APPENDICES
APPENDIX A

List of reagent preparation

Urine collection

Preparation reagent for urine collection

0.5N NaOH

- NaOH 100g

Adjust volume to 250ml with deionized water

N-acetyl-D-glucosaminidase (NAG)

Preparation reagents for NAG analysis

Buffer reagent

Prepare the buffer reagent by adding distilled water 55ml into the buffer bottle and mix well.

Substrate reagent

Prepare the substrate reagent by adding one bottle of buffer into substrate reagent bottle and mix well.

Stop solution

Prepare the stop solution by adding distilled water 110ml into stop bottle and mix well.
β₂-microglobulin (β₂-MG)

Preparation reagents for β₂-MG analysis

*Buffer reagent*

Prepare the buffer reagent by diluted Conc. buffer 20 times by adding distilled water 1,425ml and mix well.

*Reagent for dissolve antibody*

Prepare the reagent for dissolve antibody by adding distilled water 50ml in this bottle and mix well.

*Enzyme antibody reagent*

Prepare the enzyme antibody reagent by adding one bottle of reagent for dissolve antibody into this bottle and mix well.

*Color reagent*

Prepare the color reagent by adding 20ml of reagent for dissolve color reagent into each bottle (4 bottles).

*Stop solution*

Prepare the stop solution by adding distilled water 110ml and mix well.

**Cadmium measurement**

Preparation reagents for metal measurement

*5% HNO₃*

- Conc.HNO₃ 5 ml

Adjust volume to 100 ml with deionized water

*10 mg/l Cd standard*

- Stock Cd standard (1,000 mg/L) in 5% HNO₃ 1 ml
- 5% HNO₃

1 mg/l Cd standard

- 10 mg/l Cd standard in 5% HNO₃

- 5% HNO₃
Appendix B

Questionnaire

Date ………………..   No. ………

Please check √ or fill the word in the blank of each article that is proper to you

Part I. General information
1. Name ........................................... Surname ...........................................
2. Gender     Male       Female
3. Birth date    ………../………./…………….. age………….years
4. Occupation
   1. No work    2. Farmer    3. Employee
   4. Merchant   5. Other……………………………….
5. How long have you lived in Mae Sot …………………..years

Part II. Smoking/Drinking/Medicine using history
6. Which medicine did you use within this month?
   1. Joint pain    Yes   No
   2. General pain   Yes   No
   3. Hypertension   Yes   No
   4. Diabetes    Yes   No
   5. Heart ischemia   Yes   No
   6. Others……………………………………..
7. Do you smoke?
   1. Never smoke    2. Had quit smoking
   3. Intermittently smoke   4. Regularly smoke
8. Do you drink alcohol?
   1. Never drink  2. Had quit drinking
   3. Intermittently drink  4. Regularly drink

Part III Underlying disease
9. Do you have hypertension?
   1. No  2. Yes
   3. Never check
10. Do you have diabetes?
    1. No  2. Yes
    3. Never check
11. Do you have hypercholesterolemia?
    1. No  2. Yes
    3. Never check
12. Do you have heart ischemia?
    1. No  2. Yes
    3. Never check
13. Do you have bone fracture history?
    1. No  2. Yes in……………(bone) since…………………
14. Do you have regular bone/joint pain?
    1. No  2. Yes in……………(pain area)
       When was it happen……………………
       (e.g., during work, lifting heavy item, sleep)
15. Do you have regular heavy weight item lifting?
    1. No  2. Yes
16. Do you have regular exercise within this month? (e.g., cycling, aerobic, swim)
    1. No  2. Yes ……………mins/time…………….days/week
17. Do you have any work with movement such as carry heavy item, cycling,
    gardening, laundry, house cleaning, etc.?
    1. No  2. Yes ……………mins/time…………….days/week
Part IV Food consumption

18. Which kind of rice do you have for your meal?
   Plain rice …………………meals/day……………….g/meal
   Sticky rice …………………meals/day……………….g/meal
   (Plain rice model size 180/240 g, Sticky rice model size 250/500 g)

19. How many eggs did you have in last week? …………………eggs
20. How many bird eggs did you have in last week? …………………eggs
21. How much amount of following kind of food did you have in last week?

<table>
<thead>
<tr>
<th>Days</th>
<th>Meals</th>
<th>Amount/meal</th>
</tr>
</thead>
<tbody>
<tr>
<td>21.1 Milk</td>
<td></td>
<td>box/meal</td>
</tr>
<tr>
<td>21.2 Chicken</td>
<td></td>
<td>table spoon/meal</td>
</tr>
<tr>
<td>21.3 Pork/Beef</td>
<td></td>
<td>table spoon/meal</td>
</tr>
<tr>
<td>21.4 Intestinal organ</td>
<td></td>
<td>table spoon/meal</td>
</tr>
<tr>
<td>21.5 Fish</td>
<td></td>
<td>table spoon/meal</td>
</tr>
<tr>
<td>21.6 Small fish</td>
<td></td>
<td>table spoon/meal</td>
</tr>
<tr>
<td>21.7 Vegetable</td>
<td></td>
<td>ladle/meal</td>
</tr>
<tr>
<td>21.8 Fruit</td>
<td></td>
<td>fruit/meal</td>
</tr>
<tr>
<td>21.9 Black sesame</td>
<td></td>
<td>tea spoon/meal</td>
</tr>
</tbody>
</table>

Part V Anthropology information

22. Weight……………kg  Height………….cm  BMI…………….kg/m²
23. Blood pressure……………..mm.Hg
Bone resorption acceleration and calcium reabsorption impairment in a Thai population with high cadmium exposure

Kowit Nambunmee¹, Ryumond Honda², Muneko Nishijo³, Wittaya Swaddiwudhipong³, Hideaki Nakagawa³, and Werawan Ruangwittikarn⁴

