CHAPTER II

MATERIALS AND METHODS

2.1 Instruments

1. Atomic absorption spectrophotometer (Shimadzu Model AA-6300, Japan) equipped with a hollow cadmium cathode lamp and autosampler (Shimadzu ASC-6100, Japan)
2. Hematology auto-analyzer (Beckman Coulter® Model Coulter® Hmx, USA)
3. Clinical chemistry auto-analyzer (Beckman Coulter® Model Synchron CX3, USA)
4. CO_2 incubator (Thermo Sciencetific Model Forma Steri-Cycle® CO_2 Incubators, USA)
5. Class II biohazard safety cabine (ESCO® Model LA2-4A1)
6. Microplate reader (Bio Rad Model 3550, USA)
7. Real time PCR machine (Applied Biosystems Model ABI 7500, USA)

2.2 Chemicals

1. 3-(4,5-Dimethylthiazol-2-yl)- 2,5-diphenyltetrazolium bromide, a tetrazole, MTT dye (USB Corporation, USA)
2. 0.25% Trypsin-EDTA, 25200 (GIBCO®, USA)
3. Certified urinary standard reference material (The National Institute of Standards,USA)

4. Dulbecco's Modified Eagle Medium (DMEM):Ham's F12 (1:1), 11039(GIBCO®,USA)
5. Foetal bovine serum EU approved origin, 10270-098(GIBCO®, USA)
6. GLAZYME, β_2-microglobulin-EIA (Sanyo Chemical Industries,Ltd., Japan)
7. Human Osteocalcin Immuno radiometric assay (IRMA) kit (Immunotopics, Inc.,USA)
8. Latex particle enhanced turbidimetric immunoassay PET kit (Dako, Glostrup, Denmark)
9. Metra™ DPD, EIA kit (Quidel Corporation, USA)
10. NAG kit test Shionogi (Shionogi Pharmaceuticals, Japan)
11. Nitric acid (HNO_3, Wako Pure Chemical Industries, Ltd., Japan)
12. Nucleospin® RNA II kit (Macherey-Nagel GmbH&Co., Germany)
13. Osteomark® NTx kit (Inverness Medical, USA)
14. Palladium solution (Wako Pure Chemical Industries, Ltd., Japan)
15. Recovery™ – Cell culture freezing medium, 0070(GIBCO®, USA)
16. RevertAid H minus first strand cDNA synthesis kit (#K1631, Fermentas Life Sciences, USA)
17. Standard solution of cadmium concentration 1,000 mg/l (Wako Pure Chemical Industries, Ltd.,Japan)
18. Standard solution of calcium concentration 100 mg/l (Wako Pure Chemical Industries, Ltd., Japan)
19. SYBR® GreenER™ qPCR SuperMix Universal, 11762-100 (Invitrogen, USA)

2.3 Study area and sample population

Target population was 700 out of 7,697 inhabitants living in the cadmium polluted area, Mae Sot district, Tak province (Figure 1) who had been reported with high urinary cadmium in the 2004 survey conducted by Department of Community and Social Medicine of the Mae Sot General Hospital. The high exposure level of 5 μg/g Cr was used as a cutoff value associated to 5 fold increase of the renal dysfunction risk[20].

The study protocol was approved by the Institutional Review Board and Ethical Committee of the Faculty of Medicine, Chiang Mai University and the Ethical Committee of the Kanazawa Medical University, Japan.

Project aims and methods were advised and explained to the subjects before entering the survey. The subjects were suggested to fast after 11.00 pm on the night before the survey day. The informed consent forms were introduced to the subjects for the permission of biological sample collection. A random morning urinary sample and 10 ml of venipuncture blood were collected. The subjects were interviewed for demographic, health, nutrition information, underlying diseases and bone fracture history using a questionnaire.

