Cadmium Bioavailability from Vegetable and Animal-Based Foods Assessed with *In Vitro* Digestion/Caco-2 Cell Model

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Background: Chronic dietary cadmium (Cd) exposure results in kidney dysfunction and decrease in bone mineral density. **Objective:** To determine and compare the bioavailability of Cd from vegetable and animal-based foods.

Material and Method: Caco-2 cells were exposed to Cd in boiled pig kidney, ark shell, kale, raw kale, mixed boiled pig kidney with raw kale and CdCl₂ after in vitro digestion. Then cellular Cd uptake from the digests and reference CdCl₂solution was measured by atomic absorption spectrometry.

Results: Cd bioavailability from animal-based foods was higher than that from vegetable-based foods. In addition, raw kale exhibited an inhibitory effect on Cd bioavailability when mixed with boiled pig kidney. However, Cd in kale was increasingly absorbed after boiling.

Conclusion: Cd binding to different molecular species, other food components in vegetable and animal-based foods, food combination, as well as cooking processes influenced the uptake of dietary Cd. A relative bioavailability factor accounted for the food matrix might be necessary for exposure assessment and consequently for estimation and prevention of the risk of dietary Cd.

Keywords: cadmium, bioavailability, Caco-2 cells, vegetable, animal-based foods

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Cadmium (Cd) is a highly toxic metal of worldwide concern in the environment. Non-smoking general population are exposed to Cd via food. Chronic dietary Cd exposure leads to kidney dysfunction and decrease in bone mineral density⁽¹⁾. Crops grown in Cd-contaminated areas in Tak Province, Thailand, had been found to contain elevated cadmium contents. The higher urinary Cd was found in the residents who mainly consumed rice grown locally in the polluted area compared to those who did not⁽²⁾. A positive correlation between levels of urinary cadmium and markers of renal dysfunction, β_2 -microglobulin (β 2-MG), N-acetyl- β - D-glucosaminidase (NAG) and

Kaojarern S, Department of Medicine, Faculty of Medicine at Ramathibodi Hospital, Mahidol University, Rama VI Rd, Bangkok 10400, Thailand. Phone: 0-2201-1610 E-mail: raskj@mahidol.ac.th total protein in adult populations exposed to high level Cd had been reported^(3,4). Cereals, vegetables and shellfish contribute to more than 80% of the estimated total cadmium intake⁽⁵⁾. Total Cd intake may exceed provisional tolerable daily intake (PTDI) for consumers who have high total food consumption or for some individuals who live very close to contaminated areas or who habitually consume diets high in Cd⁽⁶⁾. Meat products, especially liver and kidney, are high in Cd and are able to contribute to Cd intake approximately twice the provisional tolerable weekly intake (PTWI)⁽⁷⁾. However, not only the quantity but also the bioavailable fraction of Cd from diet determines Cd body burden that is relevant to Cd toxicity⁽⁸⁻¹¹⁾.

In order to increase confidence in the estimation of risk due to dietary Cd exposure, studies should be performed on the bioavailability of cadmium from specific foods and on the factors that affect bioavailability⁽¹²⁾. As the information regarding

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to effect of food matrices on Cd bioavailability from food is very limited, it is of interest, to assess and compare the bioavailability of Cd from vegetable and animal-based foods by means of *in vitro* digestion combined with Caco-2 cell uptake system. In addition, the effects of mixed food and cooking process on Cd bioavailability were also investigated.

Material and Method

Preparation of food samples

Ten different food items selected from the three major food groups of Cd contributors, including vegetable, offal, and shellfish, were determined for Cd contents. Kale, pig kidney and ark shell were selected as representative of each Cd contributing food group due to their high Cd levels. Two samples of pig kidney and ark shell were purchased locally from each of five markets. The edible portion of pig kidney and the meat of ark shell were washed thoroughly and boiled in de-ionized water for 2.5 min before homogenizing with a kitchen blender. The same quantity of ten slurries of boiled pig kidney and ark shell were mixed to make a pooled sample of each kind of food. These pooled samples were freeze-dried, re-homogenized in a Waring blender, and subsequently passed through a 0.4 mm Teflon sieve. The powder samples were kept in an acid-washed polypropylene bottle.

