Increased levels of metallothionein in placenta of smokers

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Abstract

Experiments were designed to evaluate and compare metallothionein (MT), zinc and cadmium levels in human placentas of smoking and non-smoking women. Smoking was assessed by self-reported cigarette consumption and urine cotinine levels before delivery. Smoking pregnant women with urine cotinine levels higher than 130 ng/ml were included in the smoking group. Determination of placental MT was performed by western blot analysis after tissue homogenization and saturation with cadmium chloride (1000 ppm). Metallothionein was analyzed with a monoclonal antibody raised against MT-1 and MT-2 and with a second anti mouse antibody conjugated to alkaline phosphatase. Zinc and cadmium were determined by neutron activation analysis and atomic absorption spectrometry respectively. Smokers showed higher placental MT and cadmium levels, together with decreased newborn birth weights, as compared to non-smokers. The semi-quantitative analysis of western blots by band densitometry indicated that darker bands corresponded to MT present in smokers' samples. This study confirms that cigarette smoking increases cadmium accumulation in placental tissue and suggests that this element has a stimulatory effect on placental MT production.

Keywords: Metallothionein; Placenta; Cadmium; Birth weight; Smoking

1. Introduction

Cigarette smoking is one source of human exposure to cadmium and causes increased levels of this toxic element in the body (Roquer et al., 1995). Smoking during pregnancy results in elevated cadmium concentrations in placental tissues (Piasek et al., 2001) and is known to cause a wide range of deleterious effects during fetal growth and development (Ferm, 1971) which leads to low birth weight (Abel, 1980). This latter effect has been subsequently related to the ratio of placental zinc to placental cadmium, (Kuhnert et al., 1987a), thereby linking both metals in the mechanisms involved in fetal growth. It has been suggested that the reduced birth weight found in neonates delivered by smokers may be related to a deficient trans-

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ference of zinc from the placenta to the fetus (Kuhnert et al., 1987b, 1988). Zinc is an essential micronutrient for fetal growth and for proper immune system function, stressing the importance of balanced zinc levels during pregnancy (Wellinghausen, 2001). When a mother has reduced zinc reserves, newborn zinc deficiency could occur during pregnancy and nursing due to reduced transference of this metal through the placenta and in breast-feeding respectively; therefore, improving maternal zinc nutrition is key for the infant's zinc nutritional status (Dorea, 2002). Zinc supplements during pregnancy do indeed reduce childhood morbidity in populations where zinc deficiency is common (Hamadani et al., 2002).

Different factors are known to influence the transference of zinc to the fetus including: gestational age, levels of maternal plasma zinc and zinc binding proteins in maternal and fetal circulation and in tissues (Simmer et al., 1985; Paterson et al., 1991). Interestingly, it has been reported that, in addition to having elevated placental cadmium concentrations, smokers also show increased placental zinc levels (Kuhnert et al., 1987a). Thus, the low birth weight of neonates born to smoking mothers could be due to placental zinc accumulation through a mechanism involving placental cadmium and metal binding proteins (Torreblanca et al., 1992).

At the cellular level, one of the most important bivalent metal (zinc, cadmium and copper) binding proteins is metallothionein (MT). This low molecular weight (6000–7000 Da), cysteine-rich protein may be crucial for the regulation of zinc homeostasis and metabolism (Richards, 1989; Kägi, 1991). It has been proposed that MT maintains zinc homeostasis by controlling cellular zinc uptake, distribution and excretion, and by acting as a short-term storage reservoir for this metal (Richards, 1989; Klaassen et al., 1999). Metallothionein synthesis may be induced by some of the metals to which it binds (Klaassen et al., 1999). Thus, cadmium and zinc, in addition of showing high affinities for MT, are potent inducers of MT synthesis (Harford and Sarkar, 1991). In this sense, high levels of cadmium in smokers' placentas and cadmium-zinc-induced MT synthesis in cultured human trophoblasts have been previously reported (Lehman and Poisner, 1984). The aim of this study was to evaluate and compare MT levels in placentas from smokers and non-smokers and correlate these results to placental zinc and cadmium concentrations. According to the present findings, a possible mechanism leading to low birth weight related to maternal smoking is suggested.