¹Doctor of Philosophy Program in Biomedical Sciences, Faculty of Associated Medical Sciences, Chiang Mai University, Thailand, ²Department of Public Health, Kanazawa Medical University, Ishikawa, Japan, ³Department of Community and Social Medicine, Mae Sot General Hospital, Tak Province, Thailand, and ⁴Department of Forensic Medicine, Faculty of Medicine, Chiang Mai University, Thailand

Abstract
Some residents of the Mae Sot district in Thailand have suffered long-term exposure to elevated dietary levels of cadmium. To test the hypothesis that chronic dietary cadmium exposure can cause imbalance in calcium dynamics and accelerate bone resorption, a group of these residents (156 men and 236 women aged 50) were selected on the basis of previous records of elevated urinary cadmium and tested for urinary and blood cadmium, bone formation and resorption markers, and the renal tubular dysfunction markers. Both genders had high levels of blood and urinary cadmium and high urinary levels of the markers for renal dysfunction and bone resorption in a dose–response relationship to urinary cadmium. The excretion of bone resorption markers was positively correlated to the ratio of excreted calcium and urinary cadmium. The results of a multivariate regression analysis indicated that bone resorption was accelerated by impaired calcium reabsorption in renal tubules.

Keywords: Cadmium; bone resorption; calcium reabsorption; Mae Sot

Introduction
High levels of cadmium in rice grown in the Mae Sot area of Thailand have recently been reported (Simmon et al. 2005). Cadmium concentrations in 524 rice samples ranged from 0.05–7.7 mg/kg and over 90% of the samples contained cadmium concentrations greater than the Codex Committee on Food Additives and Contaminants (CCFAC 2002) draft Maximum Permissible Level for rice of 0.2 mg cadmium/kg (Simmon et al. 2005) or above the level considered to be ‘safe’ for a staple food, of 0.1 mg/kg (Satarug et al. 2000; Satarug and Moore 2004). Surveys between 2001 and 2004 found that soil from rice paddy fields in the Mae Sot district contained markedly elevated cadmium levels (Simmon et al. 2005). The cadmium contaminated fields had been irrigated from two creeks which drained a catchment in which a large zinc mine had operated for more than 20 years.

The Mae Sot Hospital and the Bureau of Occupational and Environmental Diseases, Ministry of Public Health have regularly monitored cadmium levels and markers for renal toxicity in the affected population since 2001. An epidemiological study revealed that persons who mainly consumed rice grown in contaminated fields around Mae Sot had higher urinary cadmium than those who did not (Limpatanachote 2007; Swaddiwudhipong et al. 2007). Teeyakasem et al. (2007) also investigated 224 subjects, 30–87 years of age, who were residents of the cadmium polluted area in Mae Sot District, and reported that the excreted urinary cadmium ranged between 1–58 µg/g creatinine with a geometric mean of 0.2 µg/g creatinine. This was 10 fold

Address for Correspondence: Werawan Ruangwittikarn, Division of Toxicology, Department of Forensic Medicine, Faculty of Medicine, Chiang Mai University, Chiang Mai 50200, Thailand. Tel: 66-653-945433, FAX: 66-653-945434. E-mail: werawan52@gmail.com

(Received 30 July 2009; revised 28 October 2009; accepted 30 October 2009)

ISSN 1537-6516 print /ISSN 1537-6524 online © 2010 Informa UK Ltd
DOI: 10.3109/15376510903452941

http://www.informahc.com/txm
greater than the average reported for the general Thai population by Sirivarsri et al. (2002). In 2004, the Thai Ministry of Natural Resources and Environment asked farmers in the affected area to grow alternative crops, and warned against eating rice grown in that area.

The most severe manifestation of cadmium toxicity in humans is Itai-Itai disease, documented in patients in Japan who suffered chronic exposure to extremely high cadmium concentrations in contaminated water and rice (Nogawa et al. 1987; Aoshima et al. 1988; Kido et al. 1990). The effects of Itai-Itai disease are renal tubular dysfunction and osteomalacia combined with severe osteoporosis (Kasuya 2000; Takebayashi et al. 2000; Aoshima et al. 2003; Nishijyo et al. 2006; Omote et al. 2006). Decreased bone density associated with elevated levels of cadmium in urine and blood has also been reported from populations exposed to cadmium in China (Wang et al. 2003), Belgium (Staessen et al. 1999), and Sweden (Alfvén et al. 2000; Åkesson et al. 2006).

Decreasing bone density and increasing bone fracture risk without evidence of renal tubular dysfunction has been reported in subjects exposed to low levels of dietary cadmium in Japan (Honda et al. 2003) and in Belgium (Staessen et al. 1999). Some proposed etiologies for bone damage induced by cadmium exposure are the direct (impairment) of bone metabolism (Kjellström 1992; Reg nanathan et al. 2003) or indirect effects like reduced calcium reabsorption, via impaired function of renal tubules (Kido et al. 1993; Hayashi et al. 2003; Schutte et al. 2008).

The objective of this study was to investigate whether human exposure to elevated levels of dietary cadmium correlated with an increased risk of osteopathy, specifically by investigating relationships between cadmium exposure, calcium reabsorption impairment, renal tubular dysfunction, and/or bone resorption acceleration.

Methods

Study area and sample population

Four hundred and twelve subjects aged over 50 years from Mae Sot district, Tak province, Thailand, who were reportedly affected by environmental cadmium exposure, were chosen for this study. They were selected from 554 people with urinary cadmium over 5 µg/g creatinine, who were identified in a survey in 2004 conducted by the Department of Community and Social Medicine of the Mae Sot General Hospital. That survey of 7,697 people older than 15 years found 93% had urinary cadmium excretion of less than 5 µg/g creatinine, 377 people (4.9%) had urinary cadmium excretion levels between 5-10 µg/g creatinine, and 177 people (2.3%) had urinary cadmium excretion levels greater than 10 µg/g creatinine (Suwaddivudhipong et al. 2007).

