second half of pregnancy. Over the years, there have been many different theories about aetiology and pathogenesis of this disease (Dekker and Geijn, 1996). However, until now there has been no specific therapy apart from the delivery of the infant. An imbalance of vasoactive prostaglandin production, i.e. the vasoconstrictory and platelet aggregating thromboxane (TXA2) and its functional antagonist prostacyclin (PGI2), are thought to be involved in the pathophysiological changes during PIH and pre-eclampsia (Ylikorkala and Viinikka, 1992). Studies in vivo (urinary metabolites) and in vitro (placental tissue culture), although showing partly contradictory results, have led to the view that pre-eclampsia is accompanied by a decreased PGI2 production followed by a decreased PGI2/TXA2 ratio (Zahradnik et al., 1991; Ylikorkala and Viinikka, 1992). Recent studies investigating the expression of the specific synthesizing enzymes of PGI2 and TXA2, prostacyclin synthase (PCS) and thromboxane synthase (TXS), found either an increased expression of TXS in placenta (Woodworth et al., 1994) or no change in the expression of both enzymes in placenta and placental bed (Wetzka et al., 1996). After the release of arachidonic acid (AA) from membrane phospholipids, cyclooxygenase (COX) action constitutes an important regulatory step of PGI2 and TXA2 biosynthesis (Marshall et al., 1987). The activity of COX converting AA to prostaglandin H2 (PGH2), the common precursor of all prostaglandins, is regulated directly by auto-inactivation, the concentration of hydroperoxide and the activity of glutathione synthase in tissues (Marshall et al., 1987). Further, the existence of two genes encoding different forms of COX have been described recently (Yokoyama and Tanabe, 1989; Appleby et al., 1994). COX-1, encoded by a gene showing characteristics of a ‘house-keeping gene’, is expressed constitutively in many cell types and is thought to be involved in rapid physiological responses (Smith and DeWitt, 1995). COX-2, the product of an immediate early gene, is induced in certain cell types in response to stimuli such as lipopolysaccharide, cytokines and growth factors, and probably plays a role in prolonged physiological or inflammatory reactions (Hla and Neilson, 1992; Appleby et al., 1994). The enzyme characteristics of both cyclooxygenases are similar, but their pharmacological properties regarding inhibition by non-steroidal anti-inflammatory drugs (NSAID) are somewhat different (Smith and DeWitt, 1995).

We performed the present study to address the following questions concerning pathophysiology and treatment of pre-eclampsia: (i) Which cells in placenta and placental bed biopsies express COX-1 and/or COX-2 and is there a difference in expression after pre-eclamptic pregnancies? If COX activity is decreased in the PGI2 (endothelial cells) and/or increased in the TXA2-producing cells (macrophages), altered prostaglandin concentrations will result. (ii) Does one of the COX isoforms...
show a different level of expression in pre-eclampsia and therefore is it inhibited selectively with new COX-1- or -2-specific NSAID currently under development?

**Materials and methods**

**Materials**

Anti-human cytokeratin (Clone MNF 116), anti-human CD68 (KP1), mouse and rabbit immunoglobulins (irrelevant antibody), goat serum, biotinylated goat-anti-mouse immunoglobulin and fluorescein isothiocyanate (FITC)-conjugated goat-anti-mouse immunoglobulin were bought from Dako, High Wycombe, UK and anti-human CD14 (Leu M3) was obtained from Becton Dickinson, San Jose´ , CA, USA. Biotin–streptavidin–peroxidase complex (Vectastain Elite™) was purchased from Vector Laboratories, Peterborough, UK, and Immobilon™ (PVDF) from Millipore, Bedford, MA, USA. Enhanced chemiluminescence system (ECL™-Kit) and biotinylated molecular weight markers were bought from Amersham International, Little Chalfont, Buckinghamshire, UK. Guanidinium thiocyanate and Superscript reverse transcriptase were obtained from Gibco-BRL, Uxbridge, UK. Amplitaq polymerase was bought from Perkin Elmer, Weiterstadt, Germany. Tetramethylrhodamin isothiocyanate (TRITC)-conjugated goat anti-rabbit immunoglobulin, diaminobenzidine (DAB), aprotinin, pepstatin, leupeptin and trypsin inhibitor were purchased from Sigma, Poole, UK. All other reagents were obtained from BDH, Poole, UK.

