

Supplemental Material

Arsenic-Associated Oxidative stress, Inflammation, and Immune Disruption in Human Placenta and Cord Blood

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Supplemental Material

Computerized Image Analysis for Detection of Immunostaining.

Acquired computerized image analysis was used to quantify immunohistochemical staining *in situ* by transferring digital images of the stained tissue samples from a microscope to a computerized image analysis system Leica Qwin Runner (version 3) through a three-chip charged-coupled device (CCD) color camera DCF295 (Leica Microsystems CMS GmbH, Wetzlar). Special software (tissue-includer) was written in the high level language, QUIPS (Bjork 1996) which is capable of distinguishing positive and negative cells and measuring the cell size (μm^2) and intensity (256 grey scale) of each cell in the analysis.

The acquired image was divided into 512 by 512 pixels and each pixel was expressed in square micrometers (area) after calibration with the magnification being used. The threshold for red, green, and blue (RGB) values were initially defined, each at 1–256 levels, allowing separation of 16.7×10^6 color combinations. The color and morphology of the hematoxyline counterstained cells were set as a standard (Bjork 1996).

To measure cytokine or cell phenotype immunoreactivity at the single cell level out of total cell population, two special binary planes were applied. Binary plane 1 and 2 were detected on the processed image which gave the threshold values of the positive and total cell population respectively. The two binary planes were displayed by color coded contour lines; positive cells were displayed with green lines and total cells with red (Supplemental Material, Figure 1).

The average size of each placental tissue section was $5.25 \times 10^6 \pm 3.0 \times 10^6 \mu\text{m}^2$ (mean \pm SD). For each section, at 400X magnification about 400 ± 25 fields were read that covered the total tissue area and the average of all fields was used for quantification of immunostaining in

each tissue section. Standard positive and negative cells were set by the Leica software (Leica QWin) and the positive staining of markers in placental tissue sections was defined. The results were expressed as the total positively stained area measured x total mean intensity (1-256 levels/per pixel) of the positive area divided by total cell area measured and the unit was termed as ACIA (acquired computerized image analysis) scores.

Localization of immune cells and inflammatory markers in the placenta.

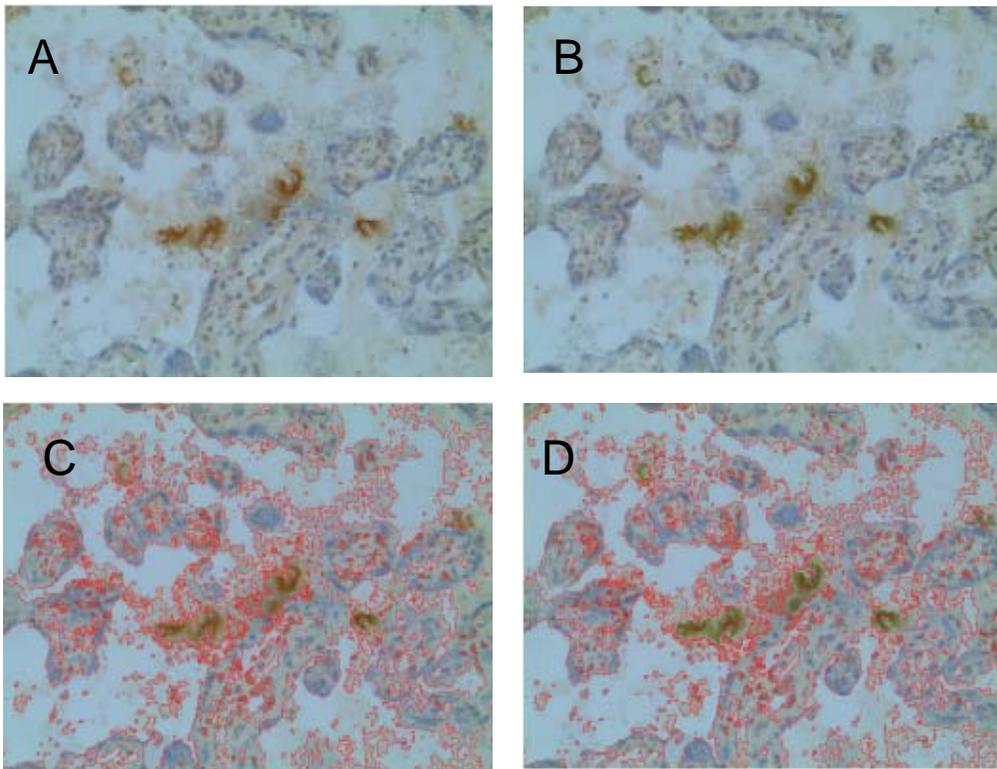
Clusters of CD3 (Supplemental Material, Figure 2, A & B) and CD8 cells (Supplemental Material, Figure 2, C & D) were noted in the chorionic villi only. Macrophages and granulocytes expressing CD64 and CD68 phenotypes, along with MPO expressing neutrophils were distributed randomly within the chorionic villi, the intervillous space, and the vascular beds (not shown). Localization of 8-oxoG was primarily in the cytotrophoblast lining in the chorionic villi and to some extent in syncytiotrophoblast cells and capillary endothelium (Supplemental Material, Figure 2, E & F). Leptin expression was present in almost all areas of the placental tissue (Supplemental Material, Figure 2, G & H). Expression of pro-inflammatory cytokines IL-1 β (not shown), TNF- α (Supplemental Material, Figure 2, I & J) and IL-6 (not shown) was mostly located in the capillary endothelium, within the chorionic villi, and in neutrophils. Expression of IFN- γ (Supplemental Material, Figure 2, K & L) and IL-10 (not shown) was mostly localized to syncytiotrophoblast cells. IFN- γ expression was also noted in the endothelial lining.

