CHAPTER III

RESERCH METHODOLOGY

Study design

This study investigated the localization of DMT-1 in term human placenta and the differential of DMT-1 mRNA and protein expressions in the term placentas of women who live in non-Cd and Cd contaminated areas.

Subjects

All healthy pregnant women from both groups had to live in such selected areas for longer than 5 years and giving a normal birth at Mae Sot hospital, Mae sot, Tak. The experimental group was 23 pregnant women living in Cd contaminated area (Mea Tao, Mea Ku and Pratat Pha Daeng subdistricts), Mae Sot, Tak, Thailand. The control group was 28 pregnant women living in non-Cd contaminated area, other sub district, Mea Sot, Tak Thailand. All subjects were informed consent for participate in this study.

Cadmium elements determination

The maternal blood and urine samples were collected at 4-8 week (32-38 weeks of gestational ages) prior to delivery whereas the placental tissue was collected immediately after delivery at Mae-Sot hospital. The serum ferritin was also determined by Inductively Couple Plasma-Optical Emission Spectrometer (ICP – OES) using serum ferritin test kit for maternal anemia condition analysis. The result of serum ferritin was obtained from Mae-Sot hospital. Cd levels in maternal blood, urine were analyzed by Graphite Furnace Atomic Absorption Spectroscopy (GFAAS). Moreover Cd levels in placental and umbilical cord blood tissue were analyzed by Inductively coupled plasma mass spectrometry (ICP-MS). These results were obtained from co-worker in Toxic Metal Research Group (TMRG). The maternal blood and urine samples were collected at 4-8 week (32-38 weeks of gestational ages) prior to delivery whereas the placental tissue and cord blood was collected immediately after delivered at Mae-Sot hospital.

DMT-1 expression determination

Among of them, we selected 6 of placenta from each group by using Cd in maternal blood in criteria. A low-Cd group had maternal blood Cd $\leq$ 0.6 (0.2-0.6 μg/l). A high-Cd group had maternal blood Cd > 0.6 μg/l (0.8-3.7 μg/l).

Materials and instruments

1. Instruments

1.1 Autoclave (Hl-325, Gemmy industial corporation)

1.2 Beaker

1.3 Centrifugation machine (Z160M, Hermle)

1.4 Cold plate (kunz instruments CP-4, Shandom company)

1.5 Cylinder

1.6 Digital camera (DXM 1200c, Nikon)

1.7 Flask

1.8 Heat block (HB-1, Weal tec corporation)

1.9 High voltage power supply (Elite 200, Weal tec corporation)

1.10 Homogenizer (fastprep FP120, Bio-active co.,Ltd.)

1.11 Hood (Easy lab)

1.12 Hot air oven (D 06062 medell 600, Memmert GH+CO.KG)

1.13 Hot plate & stirrer (CB162, Stuart)

1.14 Hot plate (HP-3, Shandom company)

1.15 Kodak medical X-ray cassette (Eastman Kodak company)

1.16 Light microscope (Eclipse 80i, Nikon)

1.17 Microtome (As 325 reaction, Shandom company)

1.18 Operating set

1.19 pH meter (WPA CD-500, WPA company)

1.20 Shaker (Compact rocker CR300, FinePCR)

1.21 Spectrophotometer (UV-1650 PC)

1.22 Timer (Canon)

1.23 Tissue embedding machine (kunz instruments WD-4, Shandom company)

1.24 Tissue processing machine (Citadel 1000, Shandom company)

1.25 Vertex (Vertex-2 genie, Scientific industries)

1.26 Water bath (Histo-float, Shandom company)

1.27 Weighing machine (AND HP-200, A&P company limited)

1.28 Western blotting electrophoresis set (miniVE vertical electrophoresis system, Amersham bioscience)

2. Materials

2.1 2-mercaptoethanol (Sigma)

2.2 2-proponol (Sigma)

2.3 37% paraformaldehyde (Scharlau)

