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Short Communication

Parenchymal Calcifications in Third Trimester Placentas Contain Little or No Detectable Lead As Determined by Atomic Absorption Spectrometry (AAS) and X-ray Spectrophotometry (XRS)

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Abstract

Background: Placental calcifications are often identified in third trimester placentas. It is hypothesized that placental calcifications may sequester lead in the third trimester when there is the greatest risk for resorption from maternal bone.

Design: Five 3rd trimester placentas with large numbers of parenchymal calcifications and five 3rd trimester placenta controls were chosen to undergo analysis of lead content by both AAS and XRS. For both assays, multiple cores were extracted from formalin-fixed paraffin-embedded tissue blocks. In test cases, areas with the most calcifications were sampled and for controls, random cores were taken. For AAS, nitric acid digests were prepared from the tissue cores and analyzed for lead with a Model AA600 Perkins-Elmer graphite furnace atomic absorption spectrophotometer. Lead levels in µg/g dry weight were calculated for test cases and controls then compared using an unpaired *t* test. For Electron Dispersive X-ray Spectrometry (EDS, EDAX system), 2 cases were analyzed and underwent deparaffinization, rehydration, fixation in glutaraldehyde, post-fixation with osmium tetroxide, dehydration and embedment in epoxy resin prior to being analyzed using Transmission Electron Microscopy (TEM).

Results: By AAS there was no significant difference in lead levels between cases (range 0.30-0.73 µg/g) and controls (range 0.32-1.21 µg/g) ($p=0.46$). No lead was detected in 2 cases using the XRS method, although calcium was detected.

Conclusion: Minimal to no lead was detectable in third trimester placental calcifications using 2 different methods. The presence of calcifications, therefore, does not appear to be a marker for sequestration of lead from bone during the 3rd trimester of pregnancy.

INTRODUCTION

Several studies have shown that lead can concentrate in the placenta [1,2]. The mechanism may involve trophoblast transport or, more likely, precipitation with calcium especially in aging term placentas [1]. Some studies have used maternal and cord blood, bone, and meconium lead measurements to show that prenatal exposure to lead can increase risk of preterm delivery, intrauterine growth restriction (IUGR), impaired neurologic development, and altered postnatal growth parameters [3-6]. Other investigators have studied whether elevated placental lead levels are also related to these outcome measures. The literature contains mixed results: one group found that elevated placental lead levels are associated with both stillbirth and neonatal death [7] while another group found higher placental lead levels in cases with premature rupture of membranes and preterm labor but no association with IUGR [8]. The distribution of placental calcifications has also been studied [9] and their association with increasing gestational age [10], maternal smoking [11,12], pre-eclampsia, hypertension [13], and IUGR [14] have been identified. A recent meta-analysis of studies that quantitated total lead and other heavy metals in placentas using a systematic search in accordance with the PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) statement criteria found that the placenta was not well developed to be a biomarker for heavy metal exposure because of heterogeneity among studies [15]. With the significant overlap of associations between the prenatal risk of exposure to lead and the presence of placental calcifications, this study hypothesizes that placental calcifications may sequester lead in the third trimester when the

majority of calcifications form and when, next to lactation, there is the greatest risk for resorption of lead from maternal bone [16]. A focus on calcifications could also possibly lead to identifying a better biomarker within placentas as a surrogate for exposure to lead in pregnancy.