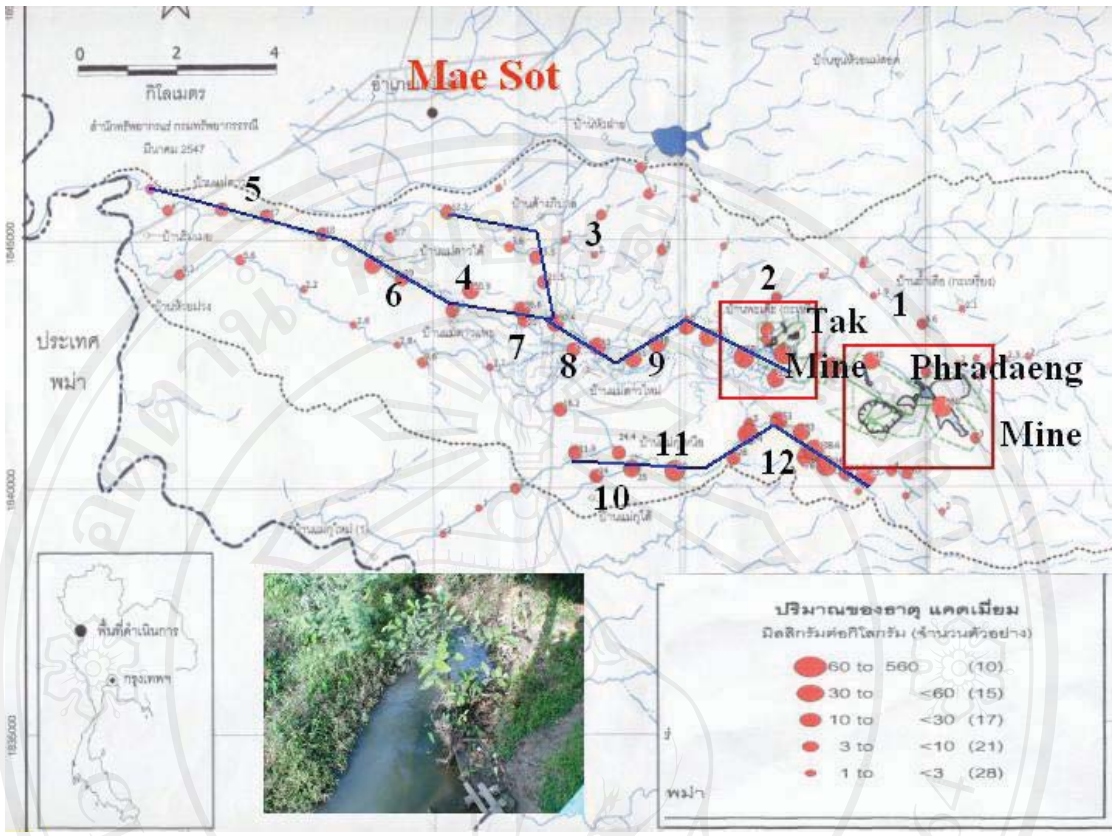


Figure 1 Cadmium polluted area in Mae Sot District, Tak Province. There are 12 villages and 2 big zinc-mines located upstream of water irrigation system. Red rectangle indicates location of zinc mines whereas blue line represents water irrigation path. Red spot indicates the soil sampling site which was performed by the Department of Mineral Resources, Ministry of Natural Resources and Environment in 2004[136].

The minimal sample size was calculated using the following equation[137]

$$n = \left(\frac{Z_{\alpha} + Z_{1-\beta}}{Z_{\rho}} \right)^2 + 3$$

In which; n = sample size

Z_{α} = critical Z value of alpha $Z_{1-\beta}$ = critical Z value of power of the test

Z_{ρ} = critical Z value of correlation coefficient

The assumption was alpha of 0.05 (two-sided), which $Z_{\alpha} = 1.96$ and beta of 0.20 (one-side) in which $Z_{1-\beta} = 0.84$. Sample size calculation refered to the correlation coefficient between urinary cadmium and β_2-microglobulin from our preliminary study with 56 subjects or approximately 10% of the previous study sample number. The obtained Spearman's correlation coefficient was 0.202.