2. Materials and methods

2.1. Preparation of placentas

Placentas were obtained upon delivery in the maternity ward at Sótero del Río Hospital in southern Santiago. Inclusion criteria included healthy young parturients, with normal pregnancies and without history of alcohol or drugs. All mothers had normal nutritional status evaluated as previously described (Atalah et al., 1997). The ethical committee of our institution approved the research project and the questionnaire, which included medical and dietary history as well as data on occupational and possible environmental sources of metal exposure. The assessment of smoking was based on self-reported individual cigarette consumption and urine cotinine determination immediately before delivery. Parturients were divided into two groups: women who had never smoked (non-smokers) and women who smoked throughout the entire pregnancy (smokers).

Immediately after delivery, the entire placenta was weighted and placed in a plastic bag and frozen at −70 °C until laboratory transport. To determine trace elements, half of the partially thawed placenta was thoroughly washed and lyophilized using a programmed cycle of temperature and pressure: −25 °C; −20 °C (1.03 mbar); −10 °C; 20 °C (0.02 mbar) and 30 °C for 32 h (Christ Delta 1–20 KD). Finally, samples were ground and homogenized, constituting the stock placental material for metal element determinations.

2.2. Urine cotinine

A urine sample was taken just before delivery and maintained frozen until cotinine evaluation, which was carried out by radio-immunoanalysis (RIA; Diagnostic Product Company, Los Angeles, CA, USA).

2.3. Determination of placental levels of zinc and cadmium

Zinc was determined by instrumental neutron activation analysis (INAA) at the Neutron Activation Anal-

ysis Laboratory of the Chilean Commission for Nuclear Energy. The samples were encapsulated in quartz vials and heat-sealed for irradiation. The samples and the standards were irradiated for 24 h at thermal neutron flux of 1×10^{13} neutrons s^{-1} cm^{-2}. Twenty days after irradiation, the samples and the standards were measured with a high-resolution gamma ray spectrometer. The gamma ray spectra were later processed using a SAMPO computer program. The 1220 KeV line of ^{65}Zn (half life = 244 d) was used for zinc determination. The reference sample was pig kidney (trace elements) (BCR 186, Certified Reference Material, Sigma–Aldrich, MO, USA) with a zinc concentration of 128 μg/g. Results were expressed as μg/g of dry weight tissue.

Cadmium determinations were carried out by atomic absorption spectrometry (AAS 5 E-A) with a graphite furnace device for solid samples (SS-GFAAS, Carl Zeiss Technology) at the Chilean Commission for Nuclear Energy. Lyophilized and homogenized samples were analyzed without previous preparation. They were introduced directly on the platform of the graphite furnace. A deuterium pump was used to correct the background. Lectures were done at 228 nm with a lamp current of 5.0 mA. The reference sample was dorm-2 dogfish muscle (National Institute for Standards and Technology, MD, USA) with a cadmium concentration of 0.0443 ± 0.008 μg/g. Results were expressed as μg/g of dry weight tissue.

2.4. Determination of placental metallothionein by western blots (WB)

2.4.1. Preparation of placental tissue

Placentas (3 g) were weighted, homogenized in buffer Tris–HCl (10 mM, pH 7.4) and centrifuged at $10{,}000 \times g$ for 15 min at 4 °C. Supernatants were then ultracentrifuged at $100{,}000 \times g$ for 1 h, (Nostelbacher et al., 2000). After this procedure, 1 ml aliquots were saturated with 50 μl of $CdCl_2$ (1000 ppm) and heat-denatured for 1 min in boiling water. Then, samples were centrifuged at $10{,}000 \times g$ for 10 min and membrane-filtered. Clear supernatants from placentas of non-smokers and smokers were analyzed by WB as previously described (Yasunobu and Suzuki, 1991; Mizzen et al., 1996). Protein determination was accomplished by BioRad (Bio-Rad, Richmond, CA, USA).