To obtain a vegetable with high level of intrinsic Cd as in animal-based foods, kale was grown by using the floating hydroponic system in 0.15 ppm Cd supplemented nutrient solution for two weeks. The edible portions of kale were washed with de-ionized water before dividing each part of the vegetable into two identical halves. The first half was prepared as the raw sample and the other was boiled in de-ionized water for five minutes. All vegetable samples were prepared similarly to the pig kidney and the ark shell. Finally, Cd-supplemented and non-Cd supplemented freeze-dried hydroponic kales were mixed in the ratio of 1:1 to achieve a comparable cadmium concentration in vegetable and non-vegetable foods.

The pooled samples were examined for homogeneity by analyzing Cd concentrations of the sample aliquots from vegetable and animal-based foods.

In vitro digestion procedure

The protocols were done according to the method of Miller et al⁽¹³⁾ with a modification in amount of digestive enzymes to provide food digests containing low enzyme residues and not causing any damage to the cells.

Approximately 0.5 g of food sample was weighed into a 50 ml polypropylene tube containing 3 ml of de-ionized water. The pH of the mixture was brought to 2 with 5 M HCl before adding 0.25 ml of pepsin solution (2 g pepsin dissolved in 50 ml of 0.1 M HCl) and then incubated for 3 hours at 37°C in a shaking water bath at 120 rpm to simulate gastric digestion. Following incubation, pH was raised to 5 by slow addition of 1 M NaHCO, drop wise and 1.25 ml pancreatin-bile mixture (0.5 g pancreatin and 3 g bile extract dissolved in 250 ml of 0.1 M NaHCO₂) was added. The pH was adjusted to 7.4 by addition of 1M NaOH and the final volume was made up to 10 ml with deionized water. The sample was again incubated at 37 C for 4 hours to mimic duodenal digestion. Shortly after removal from the shaking water bath, each digest sample was placed on ice to stop pancreatic activity and then was subjected to centrifugation at 10,000g, 4°C for 1 hour. The supernatant was diluted 1:2 with the Hank's balanced salt solution (HBSS) and de-ionized water to adjust the osmolarity to $290 \pm 10 \text{ mOsm/ kg H}_{2}$ O. The centrifugation was repeated; the final supernatant was collected and sterilized through the 0.22 µm filters. The aliquots of all digest supernatants were analyzed for Cd concentrations by Zeeman atomic absorption spectrophotometer Spectr AA-400 (Varian, Victoria, Australia).

Cell culture

Human intestinal Caco-2 cells were grown and maintained in Dulbecco's Modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% non-essential amino acids and 1% penicillinstreptomycin at 37°C, 95% air and 5% CO₂. Cells of passage 44-45 were used. At 80-90% confluence, cells were trypsinized and seeded onto 60 mm culture dishes at a density of 15,000 cells/cm². At 21 days post-seeding, Caco-2 cells differentiated into enterocyte-like cells that were used in Cd bioavailability (uptake) experiments⁽¹⁴⁾. All cell culture reagents were purchased from GIBCO (Grand Island, NY, USA).

Cadmium uptake by Caco-2 cells

Cd uptake experiments were carried out with the 21 days post-seeding Caco-2 cells. Culture medium was removed and the cell monolayers were washed once with HBSS. Then the food digest supernatant was applied onto the apical surface of the monolayers. The 150 μ l aliquots of supernatant were taken to determine lactate dehydrogenase (LDH) activity using the TOX-7 cytotoxicity kit (Sigma, MO, USA) immediately after applying on cell monolayers. After 120 min of incubation at 37°C in an incubator shaker, the 150 μ l aliquots of applied solution were collected again for LDH activity at the end of the experiment, the remainders were discarded and the monolayers were washed twice with ice-cold HBSS. The cells were then detached from dishes by adding 1.2 ml of 0.1% Triton-X and pipetting up and down. Two hundred μ l of 1 M NaOH was added into 800 μ l of cell solution to solubilize the cells. The cell solutions were analyzed for Cd concentrations by graphite-furnace atomic absorption spectrometer. The remaining cell solution was used for protein determinations by Bradford assays (Sigma) with bovine serum albumin as the calibration standard.