Rabbit polyclonal antibodies (pAb) against COX-1 and COX-2 were raised at the Department of Pediatrics, Philipp's University, Marburg, Germany (Kömhof et al., 1997).

**Tissue collection**

Placenta and placental bed biopsies were obtained from patients undergoing a Caesarean section before the onset of labour. Preliminary experiments showed increased COX-1 and -2 protein concentrations in placenta during labour, therefore only tissue specimens from patients undergoing a Caesarean section before the onset of labour were included in this study. Written informed consent was obtained in all cases and the study was approved by the Ethical Committee of Addenbrooke’s Hospital NHS Trust. In the ‘control (C) group’ (*n* = 7 for placenta, *n* = 6 for placental bed), Caesarean sections were performed because of breech presentation or previous Caesarean section. In the ‘pre-eclampsia (PE) group’ (*n* = 7 for placenta, *n* = 6 for placental bed), all patients had an elevated blood pressure of >140 mmHg systolic and >90 mmHg diastolic and proteinuria of >0.5 g/24 h. Clinical details of the patients belonging to both groups are shown in Table I.

**Immunohistochemistry**

Frozen sections (5 µm) were mounted onto silane-treated microscopic slides and fixed in cold acetone. Incubation with pAb against COX-1 (1:1000) and COX-2 (1:500), respectively, was performed for 1 h at room temperature. The bound antibody was detected with the biotin–streptavidin–peroxidase system with DAB as chromogen. After counterstaining with haemalum, the slides were mounted with DePeX. All sections of either placenta or placental bed were processed simultaneously. The sections were evaluated by conventional light microscopy and scoring was performed in a relative manner (strong, medium, weak) as described previously (Wetzka et al., 1994). To confirm the identity of the trophoblast cells and processed in the same way as described above.

For identification of macrophages, double immunofluorescence staining was performed on representative placental sections by incubation with both anti-COX-1 (1:200) and anti-CD68 (1:100) or anti-COX-2 (1:200) and anti-CD68 overnight at 4°C. Placental bed sections were incubated with anti-COX-1 or anti-COX-2 and anti-CD14 (1:25). Trophoblast cells in placental bed were localized by applying both anti-COX-1 or anti-COX-2 and anti-cytokeratin (1:25) onto serial sections. The bound antibodies were detected by incubation with FITC-conjugated goat anti-mouse and TRITC-conjugated goat anti-rabbit immunoglobulins. The slides were viewed in a UV microscope (Axioskop from Zeiss, Oberkochen, Germany) with filters for selective red or green fluorescence.

Negative controls were performed by incubating serial sections with an irrelevant antibody in an equivalent concentration and by omitting the first antibody.

**Western blotting**

Frozen tissue pieces of 200–500 mg were homogenized by Polytron homogenization on ice in 10 mM potassium phosphate buffer, pH 7.4, containing 2 mM EDTA, 1% Triton X-100, aprotinin (0.01 U/ml) and pepstatin (2 mg/ml), leupeptin (2 mg/ml) and trypsin inhibitor (2 mg/ml) followed by centrifugation at 10 000 g for 10 min. 100 and 200 µg of protein were submitted to 10% sodium dodecyl sulphate–polyacrylamide gel electrophoresis, then transferred electrophoretically by semi-dry blot onto PVDF membranes. All specimens (control and pre-eclamptic group) of placenta and placental bed, respectively, were run on one gel. The membranes were blocked with 5% fat-free milk powder in PBS for 2 h. Immunodetection was performed with either anti-COX-1 (1:1000) or anti-COX-2 (1:500) o/w to confirm at 4°C, followed by incubation with biotinylated anti-rabbit antibody. The bound antibodies were visualized by enhanced chemiluminescence according to the instruction of the manufacturer. Documentation was performed by exposure to X-ray film (Fuji, Japan) and the intensity of film staining was evaluated by computer densitometry (Computing Densitometer Model 300A, Molecular Dynamics, Kent, UK).

