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Cadmium Contamination and Health Assessment in Frog *Microhyla fissipes* Living Downstream of Zinc Mining Area in Thailand

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Abstract

In Mae Sot District, Tak Province, Thailand, concerns have been raised over cadmium contamination, potentially due to zinc mining activities. Although there is no report of acute toxicity on animals in this area, the impact of long-term environmental exposure to cadmium on their health are of attention. Water and sediment samples collected from two field sites (low-Cd and high-Cd sites) in Mae Sot during 2008 were analyzed by GFAAS. Year round cadmium contamination in water ranged from 0.0015-0.002 mg/L in low-Cd site to 0.0019-0.0023 mg/L in high-Cd site, while higher levels were found in sediment ranged from 0.1013-0.2206 mg/kg in low-Cd site to 2.9260-3.2888 mg/kg in high-Cd site. *Microhyla fissipes* was collected from each habitat in 2-month interval during wet season. Detectable level of cadmium residue was found only in the frog collected from high-Cd habitat. Gravimetric analysis showed that hepatosomatic indices were significantly higher in high-Cd habitat. Histopathology showed several similar alterations in the liver, however higher number of melanomacrophage center was found in high-Cd habitat. Renosomatic indices and kidney tissue alterations were not significantly different between two sites. Reproductive health in term of gonadosomatic indices (GSI) was not significantly different between male frogs from both habitats. But in the females living in high-Cd habitat, significantly lower GSI were observed. The results indicate that exposure to environmentally relevant dose of cadmium may interfere with the frog health. Using the frog as a sentinel species in this study suggests an important implication for overall health of animals/human in this area.

Keywords: metal; histopathology; kidney; liver; sentinel species

1. Introduction

Cadmium is a heavy metal contaminated in environment and dispersed as a result of pollution from activities such as industries and mineral mining (Liu *et al*., 2008). Cadmium can be accumulated and cause toxic effects on aquatic and terrestrial ecosystems (Muntau and Baudo, 1992). It is a toxic metal known to cause health effects, antifertility effects, carcinogenic effects, teratogenic effects and mutagenic effects in animals (Degraeve, 1981). Among different target organs, liver and kidney have been frequently reported as the organs adversely affected by cadmium (Friberg, 1984; Dudley *et al*., 1985). Moreover, cadmium is a known endocrine disruptor (Henson and Chedrese, 2004; Vetillard and Bailhache, 2005). Exposure to low levels of cadmium may induce changes in reproductive endocrine axis resulting in effects on animal reproduction. Animals exposed to or lived in a cadmium contaminated environment are thus susceptible to be affected. Reproductive-endocrine effects of cadmium on many species have been reported (Vetillard and Bailhache, 2005).

In Mae Sot District of Tak Province, northwestern part of Thailand, people are facing health and economic problems caused by cadmium contamination in their agricultural areas. The contaminated sediment plume of cadmium is resulted from runoff and natural irrigation system that leach cadmium from zinc mining area. Levels of cadmium contamination in soil ranged from 0.5 to 284 mg/kg which is 0.17-94 times of the EU maximum permissible soil cadmium concentration of 3.0 mg/kg (Simmons *et al*., 2005). Since contamination in the area is associated with suspended sediment transported to the field by irrigation supply, this could lead to contamination and compromised health of aquatic and semi-aquatic animals in the affected stream. A previous study reported that cadmium residues (0.180-0.549 mg/kg) were found in tissues of rice frog *Fejervarya limnocharis* caught from a potentially contaminated area in Mae Sot District (Othman *et al*., 2009). Although there is no report of acute toxic effect on animals in this area, the impact of long-term exposure to environmental cadmium contamination on their health are of attention.

This study aims to determine the levels of cadmium contamination in environment and in the frog *Microhyla fissipes* living in the cadmium contaminated areas of Mae Sot District, Tak Province, Thailand. This frog is a candidate sentinel species because it is a small vertebrate commonly found in this area. Frogs could be exposed to cadmium contamination throughout their lifespan via skin penetration and dietary intake (Othman *et al*., 2009). Moreover, among small vertebrates in the area, this species is abundant and can be found throughout the wet season. In this study, health status of this frog was evaluated by gravimetry and histology of vital organs including liver, kidney and gonad.