where; $$Z_{\rho} = \frac{1}{2} \ln\left(\frac{1 + 0.202}{1 - 0.202} \right)$$

$$= 0.205$$

Therefore, $$n = \left(\frac{1.96 + 0.84}{0.205} \right)^2 + 3$$

$$n = 189.90$$

$$n \approx 190$$

The minimum surveyed population number should be 190 for a significance of correlation between urinary cadmium and β_2-microglobulin. Therefore, in this study, the sample size was 700 with 261 men and 439 women which was large enough to see the correlation between cadmium exposure and renal dysfunction markers.

For the study of bone metabolism and cadmium exposure, the subjects aged ≥ 50 years old were selected. Subjects currently using contraceptives or with a medical history of bone fractures were excluded from the study after sampling and every subject was not using active vitamin D metabolite or its analogue. Therefore, there were 412 subjects (156 men and 256 women) included.

For the study of anemia prevalence and cadmium exposure, all of the subjects aged ≥ 20 years old were included. They were totally 700 subjects.

2.4 Collection of urinary and blood samples

Morning urine samples were collected in polyethylene bottles after the subjects underwent physical examination and anthropometric measurements. The samples were immediately tested qualitatively for pH, protein, glucose, occult blood, urobilinogen and ketone body using paper indicator strips (Ames test, Bayer, Germany).

Each urine sample was divided into three (3–5 ml) aliquots. First aliquot was used to determine β_2-microglobulin level and if the sample's pH was below 5 it was adjusted to pH 6–8 by 0.5 N sodium hydroxide, to prevent degradation of β_2-microglobulin. Second aliquot was used to determine renal and bone markers. Third aliquot was kept in metal free polyethylene tube and used to determine cadmium levels.

Five milliliters of venipuncture blood was drawn and collected in two separated tubes with and without heparin. All aliquots were frozen and stored at -20˚C for further analysis.

2.5 Determination of blood and urinary cadmium

Blood and urinary cadmium concentrations were used to indicate cadmium body burden as well as cadmium exposure in the subjects using a flamless atomic absorption spectrometer (AAS).

Atomic Absorption Spectrometry (AAS)

Principle of the method is an electron number in each element is specific and associated with the atomic nucleus in an orbital structure. The electrons occupy orbital position in an orderly and predictably. Ground state is the most stable electronic configuration of an atom. When we apply energy to the atom, it will absorb energy and the outer electron will be promoted to a less stable configuration which known as excited state. Excited state is unstable, the atom will immediately and spontaneously returns to ground state configuration. The electron returns to its initial, stable orbital position and radiant energy equivalent to the amount of energy initially absorbed in the excitation process.

Particular wavelength of light from hollow cathode lamp is used as an energy source in the AAS. When light radiates to atomic cloud of metal, it will be absorbed by metal atom in a concentration-related manner and the remained light intensity will provide information on the quantitation of the element[138].

Sample preparation for blood cadmium analysis

Five hundred microliters of whole blood was mixed with 1 ml of 5% nitric acid in a microtube. It was then vigorously mixed for 30 seconds and placed in room temperature for 1 hour. The microtube was centrifuged at 12,000 rpm for 5 min and the supernatant was transfered to a new microtube. The supernatant was centrifuged

again at 12,000 rpm for 5 min before pipetting into an autosampler's cup for AAS analysis.

Standard addition method was used for setting up a standard curve for blood cadmium analysis. The standard sample was prepared by mixing 500 µl of the blood from healthy people with 1 ml of $CdCl_2$ solution at concentrations of 1.25, 2.50, 3.75 and 5.00 µg/l and processed as samples.

Sample preparation for urinary cadmium analysis

Urinary sample, 250 µl, was diluted with 500 µl of 20 mg/l palladium chloride in 5% nitric acid and 250 µl distrilled water. After mixing well, the sample was introduced into the flameless-AAS.