Two bioavailability experiments were performed by the measurement of cadmium uptake from CdCl₂ solution and the digests of boiled pig kidney, ark shell, kale, raw kale, mixed boiled pig kidney with raw kale and CdCl₂. From two experiments, six monolayers per food digest were analyzed. Intracellular Cd uptake was expressed as the percentage of ng Cd loaded/mg cell protein.

Analysis of cadmium

Lyophilized food samples were digested by heating with concentrated nitric acid in Teflon vessels. Metals in digested solutions were then formed complexes with ammonium pyrroridine dithiocarbamate (APDC) before extraction by chloroform. Finally, Cd was back-extracted into diluted nitric acid. Cd concentrations in the acidic extract from foods and the cell solutions were determined by using a graphitefurnace atomic absorption spectrophotometer (GFAAS) with the Zeeman Effect (Varian). The quality control and validation of Cd analysis procedure were performed by measurement of Cd contents in reference material OAC119 (seafood mix) in parallel with the food samples. The result (mean \pm SD) from the present study was 14.70 ± 1.31 mg/kg that showed good agreement with the certified value of 13.9 ± 1.2 mg/kg.

All lab wares were washed and then soaked overnight in 15% nitric acid. After three times rinsing with de-ionized water, they were dried in a hot air oven before placed in closed containers.

Statistical analysis

The differences between Cd contents of freeze-dried food samples as well as cellular Cd uptakes from different digests and $CdCl_2$ solution were determined by one-way ANOVA test. Scheffe's multiple comparison tests were then used for testing the

differences between group means. In the integrity control experiment, the nonparametric Wilcoxon signed rank test was used to analyze changes in LDH release during 120 min of incubation period. The level of significance was set at p < 0.05.

Results

Food samples

As shown in Table 1, based on three food groups of cadmium contributors (vegetable, offal and shellfish); kale, pig kidney and ark shell contained relatively high Cd. Hence, they were selected to be used in the Cd bioavailability study.

Aliquots of each lyophilized pooled sample were determined for Cd contents. It was found that the boiled pig kidney sample had significantly higher Cd level than the others (Table 2). The mean and standard deviation of Cd concentrations of the sampling aliquots indicated that all lyophilized pooled samples were homogeneous.

Table 1. Concentrations of cadmium in different foods⁺

Samples	mg Cd/ kg (wet wt)
Cabbage	< 0.01
Kale	0.04
Lettuce	< 0.01
Ginger	< 0.01
Mushroom	< 0.01
Pig liver	0.18
Pig kidney	3.28
Sea water mussel	0.31
Ark shell	0.84
Crab meat	0.03

+ Values are averages of two determinations

 Table 2. Concentrations of cadmium in lyophilized pooled samples

Samples	mg Cd/ kg (dry wt)	
Boiled pig kidney ⁺ Boiled ark shell ⁺ Boiled kale ⁺⁺ Raw kale ⁺⁺	$\begin{array}{c} 9.39 \pm 0.67^{a} \\ 7.42 \pm 0.42^{b} \\ 8.13 \pm 0.02^{b} \\ 7.85 \pm 0.22^{b} \end{array}$	

 $^+$ Values are means \pm SD for ten determinations

⁺⁺ Values are means \pm SD for three determinations

 $^{a, b}$ Mean values with unlike superscript letters indicate significantly different: p < 0.05

Integrity of the monolayer

For Cd uptake experiments the test chemicals have to be solubilized and stabilized in a solution that is not harmful to the cells or the barrier function of monolayer. To define nontoxic conditions of Cd uptake, the effects of experimental conditions were evaluated according to cell monolayer integrity and cell injury. As shown in Fig. 1, the observation under an inverted microscope illustrated that Caco-2 cells tolerated the digest supernatants prepared by protocol described. The monolayers incubated with all digest supernatants remained intact without any signs of damage.