2. Materials and Methods

2.1. Study sites

Two potentially cadmium-contaminated sites in Mae Sot District, Tak Province, Thailand (low-Cd: Huay Luek creek and high-Cd: Mae Tao creek) were chosen in this study. Mae Tao creek (high-Cd site; Simmons *et al*., 2005) is a major irrigation stream for agricultural activities of Mae Sot District. Part of Mae Tao creek pass through a zinc mining area where cadmium is believed to be released to the aquatic environment due to mining activities. The elevated levels of cadmium in the paddy soils (0.5 to 284 mg/kg) and rice grain grown in vicinity of Mae Tao creek downstream of the zinc mining area was reported (Simmons *et al*., 2005). The sampling site at Mae Tao creek (high-Cd site) is located in the potentially contaminated area. Huay Luek creek (low-Cd site) located 10 kilometers northeast of Mae Tao creek is on a different tributary basin and not on the path of cadmium-contaminated sediment plume. By this reason this site was chosen as a reference site in this study.

2.2. Water and sediment collections

In 2008, water and sediment samples were collected from the two study sites in Mae Sot District, Tak Province. Water and sediment samples were transported on ice to laboratory at Chulalongkorn University and kept at 4°C until use.

2.3. Contaminant analysis for cadmium

Water samples were collected in plastic bottles and transported to the laboratory on ice. Fifty milliliters of the water samples were added to the microwave vessels. Then concentrated nitric acid was added into each vessel. Samples were then microwaved (980 kW) at 160°C to 165°C (US EPA Method 3015) using EthosPro Microwave Digestion Labstation. The digested samples were cooled to room temperature before they were filtered. Then distilled water was added to the sample to volume. Sample blank (distilled water) was also subjected to the same microwave procedure. Samples were then analyzed for cadmium content with graphite furnace atomic absorption spectrometer (GFAAS ZEEnit 700, Analytik Jena AG) with the digested distilled water as blank. Cadmium concentrations were determined in the unit of mg/L. Concentration of cadmium in the sample was then calculated into the unit of mg/L of sample.

Sediment samples were collected in plastic bags and put on ice before they were transported to the laboratory. The sediment samples were dried at room temperature to a constant mass. The dried sediment was homogenized by hand grinding with mortar and pestle. Extraneous organic matter was removed during grinding and sifting through a 2 mm-mesh size sifter. About 0.5 g of the dried and sifted sediment was weighed into each of the microwave vessels. Then concentrated nitric acid was added to the samples before the vessels were secured. Samples were then microwaved (980 kW) at 175^{o}C (US EPA Method 3051). The digested sediment samples were cooled to room temperature before filtering. Distilled water was added to dilute the samples to volume. Sample blank (distilled water) was also subjected to the same microwave procedure. Sediment samples were then analyzed for cadmium content with GFAAS with the digested distilled water as blank. Cadmium concentrations were determined by in the unit of mg/L. The concentration of cadmium in the sediment was then calculated into the unit of mg/kg dry weight.

Each whole-body frog sample was oven-dried at 80^{o}C overnight. A mixture of concentrated nitric acid and hydrogen peroxide in the ratio of 7:1 was added to the sample in microwave vessels. The sample was then microwaved (980 kW) at 200^{o}C. The digested tissue sample was cooled to room temperature before filtered. Distilled water was added to dilute the sample volume. Sample blank (distilled water) was also subjected to the same microwave procedure. Tissue sample was analyzed for cadmium content with GFAAS in the unit of mg/L. The concentration of cadmium in the tissues was then calculated into the unit of mg/kg dry weight.

2.4. Animal collection and gravimetry

Adult *Microhyla fissipes* was collected by visual encounter method from each site in 2-month interval during wet season (June-November 2008). The sampling periods were divided into early, middle and late wet seasons. A total of five individuals were sampled from each site for whole-body contaminant analysis for cadmium. Data of the body, liver, kidney and gonad weighs were recorded and used to calculate the hepatosomatic index (HSI), renosomatic index (RSI) and gonadosomatic index (GSI). Liver, kidney and gonad specimens were dissected and fixed in 10% neutral buffered formalin for further histological analysis.

2.5. Histology

After all organ specimens were fixed in 10% neutral buffered formalin, they were dehydrated through a series of graded ethanol and processed through a standard method for paraffin section (Humason, 1967). The tissue sections were stained with hematoxylin and eosin. Histopathological alterations in liver and kidney tissues of the frogs were examined by light microscopy.

2.6. Statistical analysis

Gravimetric data including hepatosomatic indices (HSI), renosomatic indices (RSI) and gonadosomatic indices (GSI) were tested for normal distribution and homogeneity of variance. Two Way Analysis of Variance (ANOVA) followed by Student-Newman-Keuls multiple comparison were used to compare mean HSI, RSI and GSI between frog populations. Quantitative histological data were analyzed by Student's t-test. A significant difference is reported at $p < 0.05$. Statistical analyses were done using SigmaStat for Windows version 2.0.