MATERIALS AND METHODS

Five 3rd trimester placentas from delivered women with large numbers of parenchymal calcifications and five 3rd trimester placenta controls were chosen to undergo analysis of lead content by both atomic absorption spectrophotometry (AAS) and x-ray spectrometry (XRS). For both assays, multiple cores were extracted from formalin-fixed paraffin-embedded tissue blocks (Figure 1). Calcifications are generally not quantitated in the placenta because their extent can be difficult to assess. Sectioning and staining paraffin blocks with areas of calcifications can cause the center of the calcification to "pop out", leaving only a rim of calcification. Thus blocks were chosen for analysis based on visual inspection of these rims of calcification. In cases with calcifications, areas with the most calcifications were sampled and in controls, random cores were taken. The amount of calcifications taken for the analysis was maximized by taking tissue cores instead of tissue sections. For AAS, nitric acid digests were prepared in metal-free containers from the tissue cores and analyzed for lead with a Model AA600 Perkins-Elmer graphite furnace atomic absorption spectrophotometer. Lead levels in µg/g dry weight were calculated: Assay concentration (ng lead/mL) x Total volume (mL) x 1µg/1000 ng = µg lead, then µg lead x 1/Dry weight (mg) x 1000 mg/g = µg lead/g (limit of detection for lead 0.05 µg/L = 0.00005 µg/g) [17]. Results were compared using an unpaired *t* test. For EDS (energy dispersive x-ray analysis, EDAX system), the 2 cases with the highest AAS assay concentration in ng/mL (cases 1 and 3) were analyzed by deparaffinization, rehydration, fixation in 2.5% glutaraldehyde, post-fixation in 1% osmium tetroxide, dehydration to 100% ethanol, infiltration into Spurr epoxy resin and polymerization overnight at 70°C. Blocks were sectioned onto grids without heavy metal staining prior to analysis with a Hitachi 7650 Analytical TEM.

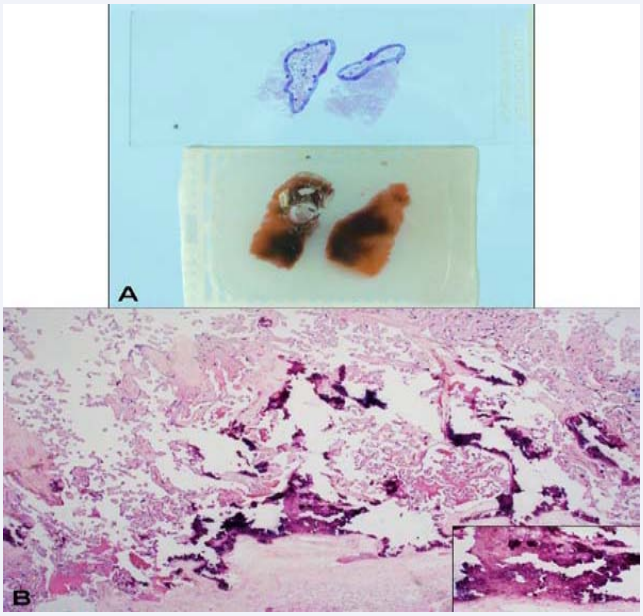

Figure 1 (A) Calcifications seen on microscopic slide (above) of Case 4 are outlined and cores are removed from corresponding region in tissue block (below).
(B) Microscopically there are abundant coarse calcifications along the maternal floor that fragmented due to sectioning artifact. Inset shows the largest and most intact focus of calcification. [original magnification: 12.5x and 100x (inset)]