Supplemental Material, Table 1. Multivariable-adjusted analysis of associations of maternal urinary arsenic concentrations (U-As, $\mu\text{g/L}$) in early and late pregnancy with placental effect markers, for CD3 and CD8 cells (cells/100 μm^2 field area) and for other cells and cytokines (ACIA score).

Dependent variables	Independent variables		Adjusted*	
	Unadjusted			
	U-As at GW8	U-As at GW30	U-As at GW8	U-As at GW30
CD68				
Beta	0.06	0.08	0.07	0.09
95% CI	(-0.18, 0.31)	(-0.18, 0.34)	(-0.25, -0.40)	(-0.25, 0.44)
<i>p</i> -value	0.62	0.54	0.65	0.58
MPO				
Beta	-0.05	0.03	-0.06	0.02
95% CI	(-0.19, 0.09)	(-0.1, 0.18)	(-0.22, 0.1)	(-0.15, 0.19)
<i>p</i> -value	0.44	0.67	0.48	0.85
IL-6				
Beta	0.43	0.30	0.43	0.30
95% CI	(-0.11, 0.98)	(-0.27, 0.88)	(-0.37, 1.23)	(-0.55, 1.17)
<i>p</i> -value	0.11	0.29	0.27	0.45
IL-10				
Beta	0.70	0.86	0.72	0.88
95% CI	(-0.17, 1.59)	(-0.06, 1.79)	(-0.68, 2.14)	(-0.56, 2.33)
<i>p</i> -value	0.11	0.06	0.27	0.20

Abbreviations: U-As, maternal urinary arsenic; GW, gestational week; IL-6, interleukin-6; IL-10, interleukin-10; Beta, standardized regression coefficient; CI, Confidence interval, Outcomes and exposure variables were ln transformed. * denotes adjusted for mother age, socioeconomic status and tobacco chewing.

Supplemental Material, Figure 1



E

Cell area measured (um ²)	93664.15
Stained area measured (um ²)	2197.12
% stained area in the cell area	2.35
Mean intensity of the positive area	129.55
ACIA Score	3.04
Field number	1

Figure 1. Computerized Image Analysis for Detection of Immunostaining. (A). IL-1beta immunoreactive placental tissue. (B) Selection of positive area, i.e. IL-1 beta immunoreactivity. (C) Selection of total cell area. (D) Positive immunoreactivity relative to total cell area. (E) Automated image analysis for the quantification of the intensity of positive immunoreactivity relative to the total cell area in one field of the tissue section.

Supplemental Material, Figure 2.