2.4.2. Western blots (WB)

Samples were first carboxymethylated and reduced by treatment with 0.05 M dithiothreitol (DTT) and 1 M fresh iodoacetamide in ethanol during 1 h at room temperature. Then, samples were suspended in a solution containing 0.125 M Tris–HCl pH 6.8, 6% SDS, 20% glycerol, 10% β-mercaptoethanol and 0.07% bromophenol blue. Sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE, 12%) was performed according to the method described by Laemmli (1970). Twenty micrograms of protein were introduced in each lane of the gel and 75–95 V was then applied. After 2 h, gels were removed and incubated at room temperature for 20 min in methanol/CAPS buffer (15% methanol in 10 mM CAPS, pH 11) supplemented with $CaCl_2$ as already described, (Mizzen et al., 1996). Proteins were then transferred to polyvinylidene fluoride (PVDF) membranes. After visualizing quality of transference with red Ponceau, membranes were treated with 2.5% glutaraldehyde. Then, membranes were incubated overnight at 4 °C with a monoclonal anti MT antibody (anti MT-1 anti MT-2, Dako Corp USA) diluted 1:1000 in 1% BSA. These membranes were subsequently washed and incubated for 1 h at room temperature with an anti-mouse antibody conjugated to alkaline phosphatase (AP). Bands were visualized using NBT-BCIP (Calbiochem; 100–50 mg/ml), to detect the product of AP activity. Finally, band intensities were evaluated using a Kodak Digital Science 1D image system and analyzed by appropriate software.

2.5. Statistics

Data are expressed as mean ± S.D. Student's *t*-test was used for statistical analyses of mother and infant characteristics with a statistical software package (Statistica for Windows, Release 4.5, Statsoft, Inc., 1993). For statistical analyses of WB bands, the non-parametric Mann–Whitney U-test was applied. Significance was considered at $p < 0.05$.

3. Results

Maternal characteristics are described in Table 1. There were no differences in age, parity, height, body mass index, placental weight and nutritional status be-

Table 1
Maternal data

	Non-smokers ($N = 6$)	Smokers ($N = 6$)	p
Age (years)	30 ± 7	28 ± 10	0.83
$Weight_i$ (kg)	59 ± 8	58 ± 11	0.92
Height (cm)	157 ± 4	153 ± 5	0.19
BMI_i (kg/mt^2)	24 ± 3	27 ± 6	0.430
$Weight_e$ (kg)	72 ± 10	66 ± 9	0.41
BMI_e (kg/mt^2)	29 ± 4	29 ± 4	0.83
Parity	1.2 ± 1.6	1.8 ± 1.3	0.55
Placental weight (g)	517 ± 41	525 ± 35	0.81
Cotinine (ng/ml)	28 ± 15	1187 ± 829	0.008

BMI_i: body mass index at initiation of pregnancy. BMI_e: body mass index at end of pregnancy. Data are expressed as mean ± S.D. Student t- test and significance at $p < 0.05$.

tween both smokers and non-smokers. However, as expected, cotinine levels in urine were significantly higher in smokers than in non-smokers. Newborn characteristics are described in Table 2 where it can be observed that gestational ages were similar in both groups. Although infants from smokers show a high dispersion of birth weights, the only significantly different parameter between both groups was precisely the birth weight. Thus, birth weights of neonates born to smoking women were significantly lower than those exhibited by newborns from the non-smoker group. Placental zinc and cadmium contents are shown in Table 3. As expected, it can be observed that, placentas from smokers have significantly higher cadmium contents. Zinc concentrations were also higher in placentas from smokers, although results were not significant. Western blot analysis to detect MT in placental samples reveals a band corresponding to this protein (Fig. 1A, arrow). Six placental samples from each, the non-smoking (Fig. 1A, NS1 to NS6) and the smoking (Fig. 1A, S1 to S6) groups were blotted. Results show fair and darker bands corresponding to samples from non-smokers and smokers respectively. This is a representative WB, since additional samples were blotted with similar results. Quantification of MT band intensities as arbitrary units corresponding to both smokers and non-smokers is shown in Fig. 1B, where the statistical analysis indicates significantly higher MT levels in placentas from smoking mothers.

Table 2
Newborn data

	Non-smokers ($N = 6$)	Smokers ($N = 6$)	p
Birth weight (g)	3488 ± 167	2792 ± 706	0.04
Height (cm)	49.3 ± 1.6	47.2 ± 3.7	0.23
Ponderal index (g/cm^3)	29 ± 3	27 ± 2	0.07
Cephalic perimeter (cm)	3.7 ± 1	33.5 ± 2	0.87
Gestational age (weeks)	39.3 ± 0.8	37.8 ± 3.7	0.34

Data are expressed as mean ± S.D. Student's t- test and significance at $p < 0.05$.