This study protocol was approved by the Institutional Review Board and Ethical Committee of the Kanazawa Medical University and the Ethical Committee of the Faculty of Medicine, Chiang Mai University. All survey candidates were advised of the aims and methods of the project before entering the survey. All consented to provide random morning urinary samples and 5-10 ml of venipuncture blood. After the first sample, demographic, health, and nutrition information on each subject was collected by interview using a questionnaire.

Subjects currently using contraceptives or with a medical history of bone fractures were excluded from the study after sampling, and no subjects were using active vitamin D metabolite or its analog.

Collection of urine 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, urea nitrogen, and ketone body using paper indicator strips (Ames test, Bayer, Germany).

Each urine sample was divided into three (3-5 ml) aliquots. In samples with pH < 5, the pH of one aliquot was adjusted to pH 6-8 by 0.5 N sodium hydroxide, to prevent degradation of β₂-microglobulin. Five-to-ten milliliters of venipuncture blood was drawn and collected in two separated tubes with and without heparin. All aliquots were then frozen and stored at −20°C for further analysis.

Determination of blood and urinary cadmium and urinary creatinine

Blood and urinary cadmium concentrations were quantified using a flameless atomic-absorption spectrometer (Shimadzu Model AAS-6300, Japan), with palladium chloride in 5% nitric acid solution as a modifier. Method validation of the analytical techniques were performed and certified by certified standard reference materials (The National Institute of Standards, Washington, DC). The urinary creatinine concentration was measured by a method based on the Jaffe reaction.

Determination of serum and urinary calcium

Serum and urinary calcium were determined by colorimetric assay using an automated analyzer (Coulter HmX, Konelab 30 and Behman Synchron CX3) at Mae Sot General Hospital. The laboratory was evaluated and certified by the Bureau of Laboratory Quality Standards, Ministry of Public Health, Thailand.

Fractional excretion of calcium was calculated, from the serum and urinary calcium concentrations, as previously described by Kido et al. (1993).

Determination of renal markers

The concentration of β₂-microglobulin in urine was determined by enzyme immunoassay (GLAZYMÉ β₂-microglobulin-EIA test kit, Sanyo Chemical Industries, Ltd., Japan) and the concentration of NAG was determined by a colorimetric assay (NAG test kit, Shionogi Pharmaceuticals, Japan).

Determination of bone markers

Serum osteocalcin (bone formation marker) was measured by immunoradiometric assay (Hauschka et al. 1989). Bone
Bone resorption, calcium reabsorption, and cadmium exposure

resorption markers, deoxypyridinoline and type I collagen cross-linked N-telopeptide (NTx), were measured by competitive enzyme immunoassay (Metry™ DPD, ELA, Hanson et al. 1992).

Data analysis

All determined variables, except age, were logarithmic transformed to correct for departure from normal distribution. Mean comparisons of age and biomarkers between genders were analyzed by independent sample t-test. The multiple comparison analysis was performed by Dunnett's T3 method. Correlations between the markers were determined by Spearman's r analysis. The relationship between cadmium and bone marker concentrations was analyzed by multivariate regression after adjustment for age, gender, and level of renal dysfunction markers. P-values of 0.05 or less were used to identify statistical significance. All statistical analyses were performed using SPSS version 11.0.

Results

The mean and the range of subject age, blood and urinary cadmium, renal dysfunction markers (β₂, microglobulin and NAG), bone resorption markers (urinary deoxypyridinoline and NTx), urinary calcium, and fractional calcium excretion (all shown in Table 1) indicated high exposure to cadmium affected renal tubular function and bone metabolism. The mean age and β₂-microglobulin concentrations were significantly higher in men than women. Whereas, urinary calcium, deoxypyridinoline, and NTx were significantly lower in men than women.

The average concentration of both bone resorption markers in female urine was higher than the bone fracture risk cut-off values proposed by Nishizawa et al. (2005) (urinary deoxypyridinoline > 7.6 nmol/mmol creatinine and NTx > 54.3 nmol BCE/mmol creatinine) (Table 1). Average concentrations of urinary cadmium and NAG, serum calcium, and fractional excretion of calcium were not significantly different between men and women. However, blood cadmium and β₂-microglobulin were significantly higher in men than in women. The mean body mass index of both genders was less than the normal level of 25 kg/m² and the body mass index for women was slightly higher than for men.

We investigated the relationship between cadmium exposure and the renal and bone marker concentrations in the urine. The subjects were grouped according to the urinary excretion of cadmium. A low level exposure group (n = 20) was defined with < 2 μg Cd/g creatinine; medium exposure groups (n = 86 and 190) with 2-5 and 5-10 μg Cd/g creatinine, respectively; and a very high exposure group (n = 116) with > 10 μg Cd/g creatinine (Table 2).

The average concentrations of the renal and bone markers increased with rising cadmium exposure and the fractional excretion of calcium also increased, especially in women. There was also a slight decrease in serum calcium in women participants, indicative of calcium reabsorption impairment. Bone resorption acceleration was also evident, especially among subjects with high urinary cadmium excretion > 10 μg Cd/g creatinine.

A comparison of excreted urinary renal and bone markers in the high (> 10 μg Cd/g creatinine) and medium (5-10 μg Cd/g creatinine) cadmium exposure groups showed NAG was significantly higher in both genders. Whereas in women, urinary β₂-microglobulin, fractional excretion of calcium, and NTx were all significantly different (Table 2).