As negative control either irrelevant rabbit immunoglobulins were applied or the incubation step with the primary antibodies was omitted.

**Quantitative reverse transcription–polymerase chain reaction (RT–PCR)**

Cellular RNA was isolated by single-step acid guanidinium thiocyanate–phenol–chloroform extraction (Chomczynski and Sacchi, 1987).
Briefly, 500 mg frozen tissue was homogenized by Polytron homogenization in 5 ml homogenization buffer containing 4 M guanidinium thiocyanate, 25 mM sodium citrate (pH 7.0), 0.5% sarkosyl and 0.1 M 2-mercaptoethanol. The resulting solution was acidified with 2 M sodium acetate (pH 4.0) prior to phenol–chloroform extraction. The RNA was then precipitated with isopropanol. The desiccated RNA was redissolved in homogenization buffer and reprecipitated. After washing twice in 70% ethanol, the total RNA was resuspended in 200 µl TE buffer (10 mM Tris, pH 7.4 and 1 mM EDTA, pH 8.0). The concentration of RNA was measured photometrically at 260 nm.

RNA (1 µg) was used for reverse transcription with specific primers for the human sequences of COX-1 (Yokoyama and Tanabe, 1989) and COX-2 (Hla and Neilson, 1992) to be analysed. To ensure amplification of cDNA, the primers were designed to span one intron–exon boundary. As a control for equivalent RNA preparation, a fragment of β-actin was amplified. Further, different RNA concentrations were used for reverse transcription to ensure linearity. In order to obtain PCR products exhibiting ethidium bromide staining of comparable intensity with the COX PCR products, the placental mRNA was diluted 1:25 and the placental bed mRNA was diluted 1:100 before amplification with the β-actin specific primers. The primers (synthesized by MWG-Biotech, Germany) were chosen to have a GC content of 50–60% and to result in an amplification fragment of similar size. 0.5 µM of the following primers were used for target specific first strand synthesis: for COX-1 PCOX1HR1 (5′-AGACGCTCCTGGAGATGCT-3′), for COX-2 PCOX2HR1 (5′-ACAGTTCATGACCAGTC-3′) and for β-actin the primer BAHR1 (5′-CTAGAAAGCATTTGCCGTTGAC-3′). After cDNA synthesis, excess primers were removed by column extraction (Qiagen, Hilden, Germany) and PCR amplification was performed using the template and the following primer pairs: for COX-1 PCOX1HR2 (5′-CCCATATCTGACTGCGAT-3′) and PCOX1F1 (5′-CAGCCCTTCAATGAAAGTACC-3′), for COX-2 PCOX2HR2 (5′-TATCATCTAGTCCCGAGC-3′) and PCOX2F1 (5′-AATGTACCGTACGACCT-3′) and for β-actin BAHR1 and BAHF1 (5′-CATACACCATGAGACTGACG-3′). The reactions were cycled 30 times in a cycle profile of 30 s at 95°C, 30 s at 52°C and 30 s at 72°C after a 5 min denaturing step at 95°C. The amplification products were analysed by 2% agarose gel electrophoresis and ethidium bromide staining. The intensity of ethidium bromide staining was measured photometrically at 260 nm.

Results of densitometric evaluation are shown as individual results of seven (placenta) and six (placental bed) tissue specimens (Figure 5), further as median and range (Table II). Since the sample number was not high enough to use parametric tests efficiently, the Mann–Whitney U-test was applied for statistical analysis with a significance level of P < 0.05. To evaluate a correlation between the duration of pregnancy and the expression of COX in placenta and placental bed, a linear regression analysis was performed. A significant correlation with r = 0.05 was assumed for a correlation coefficient r > ±0.67 (n = 7) and for r > ±0.71 (n = 6).