The LDH release before and after treatment was not significantly different (Fig. 2). These results indicated that the integrity of cell membrane was not compromised over the experimental condition for up to 120 min exposure time.

Bioavailability experiments: Uptake of Cd by Caco-2 cells

The Cd uptake in the Caco-2 cells incubated with supernatants of different foods and CdCl₂ digests varied between 0.65-3.10% ngCd loaded/mg cell protein and were significantly lower than the uptake of inorganic CdCl₂. The mean percentage of Cd uptake from boiled pig kidney was the highest but not significantly different from those of CdCl₂, mixed boiled pig kidney with raw kale and ark shell digests. A significantly higher Cd uptake was only found in the boiled pig kidney as compared to the kale digests (Table 3).

 Table 3. Cd uptake in Caco-2 cells after 2 h of incubation with CdCl, solution and supernatant of the digests

Samples	% Uptake+
CdCl,	8.13 ± 0.64^{a}
Boiled pig kidney	$3.10\pm0.47^{\mathrm{b}}$
CdCl, digest	$2.56\pm0.24^{\rm bc}$
Mixed boiled pig kidney and raw kale (1.5:1)	$1.62 \pm 0.28^{\mathrm{bc}}$
Boiled ark shell	$1.08 \pm 0.31^{\text{bc}}$
Boiled kale	$0.96 \pm 0.15^{\circ}$
Raw kale	$0.65 \pm 0.05^{\circ}$

⁺ Values are expressed as percentage of ngCd loaded/ mg cell protein; each value represented the mean <u>+</u> SEM of six monolayers from two independent experiments









Fig. 2 LDH releases into the treated solutions at the start (T_0) do not differ significantly from those at the end of 120 min incubation period (T_{120}) . Data are the means \pm SD of three monolayers

Cadmium uptake ratio demonstrates the available fraction of Cd obtained from the foods and CdCl₂ digests relative to inorganic CdCl₂. The relative Cd uptake, which reflects Cd bioavailability, was ranked from boiled pig kidney > CdCl₂ digest > mixed boiled pig kidney and raw kale > boiled ark shell > boiled kale > raw kale (Fig. 3).

Discussion

The selected food samples, pig kidney, ark shell, and kale contained high Cd contents and were commonly used ingredients in many Thai dishes. Pig kidney is the main ingredient in several Thai and Chinese recipes. Some people believed that pig kidney could promote the renal function and blood circulation.



Fig. 3 Cadmium uptakes in Caco-2 cells express as Cd uptake ratios between the food/CdCl₂ digests and the reference CdCl, solution

Cd intake from one tablespoon or 30 g of pig kidney may exceed the safe level of Cd 1 μ g/kg BW/day. Kale is a very popular vegetable that is frequently consumed by Thai people. Comparing the relative bioavailability of Cd from different sources had to be carried out under the same experimental conditions in samples containing similar Cd concentration. Growing kale by hydroponic system in Cd containing nutrient solution increased intrinsic Cd in kale to a level comparable to animal-based foods.

The human Caco-2 cell line has been extensively used as a model of the intestinal barrier because of the origin from human colon adenocarcinoma. After confluence, cells spontaneously differentiate and express several morphological and functional characteristics of mature enterocytes⁽¹⁵⁾.

From the present study, the cellular Cd uptake of inorganic Cd from $CdCl_2$ solution was significantly higher than that of the soluble Cd from the supernatants of food or $CdCl_2$ digests. It was demonstrated that organic Cd-GSH (glutathione) and Cd-hmPC₃ (phytochelatin) that exist in animal and plant based foods were taken up by Caco-2 cells more slowly than free Cd ion⁽¹⁶⁾. The tissue retention of Cd in rats fed Cd in the form of crab hepatopancreas or crabmeat and mushroom was significantly lower compared to rats fed CdCl₂ in standard diet^(17,18).