These results show that high cadmium concentrations affected the renal function, accelerated bone resorption, and impaired calcium reabsorption in elderly Thai women more than in men.

The correlation between cadmium exposure and bone markers and urinary renal tubular dysfunction markers was estimated, and Spearman's r value was used to estimate

Table 1. Comparisons of age, body mass index, urinary cadmium, blood cadmium, renal and bone markers between men and women aged 50 years and over, living in cadmium polluted area, Mae Sot district, Tak province, Thailand.

<table>
<thead>
<tr>
<th></th>
<th>Mean (SD)</th>
<th>Range</th>
<th>Mean (SD)</th>
<th>Range</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>66.40 (9.31)</td>
<td>50-86</td>
<td>61.64 (8.36)</td>
<td>50-87</td>
<td>0.003</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>20.31 (2.94)</td>
<td>12.08-30.44</td>
<td>21.54 (4.24)</td>
<td>10.96-32.09</td>
<td>0.001</td>
</tr>
<tr>
<td>B-Cd (μg/l)</td>
<td>7.20 (1.80)</td>
<td>1.44-26.05</td>
<td>5.54 (1.94)</td>
<td>0.80-33.12</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>U-β₂-MG (mg/g creatinine)</td>
<td>6.71 (1.92)</td>
<td>0.84-41.82</td>
<td>7.32 (1.89)</td>
<td>1.19-42.41</td>
<td>0.181</td>
</tr>
<tr>
<td>U-Cd (mg/g creatinine)</td>
<td>854.78 (10.38)</td>
<td>12.56-74.988.94</td>
<td>235.56 (6.49)</td>
<td>6.80-107,003.21</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>NAG (unit/g creatinine)</td>
<td>6.23 (1.77)</td>
<td>0.51-46.88</td>
<td>6.39 (1.81)</td>
<td>0.53-55.52</td>
<td>0.674</td>
</tr>
<tr>
<td>OC (mg/ml)</td>
<td>4.91 (1.85)</td>
<td>0.50-10.80</td>
<td>5.63 (1.94)</td>
<td>0.50-29.20</td>
<td>0.036</td>
</tr>
<tr>
<td>DPD (nmol/mmol creatinine)</td>
<td>6.32 (1.30)</td>
<td>2.93-14.67</td>
<td>6.30 (1.64)</td>
<td>3.13-38.40</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>NTx (mmol BCE/mmol creatinine)</td>
<td>43.50 (1.80)</td>
<td>10.40-166.50</td>
<td>50.16 (1.77)</td>
<td>11.30-323.70</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>S-Ca (mg%)</td>
<td>9.38 (1.06)</td>
<td>8.00-11.90</td>
<td>9.50 (1.07)</td>
<td>5.56-11.90</td>
<td>0.051</td>
</tr>
<tr>
<td>U-Ca (mg/g creatinine)</td>
<td>58.06 (2.60)</td>
<td>6.80-66.67</td>
<td>60.91 (2.90)</td>
<td>9.90-9900.00</td>
<td>0.011</td>
</tr>
<tr>
<td>FECA (%)</td>
<td>0.84 (0.43)</td>
<td>0.07-7.71</td>
<td>0.82 (2.59)</td>
<td>0.05-32.00</td>
<td>0.825</td>
</tr>
</tbody>
</table>

B-Cd = blood cadmium, U-Cd = urinary cadmium, BMI = body mass index, β₂-MG = urinary β₂-microglobulin, NAG = urinary N-acetyl-β-D-glucosaminidase, OC = serum osteocalcin, DPD = urinary deoxypyridinoline, NTx = urinary type I collagen cross-linked N-telopeptide, BCE = bone collagen equivalent, S-Ca = serum calcium, U-Ca = urinary calcium, FECA = fractional excretion of calcium.
Table 2. Dose–response analysis of the concentrations of renal markers (β2-MG and NAG) and bone markers (OC, DPD, and NTx) to four levels of cadmium exposure.