Standard curve was set by using standard $CdCl_2$ diluted with healthy children's urine at the concentrations of 1.25, 2.5, 5 and 10 µg/l. The standard samples were prepared by mixing 250 µl of the children's urine with 500 µl of each $CdCl_2$ concentrations and 250 µl of distrilled water.

2.6 Analysis of renal dysfunction markers

Beta2 -microglobulin (β_2-MG)

Enzyme immunoassay was used to determine urinary β_2-MG levels. The sample was incubated with polystyrene beads coated with human β_2-MG polyclonal antibody and β_2-MG antibody, a monoclonal anti-β_2-MG covalently bound to horseradish peroxidase (HRP). During incubation, β_2-MG in sample reacted with beat coated with polyclonal anti-β_2-MG then monoclonal anti-β_2-MG conjugated HRP reacted with its antigen (Figure 2). After a washing step, the bound enzyme reacted with substrate. The absorbance of the color product was correlated proportionally to

the concentration of β_2-MG in the specimen. Urinary β_2-MG was determined by the EIA test kit (GLAZYME β_2-microglobulin-EIA Test, Sanyo Chemical Industries, Ltd., Japan.

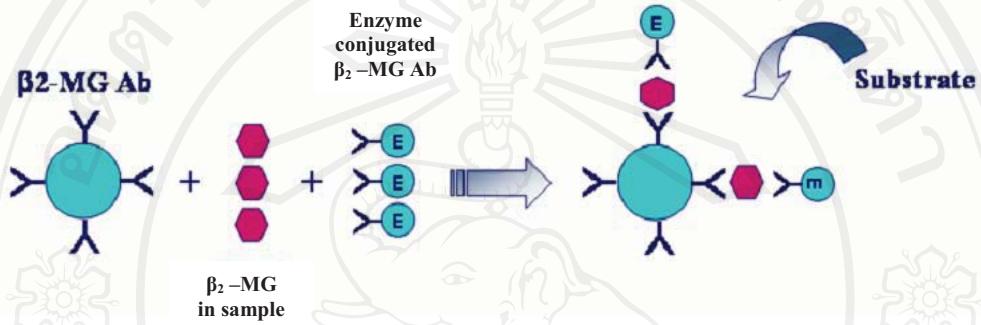
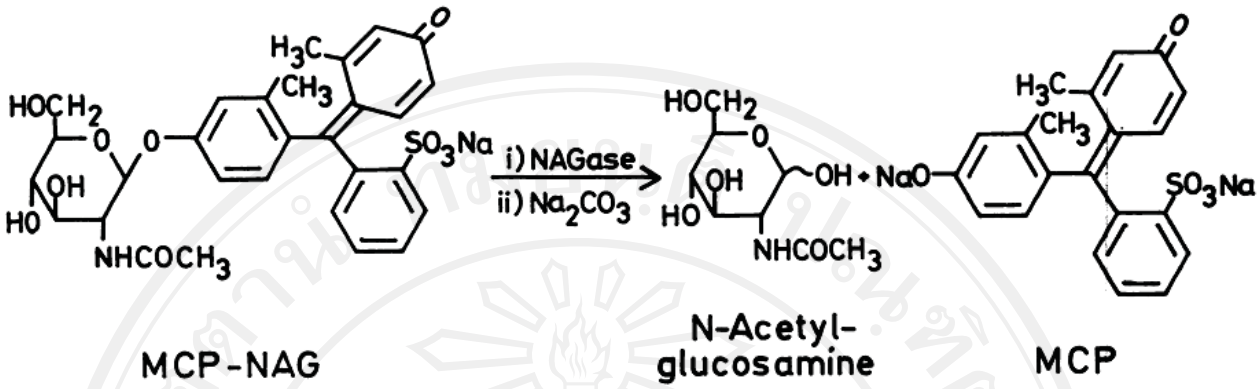


Figure 2 The reaction of β_2-MG in the enzyme immunoassay.