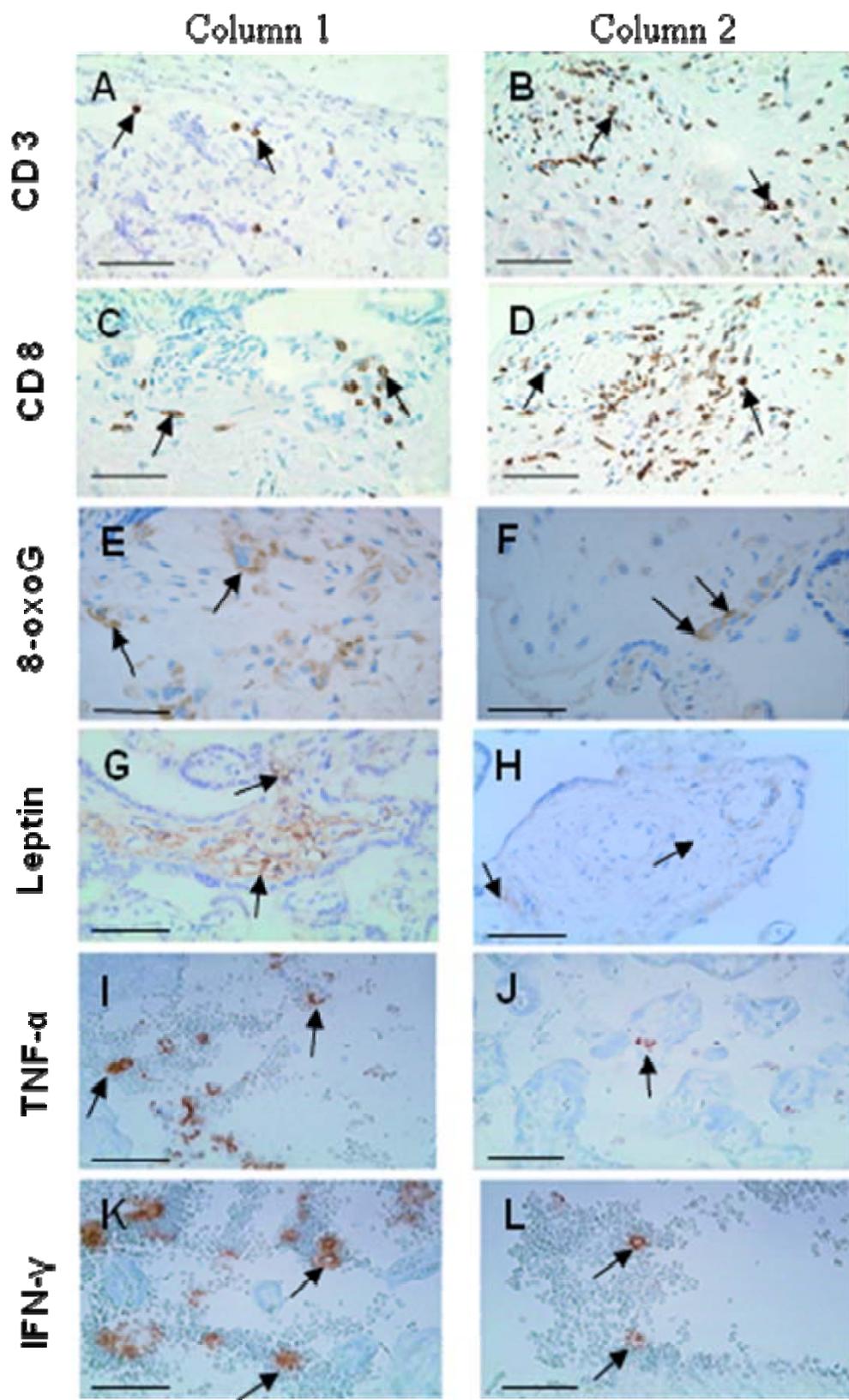


Figure 2. Immunostaining of immune cells, marker of oxidative stress, and pro-inflammatory cytokines in placental tissue. These are general expression pattern, column 1 is for high exposure (>60 µg/L) level, and column 2 is for low exposure level (<60 µg/L) of U-As at GW30. Clusters of CD3 (, A & B, arrows) and CD8 cells (, C & D, arrows) were noted in the chorionic villi. Immunostaining of 8-oxoguanine was localized in the lining of cytotrophoblasts and syncytiotrophoblasts in the chorionic villi and capillary endothelium (E and F, arrows). Leptin expression was present in almost all areas of the placental tissue (G and H, arrows). Expressions of TNF- α were typically located in capillary endothelium inside the chorionic villi (I and J, arrows). IFN- γ immunoreactivity was localized to syncytiotrophoblasts and in endothelial lining (K and L, arrows). In all figures, Bars equal to 50µm.

REFERENCES

Bjork L. 1996. Development of an Image Analysis System for the Evaluation of Cytokine Production. Stockholm: Department of Immunology, Stockholm University.