The Cd uptake ratios, which indicated the absorbed fraction of Cd from digest supernatants relative to inorganic CdCl₂, from animal-based foods and digested inorganic Cd tended to be higher than the values from boiled and raw vegetable. However, a significantly higher uptake was only found in boiled pig kidney compared to boiled and raw kale. The result of higher Cd uptake from animal food sources compared to vegetables in Caco-2 cells agrees with previous animal and human studies^(19,20).

The speciation of metals in foods in the digestive tract greatly facilitates the prediction of their bioavailability from foods⁽⁸⁾. It was shown that in cooked pig kidney, Cd bound to metallothionein (MT) like protein, which was heat stable and survived *in vitro* simulated gastrointestinal digestion⁽²¹⁾. Therefore, after the simulated gastrointestinal digestion of boiled pig kidney, free Cd²⁺ and the various forms of Cd-protein complexes including Cd bound to metallothionein (MT) like protein might present in the supernatant of the digest solution. Unlike Cd in pig kidney, Cd contaminated in shellfish such as oyster, especially from environment was bound to high molecular weight proteins, but not MT^(22,23).

Several amino acid and oligopeptide transporter proteins expressed in Caco-2 cells were similar to the native intestinal carrier proteins⁽²⁴⁾. A number of specific mechanisms involved in the absorption of different forms of Cd from dietary protein have been proposed⁽²⁵⁾. Therefore, in addition to Cd²⁺, Cd bound to non-MT or MT like protein and other Cd binding proteins may be taken up into Caco-2 cells by the mechanisms similar to those exist in the native enterocytes. However, the differences in the cellular uptake of various Cd-protein complexes in Caco-2 cells had been illustrated⁽²⁶⁾. Therefore, the difference in the residual Cd complexes following in vitro digestion may lead to the difference in fraction of Cd available for absorption of pig kidney and ark shell meat.

The concentrations of phytochelatins (PCs) which are small peptides synthesized by plants exposed to high concentration of metals increased in lettuce grown in a flow-through hydroponics (FTH) system relative to Cd levels in the nutrient solution⁽²⁷⁾. However, Cd bound to non-PCs peptides or high molecular weight proteins were also found in the Cd contaminated vegetables⁽²⁸⁾. In terms of Cd binding non-peptides or non-proteins, Cd-amino acids, Cd-glutathione, Cd-phytate and Cd bound to dietary fiber had been isolated from vegetables⁽²⁹⁾. Regarding Cd species found in vegetables, Cd-amino acid, Cd-glutathione and Cd-PCs had shown to be taken up into the enterocytes or Caco-2 cells with even less efficiency than Cd^{2+(16,25)}.

Leafy vegetables, like other plant-based diets, contain natural inhibitors of mineral absorption such as tannin and phytate. The interactions between phytate and heavy metals such as Cd and Pb had been shown *in vivo* as well as *in vitro*⁽³⁰⁾. Phytate binds minerals and reduces fraction available for absorption. Tannin forms complexes with enzyme proteins results

to decreased food digestibility and consequently minerals released from food. Thereby, these inhibitors may take part in the small uptake of Cd from supernatant of kale digests. However, after five minutes boiling, the uptake of Cd from kale tended to increase. Lewu et al⁽³¹⁾ demonstrated that five minutes boiling caused 28-61% and 17-41% reduction in tannin and phytate contents of leafy vegetables. The lower level of inhibitor, the greater fraction of Cd was available for absorption by Caco-2 cells.

The effect of food combination was evaluated in mixed raw kale and pig kidney, the Cd uptake by Caco-2 cells was much lower than expected when the fractions of uptake from the composed foods were added up. The differences between predicted and observed values indicated the interaction between Cd and the food components in pig kidney and raw kale. The value less than the predicted absorbed fraction illustrated the pronounced effects of the inhibitory factors in raw kale. This result points out the importance of vegetable foods in terms of prevention of health hazard from Cd ingested as mixed diets in a real situation.