N-Acetyl-β-D-glucosaminidase (NAG)

Urinary NAG level was determined by colorimetric assay. The principle of the colorimetric assay is hydrolyzed sodio-m-cresolsulfonphthaleinyl-N-acetyl-β-D-glucosaminidase (MCP-NAG) by NAG, with the release of m-cresolsulfonphthalein (MCP), sodium salt (m-cresol purple) in urine (Figure 3), which was measured photometrically at 580 nm. In this study, the urinary NAG was determined by a commercial colorimetric kit (NAG test Shionogi, Shionogi Pharmaceuticals, Japan). The concentration of NAG in the urine directly corresponds to the MCP and increased the purple color of the assay's medium. The reaction of N-acetyl-β-D-glucosaminidase was shown in Figure 3.



www.n-acetyl-d-glucosamine3.com/

Figure 3 The reaction of NAG in the colorimetric assay.

Serum cystatin C

Serum cystatin C concentration was measured by latex particle enhanced turbidimetric immunoassay (PET kit, Dako, Glostrup, Denmark). The principle of this measurement is cystatin C in the sample binds to the specific anti-cystatin C antibody, coated on latex particles, and causes agglutination (Figure 4). The degree of the turbidity caused by the agglutination is proportional to the amount of cystatin C in the sample.

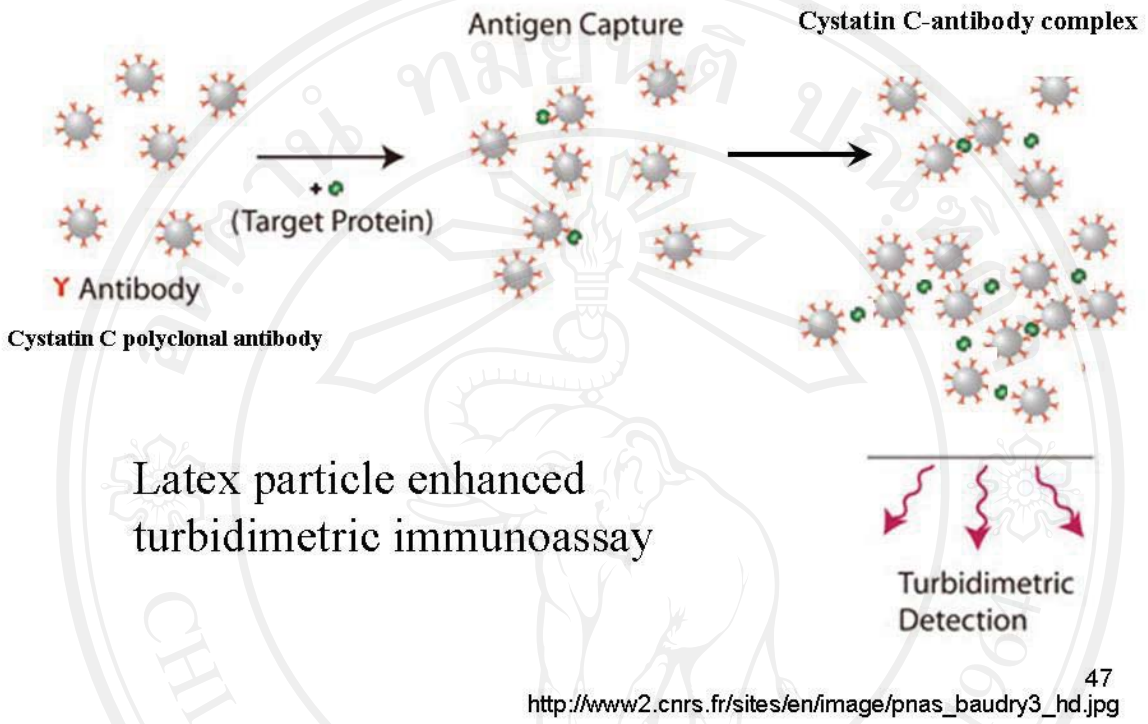


Figure 4 Reaction of serum cystatin C in the latex particle enhanced turbidimetric immunoassay.