2.4 3-aminopropyl triethoxysilane (Sigma)

2.5 40% acrylamide gel (AMRESCO)

2.6 Acetone (Fisher Scientific)

2.7 Ammonium persulfate (Applichem)

2.8 Bicin choninic acid (Pierce)

2.9 Biotinylated anti-rabbit IgG (Vector Laboratories)

2.10 Blue loading dye (Promega)

2.11 Bovine serum albumin (Gibco)

2.12 Centrifuge tube

2.13 Chemiluminescent substrate (Pierce)

2.14 X-ray Imaging Film (Kodak)

2.15 Coverslip (Menzel-glaser)

2.16 Di-Aminobenzidine tetrahydrochloride (Santa Cruz biotechnology)

2.17 Di-Sodium hydrogen phosphate dihydrate (MERCK)

2.18 Deoxycholic acid sodium salt(Fluka)

2.19 Ethanal (MERCK)

2.20 Fetal bovine serum (Pierce)

2.21 Filter paper (3M)

2.22 GBX developer and relplenisher (Eastman Kodak company)

2.23 GBX fixer and relplenisher (Eastman Kodak company)

2.24 Gloves (Supermed)

2.25 Gloves (powder free)(MICROFLEX)

2.26 Glycine (Promega)

2.27 Glycine (Fisher Chemical)

2.28 HRP conjugated streptavidine (Pierce)

2.29 Hydrochloric acid (Fisher Scientific)

2.30 Hydrogen peroxide 30% (MERCK)

2.31 Lysis buffer kit (Pierce)

2.32 Methanol (MERCK)

2.33 Micropipette (pipetman, Gilson)

2.34 Microscopic slides (Sail brand)

2.35 Microtips: 10, 100 and 1000ul (Treff Lab, Treff AG)

2.36 Microtube: 0.6 and 1.5 ml (Soreson)

2.37 Modified haematoxylin solution (C.V. Laboratories Co.,Ltd.)

2.38 Mouse anti DMT-1 antibody

2.39 Mouse anti Actin antibody

2.40 NP-40 (Fluka)

2.41 NRAMP2 rabbit polyclonal IgG (Santa Cruz Biotechmology, CA)

2.42 Nitrocellulose membrane (Pierce)

2.43 Paraplast (Oxford labware)

2.44 Permount (Fisher Scientific)

2.45 Potassium dihydrogen phosphate (MERCK)

2.46 Ponceau S (Sigma)

2.47 Protease inhibitor

2.48 Skim milk (Carnation)

2.49 Sodium chloride (MERCK)

2.50 Sodium dihydrate phosphate monohydrate (MERCK)

2.51 Sodium dodecyl sulfate (Sigma-Aldrich)

2.52 Syringe: 1 and 3 ml (Nipro)

2.53 SeeBlue® Plus2 Pre-Stained Standard(Invitrogen)

2.54 standard protein (BSA) solution

2.55 Tetramethylethylenediamine (Sigma-Aldrich)

2.56 Tris-base (Amersham Biosciences)

2.57 Tris-HCl (usb)

2.58 Tri-sodium citrate (Carlo Erba reagenti)

2.59 Triton X-100 (MERCK)

2.60 Tween-20 (Fisher Scientific)

2.61 Western Blot Signal Enhancer(PIERCE)

2.62 Xylene (Zen point)

3. Oligonucleotide primers

Table 6 Oligonucleotide primers

Genes	Accession No.	Direction	Primer sequence	Size (bp)	References
DMT-1	NM_001174130	Forward	5' GAGCCAGTGTGTTTCTATGG 3'	518	Chong, et al., 2005
		Reverse	5' CCTAAGCCTGATAGAGCTAG 3'		
β-Actin	NM_001101	Forward	5' CATCGAGCACGGCATCGTCA 3'	211	Ishii, et al., 2006
		Reverse	5' TAGCACAGCCTGGATAGCAAC 3'		