Table 3
Zinc and cadmium levels in placentas of non-smokers and smokers

Condition	Zn (μg/g dry weight)	Cd (μg/g dry weight)
Non-smokers ($N = 6$)	51 ± 14	0.024 ± 0.06
Smokers ($N = 6$)	58 ± 16	0.075 ± 0.024
p	0.49	0.001

Data are expressed as mean ± S.D. ($N = 6$). Student's t-test and significance at $p < 0.05$.

4. Discussion

Although it has been previously suggested that metallothionein (MT) may be increased in smokers' placentas due to the presence of elevated levels of cadmium (Kuhnert et al., 1987a), this is the first direct evidence of specific elevations of MT levels in placentas from smokers. This study, which corroborates the previous hypothesis, demonstrates a clear correlation between higher levels of placental cadmium and increased levels of MT in smokers. Since in vitro studies have previously demonstrated that MT is inducible by cadmium and zinc, and smokers have increased levels of placental cadmium, higher MT levels in smokers' placentas should be expected. However, clear evidence for this hypothesis had not been reported. Previous studies with isolated trophoblast cells from human placenta at term demonstrated that incubation of these cells with different cadmium acetate concentrations resulted in a dose-dependent MT accumulation in the incubation medium (Lehman and Poisner, 1984; Torreblanca et al., 1992). Metallothionein was also induced by zinc, although concentrations higher than those for cadmium were necessary to produce similar effects (Lehman and Poisner, 1984).

The presence and localization of MT in maternal and fetal cells from human placentas has been previously