In conclusion, from the present study, the Cd bioavailability determined by using in vitro digestion combined with Caco-2 model showed less bioavailability of Cd from foods than from inorganic Cd, and a higher uptake of Cd from the soluble fraction of pig kidney relative to kale. These results are consistent with reports from in vivo studies and may be due to the presence of different Cd speciations and the other components such as enhancers or inhibitors of Cd uptake in each food. The Cd uptake from the mixed food suggests that the interaction or exchange between Cd and ligands in each food digest affect the intestinal Cd uptake. Furthermore, the cooking process can alter Cd bioavailability from food. These results may be useful in the management and prevention of risk from dietary Cd exposure, especially in countries where foods with high Cd contents such as liver or kidney of animals or shellfish can contribute to the significantly high Cd intake. Further studies relating to Cd bioavailability from more varied food items, the role of essential elements, and the effects of inhibitors or enhancers or cooking processes should be carried out to additionally clarify the effects of dietary factors on Cd bioavailability.

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Potential conflicts of interest

None.

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การประเมินชีวปริมาณออกฤทธิ์ของแคดเมียมจากผักและเนื้อสัตว์โดยการย[่]อยในหลอดทดลอง และดูดซึมโดยเซลล์ Caco-2

รจนา ชุณหบัณฑิต, ทรงศักดิ์ ศรีอนุชาต, อานนท์ บุณยะรัตเวช, รัชนี คงคาฉุยฉาย, จุฑามาศ สัตยวิวัฒน์, สมิง เก่าเจริญ

ภูมิหลัง: การได้รับแคดเมียมปนเปื้อนอยู่ในอาหารเป็นเวลานานส[่]งผลให้การทำหน้าที่ของไตผิดปรกติ และ ความหนาแน่นของมวลกระดูกลดลง

วัตถุประสงค์: การศึกษาครั้งนี้มีวัตถุประสงค์เพื่อประเมินและเปรียบเทียบชีวปริมาณออกฤทธิ์ หรือ สัดส[่]วนของ แคดเมียมที่ถูกดูดซึมจากอาหารประเภทผักและเนื้อสัตว์

วัสดุและวิธีการ: ใช้เซลล์ Caco-2 เป็นแบบจำลองดูดซึมแคดเมียมจากไตหมู เนื้อหอยแครง ผักคะน้ำที่ต้มแล้ว ผักคะน้ำดิบ ไตหมูต้มผสมผักคะน้ำดิบ และสารแคดเมียมคลอไรด์ (CdCl) ที่ผ่านการย่อยเลียนแบบการย่อยอาหาร ในกระเพาะอาหารและลำไส้ในหลอดทดลอง วัดปริมาณแคดเมียมด้วยเทคนิค atomic absorption spectrometry นำเปอร์เซ็นต์การดูดซึมแคดเมียมจากอาหารและ CdCl ที่ผ่านการย่อยแล้วมาเปรียบเทียบกันหลังจากคำนวณ เป็นอัตราส่วนของการดูดซึมแคดเมียมในรูปของสารละลาย CdCl ที่ใช้เป็นสารอ้างอิง

ผลการศึกษา: เซลล์ Caco-2 ดูดซึมแคดเมียมจากอาหารเนื้อ[้]สัตว์ได้มากกว่าแคดเมียมที่อยู่ในผัก นอกจากนี้ ผักคะน้ำดิบยังให้ผลยับยั้งการดูดซึมของแคดเมียมโดยรวมเมื่อผสมกับไตหมูต[้]ม แต่ในผักคะน้ำที่ผ่านการต^{ุ้}มแล้ว แคดเมียมในผักจะถูกดูดซึมได้ดีขึ้น

สรุป: ความแตกต่างของสารประกอบแคดเมียม องค์ประกอบของสารอื่นในอาหารแต่ละชนิด ปฏิกิริยาของแคดเมียม กับสารเหล่านี้ ตลอดจนการปรุงอาหารต่างก็มีผลกับชีวปริมาณออกฤทธิ์ของแคดเมียมในอาหารทั้งสิ้น ข้อมูลเหล่านี้ อาจจะเป็นประโยชน์ในการประเมินการสัมผัสสารแคดเมียมจากอาหาร ซึ่งสำคัญต่อการประเมินความเสี่ยง และป้องกันอันตรายจากแคดเมียมที่ปนเปื้อนอยู่ในอาหาร