3. Results and Discussion

Year round cadmium contamination in water from low-Cd site (Huay Luek creek) ranged from 0.0015 to 0.0020 mg/L and from high-Cd site (Mae Tao creek) ranged from 0.0019 to 0.0023 mg/L. Higher levels of cadmium contamination were found in sediment ranging from 0.1013 to 0.2206 mg/kg in low-Cd site and ranging from 2.9260 to 3.2888 mg/kg in high-Cd site which is higher than the EU maximum permissible soil cadmium concentration of 3.0 mg/kg (Simmons *et al*., 2005). The result shows that year round cadmium contamination level in sediment from Mae Tao creek (high-Cd site) is significantly higher than that of Huay Luek creek (low-Cd site). Detectable level of cadmium residue was found only in the frog samples collected from high-Cd habitat at 0.47 ± 0.07 mg/kg.

The results from gravimetric analyses of *M. fissipes* (Table 1) showed that mean of overall HSI in male frogs was significantly higher in the frog collected from high-Cd habitat (2.0769 ± 0.2958) compared to those of the low-Cd habitat (1.4462 ± 0.0772). But in females (Table 2), HSI were not significantly different between two populations. The higher HSI induced by cadmium was also reported in Sprague-Dawley rat after chronic exposure to $CdCl_2$ (Dudley *et al*., 1985) and in the frog *Rana ridibunda* after 4-day exposure to $CdCl_2$ (Vogiatzis and Loumbourdis, 1998). Changes of HSI have been reported as a biomarker responded to various environmental contaminations. It may indicate the disturbances at cellular and tissue levels that lead to alterations in the organ gross structure. The results of RSI were not significantly different between male frogs living in both habitats (Table 1). But in the females living in high-Cd habitat, significant increase in RSI was observed in middle wet season (Table 2). The higher RSI induced by cadmium was also reported in Sprague-Dawley rat after chronic exposure to $CdCl_2$ (Dudley *et al*., 1985).

Reproductive health in term of GSI was not significantly different between male frogs living in both habitats (Table 1). But in the females living in high-Cd habitat, lower GSI were observed with significant difference in middle wet season. Mean overall GSI were also significantly different between two habitats (Table 2). In Sprague-Dawley rats exposed to $CdCl_2$, percentages of testicular weight also did not change after the exposure (Dudley *et al*., 1985). In a study in medaka *Oryzias latipes*, male GSI was reported to decrease significantly after 7-week exposure to high dose of cadmium, but not in lower dose (Tilton *et al*., 2003). In contrast, other study reported that embryonic exposure to cadmium can increase male GSI in zebrafish *Danio rerio* (Wall, 2000). The result from this study indicates that exposure to low level of environmental cadmium can not affect the male GSI of *M. fissipes*. Because of cadmium is a known endocrine disruptor with estrogenic effects (Garcia-Morales *et al*., 1994; Henson and Chedrese, 2004), some studies focused on the effects in female GSI. A study in zebrafish reported the result similar to our study that embryonic exposure to cadmium can decrease female GSI (Wall, 2000). But in contrast, a study in medaka, female GSI did not change after 7-week exposure to high doses of cadmium (Tilton *et al*., 2003). These results together with ours may indicate that the female GSI is sensitive to low level of cadmium.

Histological analyses of liver tissues of *M. fissipes* collected from low environmental cadmium habitat revealed some histopathotological alterations including white blood cell infiltration, melanomacrophage centers

Table 1. Gravimetric data (mean ± S.E.M.) of male *Microhyla fissipes* caught from Mae Sot District, Tak Province, Thailand during June-November 2008

Parameters	Periods	Low environmental cadmium habitat	High environmental cadmium habitat
HSI	Early wet season	1.2965 ± 0.0937 (n=13)	1.6145 ± 0.2049 (n=12)
	Middle wet season	1.5540 ± 0.0637 (n=16)	1.9886 ± 0.4439 (n=4)
	Late wet season	1.4881 ± 0.3183 (n=3)	2.6276 ± 0.4951 (n=7)
	Overall	1.4462 ± 0.0772	2.0769 ± 0.2958 *
RSI	Early wet season	0.4500 ± 0.0377 (n=13)	0.3629 ± 0.0363 (n=12)
	Middle wet season	0.4280 ± 0.0221 (n=16)	0.4737 ± 0.0411 (n=4)
	Late wet season	0.3371 ± 0.0986 (n=3)	0.5375 ± 0.1287 (n=7)
	Overall	0.4050 ± 0.0345	0.4580 ± 0.0510
GSI	Early wet season	0.1890 ± 0.0332 (n=13)	0.1363 ± 0.0259 (n=11)
	Middle wet season	0.1883 ± 0.0141 (n=15)	0.1578 ± 0.0349 (n=4)
	Late wet season	0.2080 ± 0.0101 (n=3)	0.2278 ± 0.0839 (n=6)
	Overall	0.1951 ± 0.0064	0.1740 ± 0.0276