Creatinine

Creatinine was determined by colorimetric assay (Jaffe method). The principle of this method is creatinine forms a yellow-orange coloured compound with picric acid in alkaline solution. The concentration of the coloured compound is a measure of the creatinine concentration which can be quantified at 520 nm spectrometrically.

2.7 Determination of fractional excretion of calcium

An increased fractional excretion of calcium (FECa) indicates impairment of calcium reabsorption capacity of renal tubule. Urinary and serum calcium levels were determined by colorimetric assay using an automated analyzer (Bechman Synchron CX3). The percentage of FECa was calculated from the serum and urinary calcium concentrations, as previously described by Kido et al. (1993)[106], using the following equation:

$$FECa(\%) = \frac{C_{Ca}}{C_{Cr}} \times 100(\%) = \frac{UV \times U_{Ca}}{S_{Ca} \times C_{Cr}} \times 100(\%)$$

C_{Ca} = Calcium clearance (ml min^{-1})

C_{Cr} = Creatinine clearance (ml min^{-1})

UV = Urinary volume (ml min^{-1})

U_{Ca} = Urinary calcium (ml min^{-1})

S_{Ca} = Serum calcium (mg l^{-1})

2.8 Determination of bone markers

Serum osteocalcin

Serum osteocalcin level was measured by immunoradiometric assay (IRMA). Two different polyclonal goat antibodies to human osteocalcin had been purified and used to recognize the 20-36 regions of the peptide. The antibody was immobilized onto plastic beads to capture the osteocalcin molecules and detected by other antibody which recognized the 1-19 region of the peptide. A second antibody is ^{125}I radiolabeled. A sample containing human osteocalcin was incubated simultaneously with an antibody coated bead and the ^{125}I labeled antibody. The osteocalcin

concentration in the sample was immunologically bound by both the bead coated antibody and ^{125}I labeled antibody to form a "sandwich" complex.

At the end of the incubation period, the bead was washed to remove any unbound labeled antibody and other components. The radioactivity bound to the bead was then measured in a gamma counter. The radioactivity of the antibody complex bound to the bead was directly proportional to the concentration of osteocalcin in the sample. A standard curve was generated by plotting the count per minute (CPM) versus the respective osteocalcin concentration for each standard on logarithmic scale. The concentration of osteocalcin in the sample was determined directly from this curve.

Urinary deoxypyridinoline (DPD)

Urinary DPD was measured by using Metra^{TM} DPD, EIA kit. The Metra^{TM} DPD assay is a competitive enzyme immunoassay in a microtiter stripwell format utilizing a monoclonal anti-DPD antibody coated on the strip to capture DPD. DPD in the sample competed with conjugated DPD-alkaline phosphatase for the antibody and the reaction was detected with a para-nitrophenyl phosphate (pNPP) substrate. pNPP was categorized by alkaline phosphatase which produced para-nitrophenolate. An increase of the para-nitrophenolate production indicated low concentration of the DPD in urine sample. Para-nitrophenolate gave yellow color which was detected at the wavelength 405 nm by spectrophotometer. The DPD levels were corrected using urinary creatinine concentration.

N-terminal cross link of type I collagen (NTx) measurement

Urinary NTx level was determined by Osteomark® test kit. The kit is a competitive-inhibition enzyme linked immunosorbant assay (ELISA). NTx in the urine sample competes with the NTx absorbed on the solid phase for binding sites on a horseradish peroxidase labelled monoclonal antibody. The amount of antibody bound is inversely proportional to the amount of NTx in the sample. The NTx concentration was determined spectrophotometrically and measured using a standard calibration curve. Tetramethylbenzidine (TMB) was used as a substrate and be oxidized during the enzymatic degradation of H_2O_2 by horse radish peroxidase. The oxidized product of TMB has a deep blue color. A clear yellow color was formed after addition of the acidic stop solution. A yellow color density was measured at 450 nm. NTx in sample was inversely proportional to the yellow color density.