Methods

1. Tissue procurement and preparation

Placental tissues were obtained from term pregnant women who were delivered to Mae Sot hospital. The 6 term human placenta tissues donated by women who living Cd contaminated area were used as the experimental group, whereas another 6 term human placenta tissues given by women who live in non-Cd contaminated area were used as the control group. Placentas were rapidly transport and fetal membrane was removed carefully from materno-fetal surface and cut umbilical cord out from each placenta. The placentas were divided into cord insertion, central and marginal areas and collected tissues from each region of placenta (Figure 27). Each region about 2 × 2 cm was cut along thickening of placentas by carbon knife and washed in normal saline and fixed in 4% paraformaldehyde in 0.1 M PBS (pH 7.2) at least 24 hour before use in localization study of DMT-1 in term human placenta. Moreover, other placental tissues were rapidly collected on ice trey after delivery. We selected a central area between fetal and maternal portion (Figure 28). A small piece of these were collected and transferred to sterile tube, and then were immediately frozen in liquid nitrogen before being stored at −80 °C for protein and mRNA expression analysis and at – 20 °C for metal analysis.

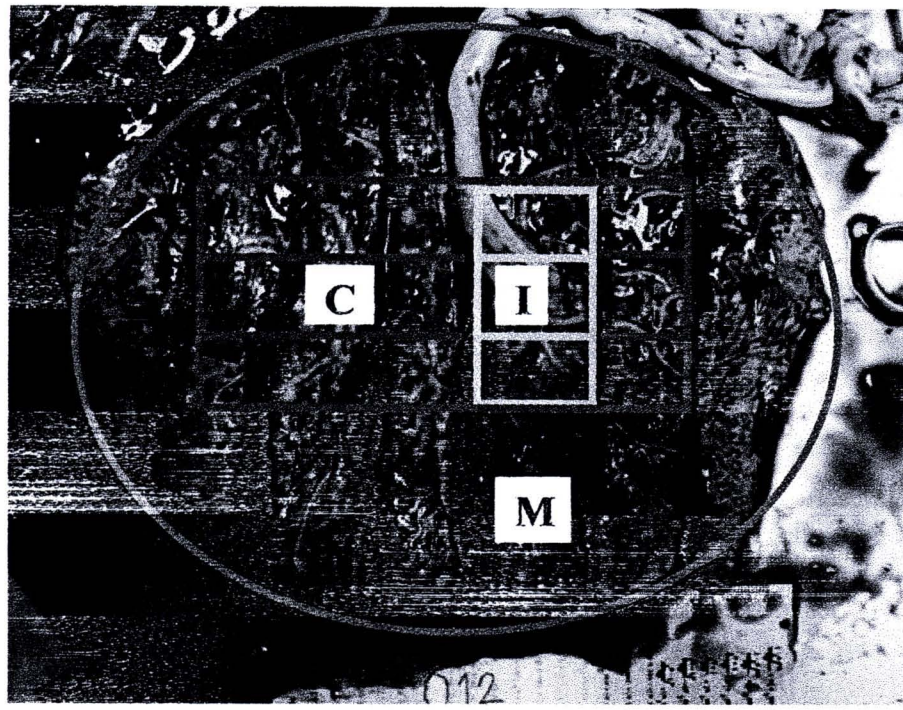

Figure 27 The placental tissue collection, I; cord insertion area (yellow line), C; central area (blue line), M; marginal area (green line)