<table>
<thead>
<tr>
<th></th>
<th>U-Cd (μg/g creatinine)</th>
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<tr>
<td></td>
<td>&lt;2</td>
<td>2–5</td>
<td>5–10</td>
<td>&gt;10</td>
<td>ANOVA</td>
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<tr>
<td></td>
<td>Mean ± SD</td>
<td>Mean ± SD</td>
<td>Mean ± SD</td>
<td>Mean ± SD</td>
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<td>n = 71</td>
<td>n = 43</td>
<td></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>Age</td>
<td>65.83 ± 13.46</td>
<td>66.56 ± 8.32</td>
<td>63.16 ± 9.32</td>
<td>64.51 ± 9.15</td>
<td>0.306</td>
<td></td>
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<tr>
<td>B-Cd (μg/l)</td>
<td>3.19 ± 1.50</td>
<td>4.02 ± 1.52</td>
<td>7.63 ± 1.58</td>
<td>12.08 ± 1.40</td>
<td>&lt;0.001</td>
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<tr>
<td>U-Cd (μg/g creatinine)</td>
<td>1.43 ± 1.28</td>
<td>3.63 ± 1.26</td>
<td>6.85 ± 1.21</td>
<td>14.06 ± 1.43</td>
<td>&lt;0.001</td>
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<tr>
<td>β2-MG (μg/g creatinine)</td>
<td>202.72 ± 7.33</td>
<td>836.49 ± 13.55</td>
<td>718.62 ± 9.38</td>
<td>1510.08 ± 9.52</td>
<td>0.112</td>
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</tr>
<tr>
<td>NAG (unit/g creatinine)</td>
<td>4.00 ± 2.52</td>
<td>5.62 ± 1.79</td>
<td>5.96 ± 1.63</td>
<td>7.89 ± 1.70</td>
<td>0.005</td>
<td></td>
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<tr>
<td>S-Ca (mg%)</td>
<td>9.41 ± 1.06</td>
<td>9.40 ± 1.05</td>
<td>9.37 ± 1.06</td>
<td>9.38 ± 1.07</td>
<td>0.991</td>
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<tr>
<td>U-Ca (mg/g creatinine)</td>
<td>40.07 ± 3.20</td>
<td>68.17 ± 2.65</td>
<td>65.90 ± 2.82</td>
<td>65.48 ± 2.34</td>
<td>0.567</td>
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<tr>
<td>FECA (%)</td>
<td>0.60 ± 0.29</td>
<td>0.89 ± 2.52</td>
<td>0.85 ± 2.63</td>
<td>0.83 ± 2.08</td>
<td>0.716</td>
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</tr>
<tr>
<td>Serum OC (mg/ml)</td>
<td>4.75 ± 1.61</td>
<td>4.63 ± 1.83</td>
<td>4.77 ± 1.82</td>
<td>5.33 ± 1.97</td>
<td>0.867</td>
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<tr>
<td>DPD (nmol/mmol creatinine)</td>
<td>4.51 ± 1.79</td>
<td>4.84 ± 1.35</td>
<td>5.12 ± 1.33</td>
<td>5.90 ± 1.30</td>
<td>0.021</td>
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<tr>
<td>NTx (nmol BCE/mmol creatinine)</td>
<td>28.75 ± 1.94</td>
<td>40.18 ± 1.93</td>
<td>43.99 ± 1.73</td>
<td>49.12 ± 1.75</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>n = 12</td>
<td>n = 52</td>
<td>n = 119</td>
<td>n = 73</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>63.83 ± 9.06</td>
<td>62.23 ± 9.11</td>
<td>61.53 ± 8.05</td>
<td>61.21 ± 8.33</td>
<td>0.870</td>
<td></td>
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<tr>
<td>B-Cd (μg/l)</td>
<td>1.71 ± 1.92</td>
<td>3.56 ± 1.61</td>
<td>5.43 ± 1.62</td>
<td>9.55 ± 1.70</td>
<td>&lt;0.001</td>
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<tr>
<td>U-Cd (μg/g creatinine)</td>
<td>1.82 ± 1.18</td>
<td>3.71 ± 1.55</td>
<td>7.37 ± 1.21</td>
<td>15.08 ± 1.38</td>
<td>&lt;0.001</td>
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<tr>
<td>β2-MG (μg/g creatinine)</td>
<td>65.48 ± 2.86</td>
<td>221.77 ± 6.61</td>
<td>188.54 ± 5.63*</td>
<td>417.25 ± 7.58**</td>
<td>0.001</td>
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<tr>
<td>NAG (unit/g creatinine)</td>
<td>4.00 ± 2.19</td>
<td>5.94 ± 1.87</td>
<td>6.06 ± 1.76</td>
<td>7.90 ± 1.99</td>
<td>&lt;0.001</td>
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<tr>
<td>S-Ca (mg%)</td>
<td>9.61 ± 1.95</td>
<td>9.63 ± 1.06</td>
<td>9.48 ± 1.05</td>
<td>9.41 ± 1.03</td>
<td>0.247</td>
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<tr>
<td>U-Ca (mg/g creatinine)</td>
<td>86.53 ± 1.93</td>
<td>65.77 ± 3.37</td>
<td>78.61 ± 2.73</td>
<td>110.03 ± 2.53</td>
<td>0.036</td>
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<tr>
<td>FECA (%)</td>
<td>0.79 ± 1.83</td>
<td>0.69 ± 2.94</td>
<td>0.73 ± 2.51</td>
<td>1.10 ± 2.40</td>
<td>0.018</td>
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<tr>
<td>Serum OC (mg/ml)</td>
<td>5.16 ± 1.59</td>
<td>5.46 ± 2.04</td>
<td>3.33 ± 1.94</td>
<td>6.40 ± 1.30</td>
<td>0.266</td>
<td></td>
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<tr>
<td>DPD (nmol/mmol creatinine)</td>
<td>7.94 ± 1.43</td>
<td>8.00 ± 1.49</td>
<td>8.64 ± 1.42</td>
<td>9.06 ± 1.44</td>
<td>0.124</td>
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<tr>
<td>NTx (nmol BCE/mmol creatinine)</td>
<td>55.59 ± 1.03</td>
<td>55.92 ± 1.95</td>
<td>61.42 ± 1.66</td>
<td>72.37 ± 1.75</td>
<td>0.006</td>
<td></td>
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Mean comparison was evaluated using Dunnett’s T3 method.

*p < 0.05, **p < 0.01, compared with the group of U-Cd < 2 μg/g creatinine; †p < 0.05, compared with the group of U-Cd 2–5 μg/g creatinine; ‡p < 0.01, compared with the group of U-Cd 5–10 μg/g creatinine.

The strength of the correlation (Table 3). Blood and urinary cadmium showed positive correlation with the bone markers. Interestingly both the bone resorption markers (deoxypyridinoline and NTx) were significantly correlated to the urinary cadmium concentrations in men, but only NTx was highly correlated to the urinary cadmium concentrations in women. Blood cadmium was significantly correlated with deoxypyridinoline in men and correlated with osteocalcin, deoxypyridinoline, and NTx in women. A highly significant positive correlation between NTx, urinary cadmium and fractional excretion of calcium in women was clear (Figure 1) with r = 0.231, p < 0.001 and r = 0.440, p < 0.001, respectively. In addition, the bone formation marker, serum osteocalcin, was well correlated to age in both men and women, but age, and bone resorption markers (deoxypyridinoline and NTx) in both genders were not correlated (Spearman’s r value was below 0.1). The renal dysfunction markers of both men and women were positively correlated with the serum osteocalcin values. However, only the NTx in men and the deoxypyridinoline in women were highly correlated with the renal markers.