**Table II. Results of densitometric evaluation of Western blot and reverse transcription–polymerase chain reaction for cyclooxygenase (COX)-1 and COX-2 in placenta and placental bed**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Control</th>
<th>PE</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Placenta</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>COX-1 Protein</td>
<td>263 (129–387)</td>
<td>246 (114–579)</td>
<td>1</td>
</tr>
<tr>
<td>mRNA</td>
<td>0.23 (0.12–0.45)</td>
<td>0.21 (0.16–0.49)</td>
<td>0.65</td>
</tr>
<tr>
<td>COX-2 Protein</td>
<td>50 (21–88)</td>
<td>43 (18–117)</td>
<td>1</td>
</tr>
<tr>
<td>mRNA</td>
<td>0.40 (0.21–0.55)</td>
<td>0.59 (0.22–1.01)</td>
<td>0.44</td>
</tr>
<tr>
<td>Placental bed</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>COX-1 Protein</td>
<td>82 (56–156)</td>
<td>175 (95–308)</td>
<td>0.04</td>
</tr>
<tr>
<td>mRNA</td>
<td>0.11 (0.04–0.17)</td>
<td>0.16 (0.14–0.19)</td>
<td>0.03</td>
</tr>
<tr>
<td>COX-2 Protein</td>
<td>53 (35–242)</td>
<td>85 (47–282)</td>
<td>0.30</td>
</tr>
<tr>
<td>mRNA</td>
<td>0.04 (0.02–0.10)</td>
<td>0.03 (0.01–0.07)</td>
<td>0.39</td>
</tr>
</tbody>
</table>

Results are expressed as median and range with n = 7 for placenta and n = 6 for placental bed biopsies. Protein: area in arbitrary units (100 µg protein used for Western blot; RNA: COXβ-actin ratio. The Mann-Whitney U-test was applied for statistical analysis.

**Results Immunohistochemistry**

COX-1-like immunoreactivity could be found in trophoblast, endothelial cells, macrophages, fibroblasts, smooth muscle cells and decidual stromal cells with the strongest staining in macrophages (Figure 1A and B). Only macrophages, endothelial cells, fibroblasts and vascular leiomycocytes stained positive for COX-2 (Figure 1C and D). The highest staining intensity for COX-2 was observed in macrophages and endothelial cells. Incubation with an irrelevant rabbit antibody resulted in the absence of staining (data not shown).

The identity of cells exhibiting COX-1- or COX-2-like immunoreactivity as macrophages in placenta and placental bed could be shown by double immunofluorescence staining with antibodies against COX-1 + CD68 or CD14 and COX-2 + CD68 or CD14, respectively (Figure 2A, B, D and E). Incubation with anti-ctyoketrerin showed extravillous trophoblast as dispersed cells and cells lining maternal blood vessels in the decidua and myometrium of placental bed sections. These cells stained positive for COX-1, but not COX-2 (Figure 2C and F). Incubation with both irrelevant rabbit and mouse immunoglobulins as first antibodies resulted in the absence of specific fluorescence staining (data not shown).

There was no obvious difference in staining intensity and the amount and distribution of positive cells between tissue sections from uncomplicated and pre-eclamptic pregnancies.

**Protein and mRNA expression**

Western blot analysis resulted in the expected immunoreactive band for COX-1 and COX-2 at 74 kDa indicating that the enzyme is in the glycosylated form (Figure 3). The results of densitometric evaluation are shown in detail in Table II. In placenta, the level of immunoreactive COX-1 and COX-2 protein was unchanged after pre-eclamptic pregnancies. Probing of placental bed homogenates with COX-1-pAb showed significantly increased COX-1 protein in this compartment.
after pre-eclampsia ($P < 0.05$), whereas the amount of immunoreactive COX-2 did not change.

The results of RT–PCR analysis are shown in Figure 4. Amplification with the specific primers resulted in a PCR product of ~400 bp. The identity of the specific PCR product was confirmed by sequencing. Densitometric evaluation of the agarose gels is shown in detail in Table II. Placenta was found to express approximately twice as much COX-1 mRNA and 10 times more COX-2 mRNA than placental bed biopsies. After pre-eclamptic pregnancies, COX-1 mRNA expression was unchanged in placenta, but significantly elevated in placental bed ($P < 0.05$). The amount of COX-2 mRNA in both tissues did not differ significantly between normal and pre-eclamptic pregnancies.