2.9 Determination of anemic markers

Definition of anemia was identified based on haemoglobin (Hgb) level which was lower than 13 mg/dl in men or 12 mg/dl in women[139]. Hgb concentration was measured based on modified cyanmethemoglobin method by automate machine (Coulter HmX, Konelab). All of the blood indices were measured at the Mae Sot General Hospital as the following equations:

- Mean corpuscular volume (MCV) = $\frac{Hct \times 10}{RBC}$

- Mean corpuscular hemoglobin (MCH)= $\frac{Hgb \times 10}{RBC}$

- Mean corpuscular hemoglobin concentration (MCHC)= $\frac{Hgb \times 100}{Hct}$

- Red blood cell distribution width (RDW) (%) = $\frac{Standard\ deviation\ of\ MCV}{Mean\ MCV} \times 100$

2.10 Expression of the human fetal osteoblast genes after cadmium chloride treatment

Cell culture

Human fetal osteoblast like cell line (hFOB 1.19, purchased from American Type Culture Collection) at the concentration of 100,000 cells/ml was cultured and harvested in phenol red free media containing Dulbecco's Modified Eagle Medium (DMEM) and Ham's F12 at the ratio 1:1 with 10% fetal calf serum at 33.5ºC and 5% CO_2 in humidified condition.

Cell viability determination

Colorimetric MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole] assay is a cytotoxicity assay was used to measure a reduction of MTT by mitochondrial succinate dehydrogenase in the cells. The yellow tetrazolium MTT salt entered the cells and passed into the mitochondria, where it was reduced to an insoluble purple formazan product. The cells were then solubilized with an organic solvent. The solubilized formazan product was spectrophotometrically measured using an ELISA plate reader. Since reduction of MTT could only occur in metabolically actived cells and the level of activity was proportional to the viability of the cells, therefore, the color product will not be measured in dead cells.

Trypan blue exclusion assay was also used to determine cell viability in comparison to the MTT assay. It was a rapid test for gross damage via permeability of the cell membran.

Cadmium chloride and hFOB cells

The LC20 of cadmium chloride ($CdCl_2$) was determined and used as the highest concentration dose of cadmium chloride in the cultured hFOB cells. LC20 is a toxicant concentration which causes 20% of cell death. The MTT assay signal from non-treated media control cells was measured and recorded as 100% cell viability. A decrease signal detected in the $CdCl_2$ (concentrations range 0.31-40 μM) treated cells were compared to the non-treated cells and expressed in cell viability ratio percentage. Cell viability standard curve was set and used to calculate the LC20.

A hundred microlitres of cell suspension (1,000,000 cell/ml) was added to each well of a 96 well plate and incubated overnight. Aspirated culture media and treated cell with various concentrations of $CdCl_2$ solution which was prepared in DMEM:HAM's F12 media without fetal caft serum supplementation and incubated for 24 hours. Ten microliters of the MTT solution at the concentration of 5 mg/ml was added to each culture wells. Cells were then cultured for another 3.5 hours at 37 ˚C. The media was then removed and the formed formazan crystal was dissolved by 100 μl of 100% DMSO. The optical density at the wavelength of 540 nm was used to determine concentrations of purple formazan product.

After hFOB cells were treated with $CdCl_2$, the optimal concentration of the RNA was extracted from the cultured hFOB cells using Nucleospin® kit. The obtained RNA was used for cDNA synthesis by RevertAid™ First Strand cDNA Synthesis Kit.