Urinary cadmium related to deoxypyridinoline and NTx, but showed no relation with osteocalcin. On the other hand, blood cadmium showed positively significant relation to all of bone markers. β2-microglobulin showed significant relation to every bone marker for both adjusting by urinary cadmium or blood cadmium. FECA was a strong explanatory variable of NTx with unstandardized regression coefficient 0.257 adjusted by urinary cadmium and 0.263 adjusted by blood cadmium (Tables 4 and 5). Unexpectedly, the deoxypyridinoline was inversely correlated to the urinary β2-microglobulin in women, whereas

Table 3. Gender dependent correlations between urinary cadmium, bone markers (rows), and renal tubular dysfunction markers (columns).

<table>
<thead>
<tr>
<th>Variables</th>
<th>Age</th>
<th>B-Cd</th>
<th>U-Cd</th>
<th>β2-MG</th>
<th>NAG</th>
<th>FECA</th>
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<td>Men</td>
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<tr>
<td>OC</td>
<td>0.216**</td>
<td>0.144</td>
<td>0.123</td>
<td>0.206***</td>
<td>0.280***</td>
<td>0.062</td>
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<td>DPD</td>
<td>0.044</td>
<td>0.209**</td>
<td>0.225**</td>
<td>0.001</td>
<td>0.145</td>
<td>0.046</td>
</tr>
<tr>
<td>NTx</td>
<td>-0.067</td>
<td>0.140</td>
<td>0.179*</td>
<td>0.337****</td>
<td>0.213**</td>
<td>0.398**</td>
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<tr>
<td>Women</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OC</td>
<td>0.209***</td>
<td>0.153**</td>
<td>0.148*</td>
<td>0.249**</td>
<td>0.278**</td>
<td>0.349**</td>
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<tr>
<td>DPD</td>
<td>0.012</td>
<td>0.128*</td>
<td>0.129</td>
<td>-0.182**</td>
<td>-0.179**</td>
<td>0.229**</td>
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<tr>
<td>NTx</td>
<td>-0.026</td>
<td>0.197***</td>
<td>0.231***</td>
<td>0.142**</td>
<td>0.078</td>
<td>0.440***</td>
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Correlation coefficients were determined by Spearman’s rho analysis. Spearman’s rho (r) values were used to estimate the probability of a chance correlation: *p < 0.05, **p < 0.01, ***p < 0.001.

β-Cd = blood cadmium, U-Cd = urinary cadmium, β2-MG = urinary β2-microglobulin, NAG = urinary N-acetyl-β-D-glucosaminidase, OC = serum osteocalcin, DPD = urinary deoxypyridinoline, NTx = urinary type I collagen cross-linked N-telopeptide, FECA = fractional excretion of calcium.
Figure 1. The elevation of urinary NTx as a function of urinary cadmium (A) and fractional calcium excretion (B) in women.

Table 4. Multivariate regression analyses of age, sex, urinary cadmium, and renal tubular dysfunction markers on bone markers.

|       | B     | SE    | t     |       | B     | SE    | t     |       | B     | SE    | t     |
|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| Age   | 0.006 | 0.002 | 3.79*** | 0.001 | 0.001 | 1.653 | -0.002 | 0.001 | -1.017 | 0.198 | 0.026 | 7.738*** |
| Sex*  | 0.109 | 0.029 | 3.621** | 0.109 | 0.027 | 4.019*** | 0.166 | 0.043 | 3.827*** |
| U-Cd  | 0.061 | 0.048 | 1.258 | -0.027 | 0.090 | -3.169** | 0.062 | 0.014 | 4.429** |
| β-MG  | 0.063 | 0.016 | 4.05** | 0.060 | 0.003 | -9.399** | -0.062 | 0.001 | -1.176 |
| Age   | 0.005 | 0.002 | 3.447*** | 0.000 | 0.001 | -9.399** | 0.062 | 0.001 | 6.332** |
| Sex*  | 0.071 | 0.027 | 2.591** | 0.017 | 0.015 | 12.868** | 0.163 | 0.025 | 6.328** |
| U-Cd  | 0.062 | 0.050 | 0.649 | 0.000 | 0.028 | 2.153** | 0.170 | 0.045 | 3.749** |
| NAG   | 0.252 | 0.066 | 4.05** | 0.060 | 0.003 | 3.499** | 0.131 | 0.051 | 2.554** |
| Age   | 0.008 | 0.002 | 5.148** | 0.060 | 0.003 | 1.019 | 0.257 | 0.027 | 9.340** |
| Sex*  | 0.079 | 0.029 | 2.859** | 0.174 | 0.023 | 7.621** |
| U-Cd  | 0.009 | 0.048 | 1.684 | 0.086 | 0.027 | 3.173** | 0.163 | 0.040 | 4.112** |
| FEECa | 0.076 | 0.034 | 1.827 | 0.031 | 0.199 | 1.827 | 0.257 | 0.027 | 9.340** |

*0 for men and 1 for women.
B = unstandardized regression coefficient, SE = standard error, t = t-score, *p < 0.05, **p < 0.01, ***p < 0.001.