Remark: Significant site-related difference is indicated by an asterisk (*)

(MMCs), lipid droplet accumulation in hepatocytes, fatty degeneration, foci necrosis and fibrosis of the liver tissues (Fig. 1). These lesions were also observed in the frog collected from high environmental cadmium habitat but in higher degree of severity for MMCs and lipid accumulation (Fig. 2). In the hepatocyte of the frog from high-Cd habitat, higher degree of lipid droplet accumulation was found as a very large droplet accumulated in the cytoplasm resulting in fatty degeneration in some areas of liver parenchyma (Fig. 2B). From our results, formation of MMCs is a major histopathological alteration observed in these frog populations. Mean number of MMCs counted is 381.87 cells/mm^2 in the frog from low-Cd habitat and 672.53 cells/mm^2 in the frog from high-Cd habitat. But this difference was not statistically significant. However, qualitative histology showed that it is relatively higher in the frog lived in high-Cd habitat (see comparison in Figs. 1C and 2C). Melanomacrophage in amphibian has many possible functions, for instance, lowering metabolic activities during hibernation, phagocytosing red blood cells leaked from blood vessel into liver, scavenging cytotoxic substances, cell debris, infectious agents and foreign substances (Loumbourdis and Vogiatzis, 2002). Because of the frogs were collected in wet season, MMC formation found in this study

Table 2. Gravimetric data (mean ± S.E.M.) of female *Microhyla fissipes* caught from Mae Sot District, Tak Province, Thailand during June-November 2008

Parameters	Periods	Low environmental cadmium habitat	High environmental cadmium habitat
HSI	Early wet season	1.8122 ± 0.2648 (n=11)	2.7026 ± 0.7962 (n=9)
	Middle wet season	1.8786 ± 0.9535 (n=2)	1.6105 ± 0.2394 (n=3)
	Late wet season	2.1300 ± 0.1659 (n=14)	2.7876 ± 0.4675 (n=6)
	Overall	1.9403 ± 0.0968	2.3669 ± 0.3790
RSI	Early wet season	0.5400 ± 0.0426 (n=11)	0.4460 ± 0.0366 (n=9)
	Middle wet season	0.4729 ± 0.0936 (n=2)	0.8729 ± 0.1131 * (n=3)
	Late wet season	0.5404 ± 0.0277 (n=14)	0.4831 ± 0.1867 (n=5)
	Overall	0.5178 ± 0.0225	0.6007 ± 0.1365
GSI	Early wet season	8.9747 ± 1.4358 (n=11)	5.7632 ± 1.1379 (n=9)
	Middle wet season	14.8659 ± 7.7079 (n=2)	1.0141 ± 0.1580 * (n=3)
	Late wet season	3.4613 ± 1.1369 (n=14)	1.7869 ± 0.9288 (n=5)
	Overall	9.1006 ± 3.2928	2.8547 ± 1.4713 *

Remark: Significant site-related difference is indicated by an asterisk (*)