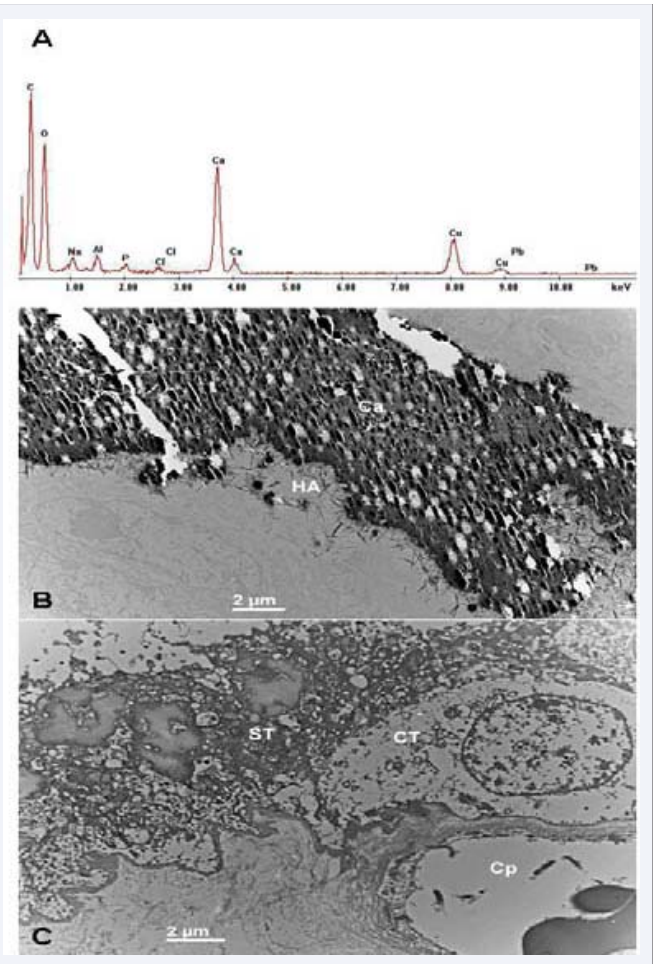

Figure 2 (A) Representative electron X-ray spectroscopy plot of tissue cores from a placenta with calcification identifies calcium (Ca) but no lead peak is present where it should be seen (Pb). Copper (Cu) and aluminum (Al) detection are from the copper grid and aluminum cap, respectively, of the grid exchange holder used for transmission electron microscopy (TEM). (B) Representative electron photomicrograph of parenchymal calcifications (Ca) with hydroxyapatite crystals (HA) at edge. (C) Representative electron photomicrograph of area of non-calcified placental tissue demonstrates the surface of a villus with syncytiotrophoblasts (ST), cytotrophoblast (CT), and subtrophoblastic fetal capillary (Cp). (original magnifications of both B and C: 7000x).

RESULTS

By AAS there was no significant difference in lead levels between cases (range 0.30-0.75 µg/g) and controls (range 0.32-1.24 µg/g) (p=0.46) (Table). No lead was detected in 2 cases using the XRS method, although calcium was detected (detection limits: lead 300-800ppm; calcium 50-1000ppm) (Figure 2). There was no significant difference in mean age between the two

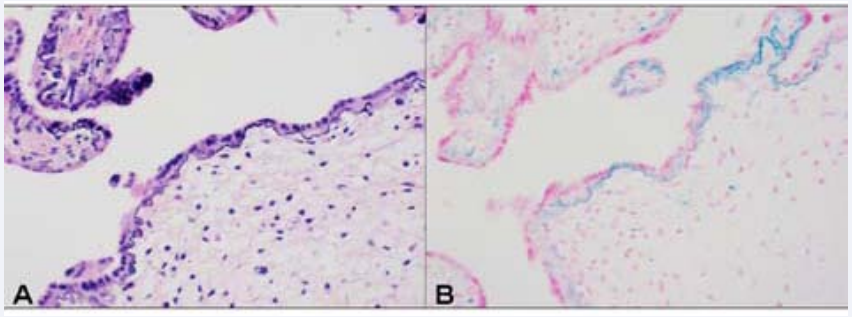

Figure 3 (A) A section from the placental tissue of a 13-week gestational age case of fetal demise in utero (not included in the present study) demonstrates the linear subtrophoblastic ribbon in a degenerating chorionic villus. (B) A Perl's Prussian blue iron stain highlights the abundant iron within this form of intravillous mineralization. (original magnifications of both A and B: 200x).

Table 1: Placenta Lead Detection by Atomic Absorption Spectrophotometry.