RNA extraction

NucleoSpin®, a total RNA isolation kit provided with a method of preventing the degradation of the RNA during the isolation procedure. The cells were lysed by incubating in a solution containing large amount of chaotropic ions. This lysis buffer immediately inactivated RNAases-which were presented in biological materials. Beta-mercaptoethanol was added to the lysis buffer as a reducing agent that irreversibly denatured RNases by reducing disulfide bonds and destroying the native conformation required for enzyme functionality. Contaminating DNA was also removed by an rDNase solution. Pure RNA was finally eluted under low ionic strength conditions with RNase-free water.

cDNA synthesis

Two hundred microliters microcentrifuge tube was used for cDNA synthesis. Every steps needs to be performed on ice to diminish RNA degradation. One microgram of the extracted RNA was used as a template for cDNA synthesis. It was mixed with 1 µl of 100 µM oligo $(dT)_{18}$ primer and top up with diethyl pyrocarbonate (DEPC)-treated water to obtain total volume of 12 µl. The microcentrifuge tube was centrifuged briefly and incubated at 65 ˚C for 5 minutes. The reaction buffer was added with 1 µl of RiboLock™ RNase inhibitor (20 unit/µl) and 2 µl of 10 mM dNTP. Mixing the solution and adding 1 µl of RevertAid™ H Minus M-MuLV Reverse Transcriptase (200 unit/µl). Then, mixed gently, centrifuged, incubated at 42 ˚C for 120 minutes and terminated the reaction by heating at 70 ˚C for 5 minutes.

Real time PCR

The obtained cDNA was used for real time polymerase chain reaction (PCR) to detect amount of the expressed RNA using SYBR® GreenER™ qPCR SuperMix Universal. SYBR Green is an asymmetrical cyanine dye used as a nucleic acid stain in molecular biology. SYBR Green I binds to double-stranded DNA, but stain single-stranded DNA at lower performance. The resulting DNA-dye-complex absorbs blue light (λ_{max}=488 nm) and emits green light (λ_{max}=522 nm). Real time PCR creates a product depends on the cDNA template concentration. SYBR binds to PCR product and creates a fluorescent signal. Cycle threshold (Ct) is a number of PCR cycle in which the signal of PCR product increase as exponential phase over background signal, the lower Ct number the higher cDNA template concentration. ΔCt method is a comparison of house keeping gene Ct to determined genes.

GAPDH was used as a house keeping gene. ΔCt method was used to determine expression level of the ALP, Col1A2, OC, OPG, and RANKL mRNAs. Their primer sequences were shown in Table 1.

Table 1 Real time PCR primer sequence for osteoblast gene expression determination

Genes	Sequence (5'-3'):	Product size (bp)	Accession (Transcription)
ALP	F:CATGGCTTTGGGCAGAAGGA R:CTAGCCCCAAAAAGAGTTGCAA	166	NM_001114107.2[140]
COL1AI	F:CAGCCGCTTCACCTACAGC R:TTTTGTATTCAATCACTGTCTTGCC	85	NM_000088.3[141]
OC	F: GAAGCCCAGCGGTGCA R: CACTACCTCGCTGCCCTCC	70	NM_199173.2[142]
OPG	F: CCTCTCATCAGCTGTTGTGTG R: TATCTCAAGGTAGCGCCCTTC	242	NM_002546.3[143]
RANKL	F: CCAAGATCTCCAACATGACT R: TACACCATTAGTTGAAGATACT	142	NM_033012.3[144]
GAPDH	F:GAAGGTGAAGGTCGGAGTC R:GAAGATGGTGATGGGATTTC	226	NM_002046.3[140]

2.11 Data analysis

All determined variables, except age, was logarithmic transformed to correct for departure from normal distribution using Kolmogorov-Smirnov Goodness of Fit test. Mean comparisons were determined by Student's t-test or Analysis of Variance (ANOVA) and Dunnett's T3 method was used to determine mean difference in ANOVA. Generalized linear model was used to calculate age adjusted mean of blood and urinary cadmium concentrations. Correlations between the markers were

determined by Spearman's rho analysis. The relationship between determined parameters was analyzed by multivariate regression or binary logistic regression after adjustment for relevant factors. P-values of 0.05 or less were used to identify statistical significance. All statistical analyses were performed using SPSS version 11.0.