Table 5. Multivariate regression analyses of age, sex, blood cadmium, and renal tubular dysfunction markers on bone markers.

|       | B     | SE    | t     |       | B     | SE    | t     |       | B     | SE    | t     |
|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| Age   | 0.006 | 0.002 | 3.736** | 0.001 | 0.001 | 1.334 | -0.003 | 0.001 | -1.394 | 0.217 | 0.026 | 8.392** |
| Sex*  | 0.128 | 0.029 | 4.26** | 0.199 | 0.016 | 12.243** | 0.217 | 0.026 | 8.392** |
| B-Cd  | 0.110 | 0.048 | 2.257* | 0.079 | 0.023 | 2.867** | 0.107 | 0.044 | 2.439** |
| β-MG  | 0.069 | 0.015 | 3.678** | -0.025 | 0.009 | -2.854** | 0.066 | 0.014 | 4.701** |
| Age   | 0.005 | 0.002 | 3.539** | 0.000 | 0.001 | -0.486 | -0.002 | 0.001 | -1.602 | 0.181 | 0.026 | 7.096** |
| Sex*  | 0.085 | 0.028 | 3.042** | 0.203 | 0.016 | 12.898** | 0.181 | 0.026 | 7.096** |
| B-Cd  | 0.140 | 0.048 | 2.256* | 0.091 | 0.027 | 2.874** | 0.121 | 0.044 | 2.721** |
| NAG   | 0.240 | 0.054 | 4.41*** | 0.124 | 0.031 | 6.019** | 0.166 | 0.050 | 3.231** |
| Age   | 0.008 | 0.002 | 5.073** | 0.210 | 0.016 | 13.183** | 0.192 | 0.023 | 8.303** |
| Sex*  | 0.098 | 0.028 | 3.473** | 0.157 | 0.019 | 2.133* | 0.115 | 0.040 | 2.658** |
| B-Cd  | 0.139 | 0.048 | 2.888** | 0.058 | 0.027 | 2.133* | 0.115 | 0.040 | 2.658** |
| FEECa | 0.075 | 0.033 | 2.251* | 0.034 | 0.199 | 1.823 | 0.263 | 0.028 | 9.495** |

*6 for men and 1 for women.
B = unstandardized regression coefficient, SE = standard error, t = t-score, *p < 0.05, **p < 0.01, ***p < 0.001.
NTx was positively related to urinary β2-microglobulin, NAG, and fractional excretion of calcium independent of blood and urinary cadmium and age. Serum osteocalcin was also positively related to urinary β2-microglobulin in both genders and only to urinary NAG in women.

Discussion

Bone formation and resorption are bone remodeling mechanisms which can be indicated using osteoblast and osteoclast cell markers. Bone marker determination is recognized as a non-invasive and comparatively inexpensive tool for assessing metabolic bone diseases. It is widely used to detect changes in bone metabolism of people at risk (Seibel 2005). In osteoporosis patients, bone markers can detect response to treatment earlier than bone mineral density measurements (Garnero 2000).

Increased serum bone alkaline phosphatase and/or osteocalcin have been reported in Japanese whose urinary cadmium was ≥10 μg Cd/g creatinine (Kido et al. 1991; Aoshima et al. 2003). In our subjects, even though serum osteocalcin levels were within the reference range of 3.1–12.7 ng/ml (Yajima et al. 2003), serum osteocalcin was positively correlated with elevated levels of urinary β2-microglobulin and NAG in both men and women (Table 3). Serum osteocalcin was also positively correlated with blood cadmium (Table 5), but showed no relation to urinary cadmium (Table 4). These findings suggest the observed increase in osteocalcin was due to renal dysfunction, independent of age and cadmium body burden.

Cadmium chronically exposed inhabitants of the Kakehashi River basin, Japan, had low serum calcium, seen with high levels of serum osteocalcin (Nishizawa et al. 1994; Tsuji et al. 1994). In this study, 43 men (27.6% of male subjects) and 73 women (28.5% of female subjects) had urinary cadmium concentrations ≥10 μg/g creatinine. These levels are similar to the highest levels recorded from Japan; however, their serum osteocalcin are still within reference range (Table 1).

A pyridinium cross-linked collagen (deoxyxypyrrolidinoline or NTx) is formed during the extracellular maturation of fibrillar collagens and is released in the degradation of mature collagens (Nishizawa et al. 2005). Measurement of deoxyxypyrrolidinoline is not influenced by the degradation of newly generated collagens, or by dietary intake, and shows a high specificity for skeletal tissues. Type I collagen cross-linked N-telopeptide (NTx) and deoxyxypyrrolidinoline have been identified as the best indicators for assessment of bone resorption (Nishizawa et al. 2005). In this study, we found that urinary deoxyxypyrrolidinoline and NTx were mainly related to an increase of urinary cadmium, blood cadmium, and/or renal tubule markers, suggesting bone resorption was related to cadmium exposure level and/or renal tubule dysfunction in both genders. There was an inverse relationship between bone urinary deoxyxypyrrolidinoline and urinary β2-microglobulin observed in women, unexpectedly. Such negative correlation between renal dysfunction and deoxyxypyrrolidinoline was also shown by Coen et al. (2000) and Aoshima et al. (2003) with undetermined etiology.

Urinary NTx, a metabolite of the N-terminal of mature collagen, was positively correlated with increased cadmium exposure levels and β2-microglobulin in both genders, this bone marker might be a more sensitive indicator of abnormal bone metabolism than deoxyxypyrrolidinoline.

Calcium reabsorption could have been affected in cadmium-exposed subjects, because renal tubules play an important role in controlling the balance of calcium (Staessens et al. 1991; Kido et al. 1993; Hayashi et al. 2003). Serum calcium and urinary calcium were used to determine the status of calcium metabolism in cadmium exposed subjects.

Serum calcium is normally tightly regulated, whilst urinary calcium is affected by the calcium intake in food and water (Limpatanachote 2007). The ratio of serum calcium to urinary calcium (fractional excretion of calcium or FECA) was used to determine the calcium handling efficiency of the kidney more precisely than serum and urinary calcium (Druke and Lacour 2003). Studies in Japan (Kido et al. 1993) showed there was a positive relationship between FECA and cadmium exposure.