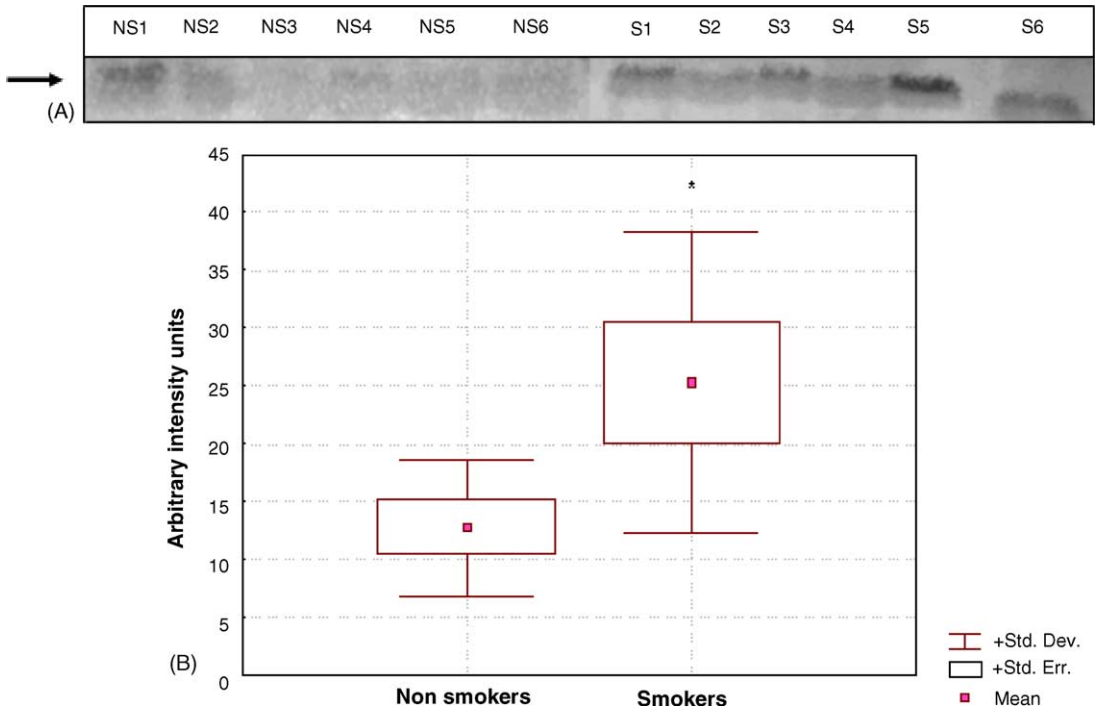

Fig. 1. Western blots of MT in placentas of non-smokers and smokers.Western blots of placental samples were performed as previously described in Methods. (A) Six samples from non-smokers (lanes NS1 to NS6) and six samples from smokers (lanes S1 to S6) were blotted. The arrow indicates bands corresponding to MT recognized with a monoclonal antibody raised against MT-1 and MT-2. (B) Densitometry was used to determine relative levels of MT from both groups of samples blotted in A. Results were expressed as mean ± S.E.M. and S.D. of arbitrary intensity units. $^{*}p < 0.05$.

reported (Wier and Miller, 1987; Goyer et al., 1992). MT synthesis in trophoblasts and decidual cells isolated from human placenta has already been demonstrated (Goyer et al., 1992). Nevertheless, in human tissues, main levels of MT have been found in epithelial, fetal liver and neoplasic cells (Nartey et al., 1987a,b). Proposed functions for MT include: regulation of essential metal homeostasis (Brady et al., 1982; Davis et al., 1998; Palmiter, 1998), protection against metal toxicity (Liu and Klaassen, 1996; Liu et al., 1996) and antioxidant activity by scavenging reactive oxygen species (Klaassen and Cagen, 1981; Sato and Bremner, 1993). All of these functions, when appropriately exerted by placental cells, may be essential to support adequate development of the embryo-fetal unit. Zinc is an essential metal for fetal growth and development and, high concentrations of this element bound to MT has been found in human fetal liver in some stages during prenatal development (Wong and Klaasen, 1979; Gallant and Cherian, 1987). On the contrary, since toxic cadmium should not be transported to the fetus, it is quite possible that the placenta accumulates cadmium by its strong binding to MT (Chung et al., 1986). Although specific placental sequestration of cadmium by MT has been reported, recent findings obtained in MT-1/MT-2 knockout mice indicate that MT does not play a major role in restricting transfer of cadmium from dam to fetus via placenta (Lucis et al., 1972; Brako et al., 2003). Further studies are required to establish whether this is also true for humans.

Increased placental zinc levels in smokers have been previously reported (Kuhnert et al., 1987b), although more recently, unaltered placental zinc concentrations in smokers were also reported (Piasek et al., 2001). In the present study, higher placental zinc concentrations were obtained in smokers than in non-smokers, however, differences between both groups were not significant.

Although the number of non-smokers and smokers included in this study was low (six pregnant mothers in each group), we found significant differences in birth weights. Thus, smoking mothers had newborns with lower birth weights than non-smokers, a finding that has been previously reported (Kuhnert et al., 1987a; Roquer et al., 1995). Although specific mechanisms for this effect have not yet been completely understood, relationships and interactions involving zinc, cadmium and MT in placental tissues could be operating.

In this study, we confirm that smokers have lower birth weight neonates and that their placentas have increased levels of cadmium and MT. Probably, these additional MT levels are required to retain cadmium in the placenta, thus contributing to protect the fetus from this toxic element. Nevertheless, this high MT level may be also responsible for binding more zinc, thereby keeping this element inside the placental cells. Under these circumstances, an inadequate amount of zinc might be transferred to the fetus. If this condition becomes chronic, it may in turn contribute to reduce birth weight of infants delivered by mothers who smoke during pregnancy.

In summary, in the present study it has been shown that placental MT levels are higher in smokers than in non-smokers. This condition is likely due to the higher levels of cadmium found in the group of smokers. Thus, abnormally elevated cadmium-induced MT levels in smokers may accomplish dual opposite functions in placental tissues: (a) a protective function, avoiding transference of cadmium from the placenta to the fetus, and (b) a negative function causing a disruptive effect on normal placental zinc homeostasis that may lead to an abnormal transference of this essential element to the fetus, a condition possibly involved in fetal growth restriction.

Additional studies are required to clearly evaluate the properties of cadmium-induced MT in relation to its zinc binding affinity and its suggested function in placental zinc availability for transference to the fetus.

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