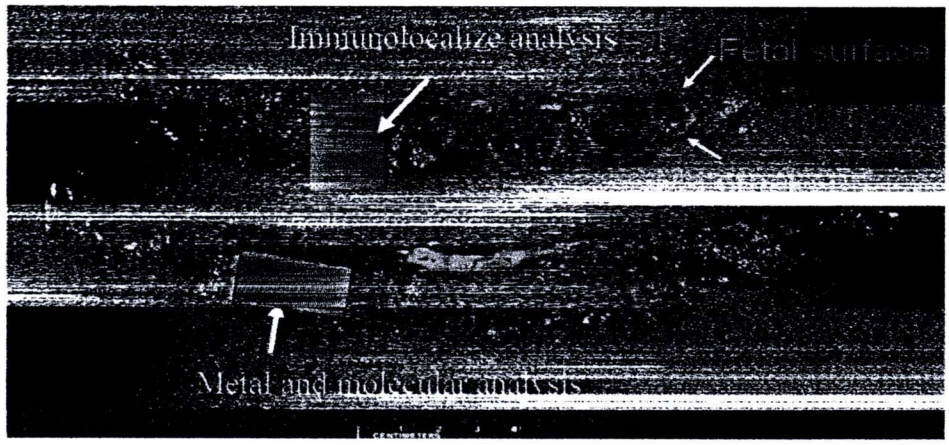

Figure 28 Cross plane of placental section for immunolocalize analysis (above) and metal and molecular analysis (below)

2. Immunohistochemistry technique (IHC)

Immunohistochemistry is a method that is based on the specificity and affinity of antibodies for the detection. The technique of immunohistochemistry is in principle composed of two steps. In a first step, a so called primary antibody is applied, which binds specifically to the epitope of a particular antigen in the tissue or cell. In a second step the binding between antigen and antibody is visualized using direct or indirect detection techniques. The experimental slides were compared with the negative control slide which omitted the primary antibody which is not expected to be found immunoreactivity and a positive tissue control is strongly recommended to ensure that the antibody is performing as expected. This tissue is a rat kidney tissue was obtained from co-worker in Toxic Metal Research Group (TMRG).

3. Western blotting technique

Western blotting or immunoblotting is used to identify specific antigens recognized by antibody. It can be used to determine the quantity and molecular weight protein of interested. Proteins are separated by size using polyacrylamide gel electrophoresis (PAGE). A small electrical current is run through the gel and the proteins migrate based on their size and electrical charge, with the smaller proteins moving faster. Sodium dodecyl sulfate (SDS) can be added to buffers to unify the electrical charge of the proteins. This results in protein separation by molecular weight only. The proteins are then transferred onto a membrane and non-specific background was blocked by using skim milk. The primary antibody followed by the HRP-conjugated secondary antibody was applied for nitrocellulose membrane incubation. Finally, this membrane was incubated in ECL substrate solution, according to expose membrane on X-ray film. The Scion image software was used for measured the intensity of all those expression bands (n=6/group).

4. Reverse Transcription Polymerease Chain Reaction (RT-PCR)

RT-PCR utilizes a pair of primers, which are complementary to a defined sequence on each of the two strands of the cDNA. These primers are then extended by a DNA polymerase and a copy of the strand is made after each cycle. RT-PCR includes three major steps. The first step is the reverse transcription (RT) where RNA is reverse transcribed to cDNA using a reverse transcriptase and primers. The second step involves the denaturation of the cDNA at 94°C, so that the two strands separate

and the primers can bind again at lower temperatures and begin a new chain reaction. The third step, when the temperature is decreased until it reaches the annealing temperature. The final step of PCR amplification is the DNA extension from the primers which is done by the Taq DNA polymerase. The PCR product is detected using agarose gel electrophoresis and ethidium bromide staining. The Scion image software was used for measured the intensity of all those expression bands (n=6/group).

5. Statistic analysis

All data are express as mean ± standard error of mean (S.E.M.) value. We used the SPSS (version 16.0) statistics package in conduct the statistical procedure. Paired Student's *t*-Test was used to determine significant differences between two groups. The one-way ANOVA was used to determined statistical significance deference levels for mean value of DMT-1 localization between three areas of placenta in both groups. Significance is accepted for $P < 0.05$. The strength of correlation between two variables was determined by Pearson's Sample Correlation Coefficient.