In this study, urinary NTx was highly correlated with FECA, independent of cadmium exposure indices (shown by the unstandardized coefficient greater than 0.200 in Tables 4 and 5). This result showed accelerated bone resorption (indicated by high NTx) in the Mae Sot population was related to impairment in calcium reabsorption (indicated by high FECA ratio).

Women are more likely vulnerable to bone metabolic dysfunction than men because of hormonal status, menstruation, and pregnancy. In this study, the mean urinary deoxyxypyrrolidinoline and NTx levels in women were significantly higher than in men and exceeded the level for increased bone fracture risk (Table 1). The unstandardized positive coefficient between gender and all bone markers indicates women are at higher risk of accelerated bone remodeling than men.

This study showed increased levels of bone remodeling markers were related to cadmium exposure. The FECA was increased in the high cadmium exposure group and showed a strong positive relation to bone resorption marker levels independent of age or cadmium exposure.

These results support the hypothesis that chronic dietary cadmium exposure caused an imbalance in calcium handling which was an explanatory factor in accelerated bone resorption, particularly in women.

Conclusion

Inhabitants of the Mae Sot district in Thailand, who had been exposed to elevated levels of dietary cadmium, were screened for blood and urinary cadmium levels. Those with elevated urinary cadmium were also shown to have impaired renal tubular function and increased bone resorption. As calcium reabsorption by renal tubular cells was reduced, this population, especially the women, face a greater than average risk
Discussion

Bone formation and resorption are bone remodeling mechanisms which can be indicated using osteoblast and osteoclast cell markers. Bone marker determination is recognized as a non-invasive and comparatively inexpensive tool for assessing metabolic bone diseases. It is widely used to detect changes in bone metabolism of people at risk (Seibel 2005). In osteoporosis patients, bone markers can detect response to treatment earlier than bone mineral density measurements (Garrero 2000).

Increased serum bone alkaline phosphatase and/or osteocalcin have been reported in Japanese whose urinary cadmium was $\geq 10$ μg Cd/g creatinine (Kido et al. 1991; Aoshima et al. 2003). In our subjects, even though serum osteocalcin levels were within the reference range of 3.1–12.7 μg/ml (Yajima et al. 2003), serum osteocalcin was positively correlated with elevated levels of urinary β2-microglobulin and NAG in both men and women (Table 3). Serum osteocalcin was also positively correlated with blood cadmium (Table 5), but showed no relation to urinary cadmium (Table 4). These findings suggest the observed increase in osteocalcin was due to renal dysfunction, independent of age and cadmium body burden.

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Serum calcium is normally tightly regulated, whilst urinary calcium is affected by the calcium intake in food and water (Limpatanachote 2007). The ratio of serum calcium to urinary calcium (fractional excretion of calcium or FECa) was used to determine the calcium handling efficiency of the kidney more precisely than serum and urinary calcium (Druke and Lecour 2003). Studies in Japan (Kido et al. 1993) showed there was a positive relationship between FECa and cadmium exposure.

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of increased risk of osteopathy. A follow-up health program should be implemented in the Mae Sot community, to explain and address this increased risk of bone disease.

Acknowledgement

We thank all subjects for their participation. Thanks also to Dr Peter R. Hawkins for proofreading the manuscript and for his valuable comments. Kowit Nambunmee is studying for a Thai doctoral degree, funded by the Strategic Scholarship for Frontier Research Network of the Commission on Higher Education, Thailand. This work was funded by the Department of Public Health, Kanazawa Medical University, Japan.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

References


CURRICULUM VITAE

Name       Mr. Kowit Nambunmee
Date of Birth     July 13, 1980

Educational Background

2005-2002       Toxicology (M.Sc.), Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand

2001-1998       Occupational Health and Safety (B.Sc.) (Second Class Honor), Faculty of Public Health, Khon Kaen University, Khon Kaen, Thailand

Scholarships

- Post-graduate Research Fellowship Scholarship
  Source: Department of Public Health, Kanazawa Medical University, Japan
  Duration: 2007-2008

- Strategic Scholarships for Frontier Research Network Ph.D scholarship
  Source: Office of the Higher Education Commission, Thailand
  Duration: 2006-2010

- Graduate Study Support
  Source: Faculty of Medicine, Chiang Mai University, Thailand
  Duration: 2003

- Outstanding Bachelor Degree Student Support Scholarship
  Source: Siam Commercial Bank, Khon Kaen, Thailand
**Professional Experiences**

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<td>Certificate in “ISO 17025: International Standard for Laboratory” Department of Forensic Medicine, Faculty of Medicine, ChiangMai University, Thailand</td>
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<tr>
<td>Sep 2007</td>
<td>Certificate in Effect of Environmental Cadmium Exposure on Bone and Calcium Metabolism in Thai and Japanese, June 25-September 21, 2007, Department of Public Health, Kanazawa Medical University, Japan.</td>
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<tr>
<td>May 2005</td>
<td>Certificate in Toxic Algae and Toxic Substance in Fresh Water, May 18-20, 2005, Faculty of Science, Chiang Mai University, Chiang Mai, Thailand</td>
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<tr>
<td>Feb 2005</td>
<td>Certificate in Biomolecular Technique in the Analysis of Human Gene Polymorphism, February 24-25, 2005, Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand</td>
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Publications

Jan 2010  

Dec 2009  

Dec 2009  

Sep 2009  


Academic Meeting of Department of Public Health, Kanazawa Medical University, Japan 2008.


Oct. 2004 Nambunmee K., Bhoopat T, Jarusuraisin N, Steger HF. “Leptin Gene Polymorphism in Alcoholic Patients: A Preliminary Study”. The 4th Joint Seminar on Biomedical Sciences with Prince of Songkla University, Chiang Mai University, Kunming Medical College and
Honours