Sample	Age	GA	Dry Weight (mg)	Total Volume Acid + Deionized Water (mL)	Assay Conc. (ng/mL)	µg Pb/g
Case 1	27	39 2/7	7.0	2.082	2.510	0.746
Case 2	33	38 1/7	4.8	2.140	0.991	0.442
Case 3	32	37 6/7	9.2	2.084	1.335	0.302
Case 4	21	41 4/7	4.6	2.129	0.990	0.458
Case 5	20	39 2/7	6.3	2.096	0.927	0.308
Control 1	39	36 5/7	3.2	1.997	0.832	0.519
Control 2	19	33 4/7	1.8	2.084	0.421	0.487
Control 3	33	36 3/7	2.3	2.074	0.445	0.401
Control 4	27	39 6/7	5.4	2.113	0.830	0.325
Control 5	34	36 6/7	2.1	2.182	1.194	1.241
GA = gestational age; Pb = lead						

groups (26.6 vs. 30.4 years) (p=0.41) but the mean gestational age between the two groups approached a significant difference (39.2 vs. 36.6 weeks) (p=0.06).

DISCUSSION

Placental calcifications were first shown in the mouse placenta to be composed of calcium hydroxyapatite crystals that contain calcium and phosphate [18]. One mechanism described for the formation of calcifications is that apoptosis of aging trophoblasts and villous stromal cells act to concentrate calcium and permit nucleation for crystal formation. Placental calcifications can be seen in otherwise normal parenchyma in third trimester placentas but they can also be seen in infarctions or within areas of perivillous fibrinoid deposition, where villi are degenerating [19]. The yolk sac, if identified grossly in the third trimester, is often calcified microscopically. In viable cells, calcium is actively transported across trophoblasts using calcium binding proteins (CBP) to allow for fetal calcium absorption [1,2]. Lead can be transported by these CBP and there is an inverse relation between amount of lead absorbed and serum calcium levels [20]. Low diet calcium may also increase serum lead levels during pregnancy [13,16].

Using 2 sensitive assays in this study, minimal lead was detected in placental calcifications in a small sample of 5 cases and the minimal lead content was not significantly more than was seen in 5 third trimester placentas without calcifications. Calcium was readily detectable but lead content was below the level of detection, below the level of quantitation, or present at a very low level. This finding is in agreement with the few prior studies of heavy metal content in calcifications in both mouse and human placentas [10,18]. The finding that the control group had an earlier mean gestational age (slightly preterm) that approached a significant difference when compared to the group with placental calcifications could be a bias in that calcifications may have formed during the last 4 weeks of pregnancy had the 4 preterm pregnancies in this control group gone to term [21]. The small number of placentas studied is a weakness of this study but the technical difficulty, time, and cost of performing AAS and EDS analyses required that the analysis was limited to a small number of cases.

The calcifications seen in third trimester placentas usually appear as irregular structures within villi of the basal plate and in chorionic septa [19]. Another form of mineralization seen in placental specimens includes the linear basement membrane mineralization [22] of villous ferrugination, which often appears to ring the stromal side of the villous trophoblast lining and are typically positive for iron deposits using a Perl's Prussian blue iron stain (Figure 3). Electron microscopy of these deposits identifies

electron dense bodies that, by X-ray spectrometry, contain iron [23]. Villous ferrugination is related to either cessation of blood flow in the fetus, as in cases of fetal demise in utero, or fetal artery thrombosis [23]. Placentas with this type of mineralization were not chosen for this study for several reasons. First, their quantity in most affected cases is small in relation to total villous area. A case report of Bartter syndrome, however, identified an unusually high density of subtrophoblastic mineralizations containing both iron and calcium using Prussian blue, Von Kossa, and Alizarin Red stains [24]. Second, incidence has been found to be higher in aneuploid stillbirth gestations than in euploid stillbirth gestations [25], suggesting a possible genetic etiology for the increased mineralizations in the villi in some cases. Of course, the presence of iron deposition does not preclude the co-deposition of other heavy metals such as lead.

In summary, minimal to no lead was detectable in third trimester placental calcifications using 2 different sensitive assay methods in a small sample of cases. The presence of calcifications, therefore, does not appear to be a marker for sequestration of lead from bone during the 3rd trimester of pregnancy.

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