Further, the correlation between the week of pregnancy from which a tissue specimen derived and its COX expression was evaluated. There was one significant negative correlation between the duration of pregnancy and the COX-2 mRNA level in placental bed biopsies from the pre-eclamptic group ($r = -0.91$; Figure 5). All other correlation coefficients did not reach the significance level.

**Discussion**

The present study showed COX-1-like immunoreactivity in nearly all cell types in placenta and placental bed, which agrees with previous studies (Woodworth *et al.*, 1994; Zuo *et al.*, 1994; Divers *et al.*, 1995). These results underline the role of COX-1 as a constitutionally expressed enzyme which is important for cell homeostasis (Smith and DeWitt, 1995). In contrast, positive staining for COX-2 could only be observed in macrophages, endothelial cells and fibroblasts, cell types in which COX-2 expression has already been described in other tissues (Hla and Neilson, 1992; Smith and DeWitt, 1995). Previous studies on human placenta and myometrium using a specific antibody against COX-2 showed positive staining for the enzyme in the same types of cells. However, immunoreactive COX-2 was also observed in myometrial leiomyocytes (Zuo *et al.*, 1994), and trophoblast and decidua (Woodworth *et al.*, 1994). This discrepancy might be caused by probing with a different polyclonal COX-2 antibody, by using paraffin instead of frozen sections and by studying tissue specimens from women during labour (Woodworth *et al.*, 1994; Zuo *et al.*, 1994) where COX-2 gene expression might have been induced.
Figure 2. Double immunofluorescence staining for cyclooxygenase (COX)-1/CD68 in placenta (A), COX-1/CD14 and COX-1/cytokeratin in placental bed (B and C), COX-2/CD68 in placenta (D) and COX-2/CD14 and COX-2/cytokeratin in placental bed (E and F). The secondary antibody against the pAb anti-COX-1 and -COX-2 was labelled with tetramethylrhodamin isothiocyanate (red fluorescence) and against mAb anti-CD14, -CD68 and -cytokeratin with fluorescein isothiocyanate (green fluorescence). Original magnification \times 400.
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Figure 3. Western blot analysis of placenta and placental bed homogenates (100 µg protein per lane) for cyclooxygenase (COX)-1 and COX-2 after normal (C) and pre-eclamptic (PE) pregnancies shown as photograph from X-ray films after enhanced chemiluminescence reaction. MW = molecular weight; kDa = kiloDalton.

Figure 4. Reverse transcription–polymerase chain reaction analysis of cyclooxygenase (COX)-1 and COX-2 in placenta and placental bed after normal (C) and pre-eclamptic (PE) pregnancies, shown on an agarose gel with ethidium bromide staining. As a control for RNA preparation, a fragment of human β-actin was amplified (dilution of RNA: placenta 1:25, placental bed 1:100). To ensure linearity of reverse transcription, different RNA concentrations were used. PCR primers were chosen to result in an amplification fragment of similar size (~400 bp). A 100 bp ladder was run between the control and pre-eclamptic group on each gel.

PCS- and TXS-like immunoreactivity was observed most strongly in endothelial cells and macrophages, respectively (Wetzka et al., 1996), which also showed an intense positive staining for COX-1 and -2. These data may encourage further studies on the regulation of PGI₂ and TXA₂ production by isolated endothelial cells and macrophages. In a recent study on isolated Hofbauer cells, the macrophages of the human placenta, we observed a shift from PGE₂ towards TXA₂ production by cells cultured under hypoxic conditions (5% O₂) (Wetzka et al., 1997).

Immunoreactive COX-1 and -2 protein and COX-1 and -2 mRNA could be found in all tissue samples from the 29th to 40th week of pregnancy. A previous study investigating the expression of COX-1 and -2 in decidua from normal and pre-eclamptic pregnancies did not observe COX-2 mRNA (Shaw et al., 1994). However, COX-2 protein and mRNA expression was found in fetal membranes and placenta beginning from early in the second trimester with a marked increase after the 30th week of pregnancy (Slater and Bennett, 1996). We did not observe a significant positive correlation between the week of pregnancy and the COX expression in our placenta and placental bed biopsies, which could be partly due to the exclusion of tissue samples of women undergoing labour from the study. Preliminary experiments showed an increased COX-1 and -2 protein expression in tissue specimens obtained after the onset of labour which were then excluded, since all biopsies from pre-eclamptic pregnancies derived from Caesarean sections before the onset of labour.

Quantitative analysis of COX-1 and COX-2 mRNA and protein expression in placenta showed no change in pre-eclamptic pregnancies, which agrees with previous observations (Woodworth et al., 1994). Therefore, the prostaglandin biosynthesis in the placenta seems to be regulated at the level of COX enzyme activity. The activity of these peroxidases can be influenced by a different metabolical environment. In the serum of pre-eclamptic women, elevated peroxide levels were found (Hubel et al., 1989). In vitro, pre-eclamptic placentae produced more peroxides and showed a lower activity of glutathione synthase (Walsh and Wang, 1993). The consecutive increased level of lipid peroxidation stimulates COX and TXS enzyme activity, whereas PCS is inhibited (Marshall et al., 1987; Walsh, 1994).

The aetiology and pathogenesis of early PIH and pre-eclampsia is unknown, but a growing number of observations points to an involvement of immunological reactions (Redman, 1992). The hypothetical immune maladaptation to pregnancy probably leads to the disturbed endovascular trophoblast inva-
sion into the uterine spiral arteries which is characteristic for PIH and pre-eclampsia (Robertson et al., 1985). Further, the clinical symptoms of severe pre-eclampsia, such as increased vascular permeability and coagulopathy, resemble a septic state. This is supported by increased serum concentrations of inflammatory cytokines, such as TNFα and IL-6 which have been found in pre-eclampsia, pointing to the involvement of activated macrophages in the pathogenesis of this disorder (Schröcksnadel et al., 1992). Greer et al. (1989) observed elevated levels of neutrophil elastase in serum of pre-eclamptic women as a marker of neutrophil activation and increased numbers of elastase-positive neutrophils in pre-eclamptic decidua (Greer et al., 1989; Butterworth et al., 1991). In our study, COX-1 protein and mRNA concentrations in the placental bed were significantly elevated in pre-eclampsia, whereas COX-2 expression was unchanged. The increased COX-1 expression was not restricted to certain cell populations. Our results suggest that an increased synthesis of COX-1 in the placental bed followed by an increased prostaglandin production might play a role in the events mentioned above. Mice having a homozygous deletion of the COX-1 gene showed a decreased response to inflammatory stimuli (Langenbach et al., 1995) which was not observed in mice lacking the COX-2 gene (Morham et al., 1995). This suggests that COX-1 activity is primarily responsible for inflammatory responses. Additionally, it was observed in a trophoblast cell culture model that the culture of the cells on a fibrin matrix increased their COX expression (Johnson et al., 1995). In pre-eclampsia, there is increased deposition of fibrin around the blood vessels in the placental bed (Hustin et al., 1983) which might stimulate extravillous and endovascular trophoblast to synthesize more COX-1.

The protein and mRNA levels of COX-2 did not change significantly in both placenta and placental bed biopsies after pre-eclamptic pregnancies. In tissue culture models, COX-2 expression was increased by cytokines such as TNFα (Smith and DeWitt, 1995), but this does not seem to be important for the pathophysiology of PIH. In pregnancy, COX-2 possibly plays a more important role for cell differentiation and development, since in mice lacking the COX-2 gene an impaired renal development was observed leading to renal insufficiency and death (Morham et al., 1995).

In conclusion, a therapy with NSAID inhibiting specifically COX-1 might be beneficial in pre-eclampsia in order to decrease COX-1 activity in placental bed. Drugs which selectively inhibit COX-2 may not be useful as a treatment of the altered intrauterine prostaglandin synthesis and may even have a disturbing effect on fetal development.

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