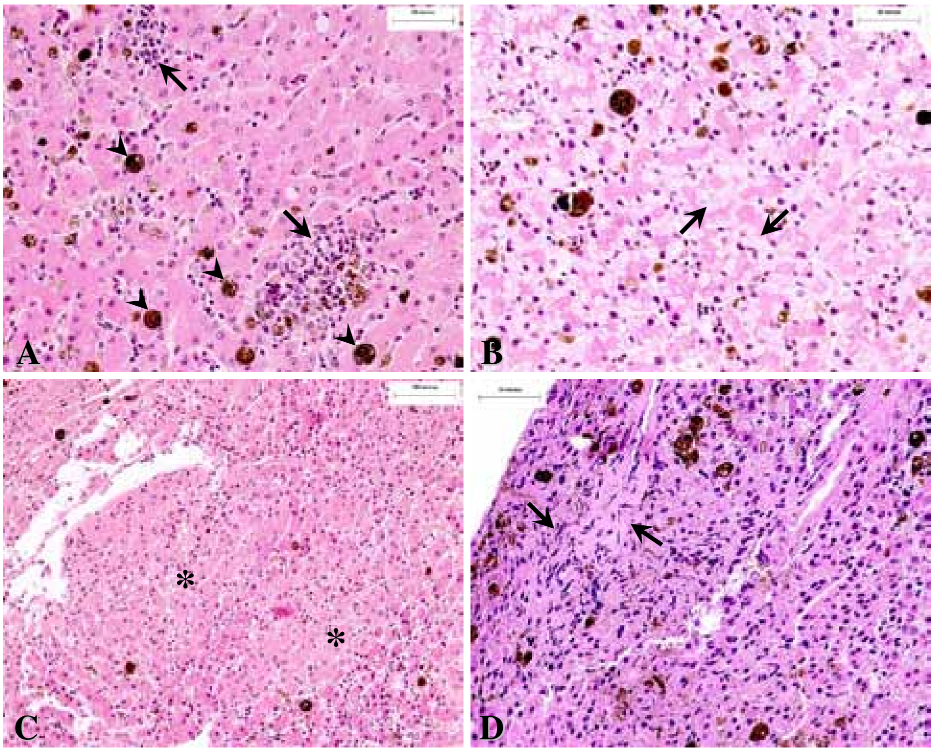

Figure 1. Micrograph of the liver tissues of *Microhyla fissipes* collected from low-Cd habitat. (A) Infiltration of white blood cells (*arrows*) and melanomacrophage centers (*arrow heads*). *Bar = 50 µm*. (B) Lipid droplet accumulation (*arrows*) in cytoplasm of hepatocytes. *Bar = 50 µm*. (C) Foci necrosis (*asterisks*) in some areas of the liver tissue. *Bar = 100 µm*. (D) Fibrosis in some areas of the liver tissue. *Bar = 50 µm. H&E*

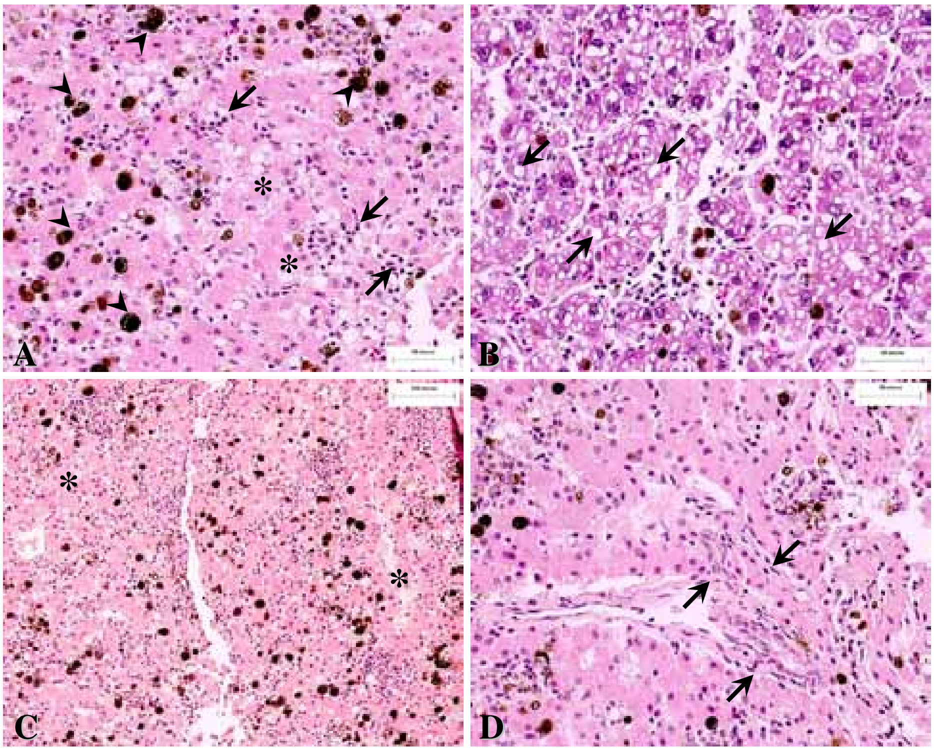

Figure 2. Micrograph of the liver tissues of *Microhyla fissipes* collected from high-Cd habitat. (A) Infiltration of white blood cells (*arrows*), melanomacrophage centers (*arrow heads*) and fatty degeneration (*asterisks*) in the liver tissue. *Bar = 50 µm*. (B) High degree of lipid droplet accumulation (*arrows*) in the cytoplasm leading to fatty degeneration of hepatocytes. *Bar = 50 µm*. (C) Necrosis (*asterisks*) in perivascular areas of the liver. *Bar = 50 µm*. (D) Fibrosis in perivascular areas of the liver. *Bar = 50 µm. H&E*

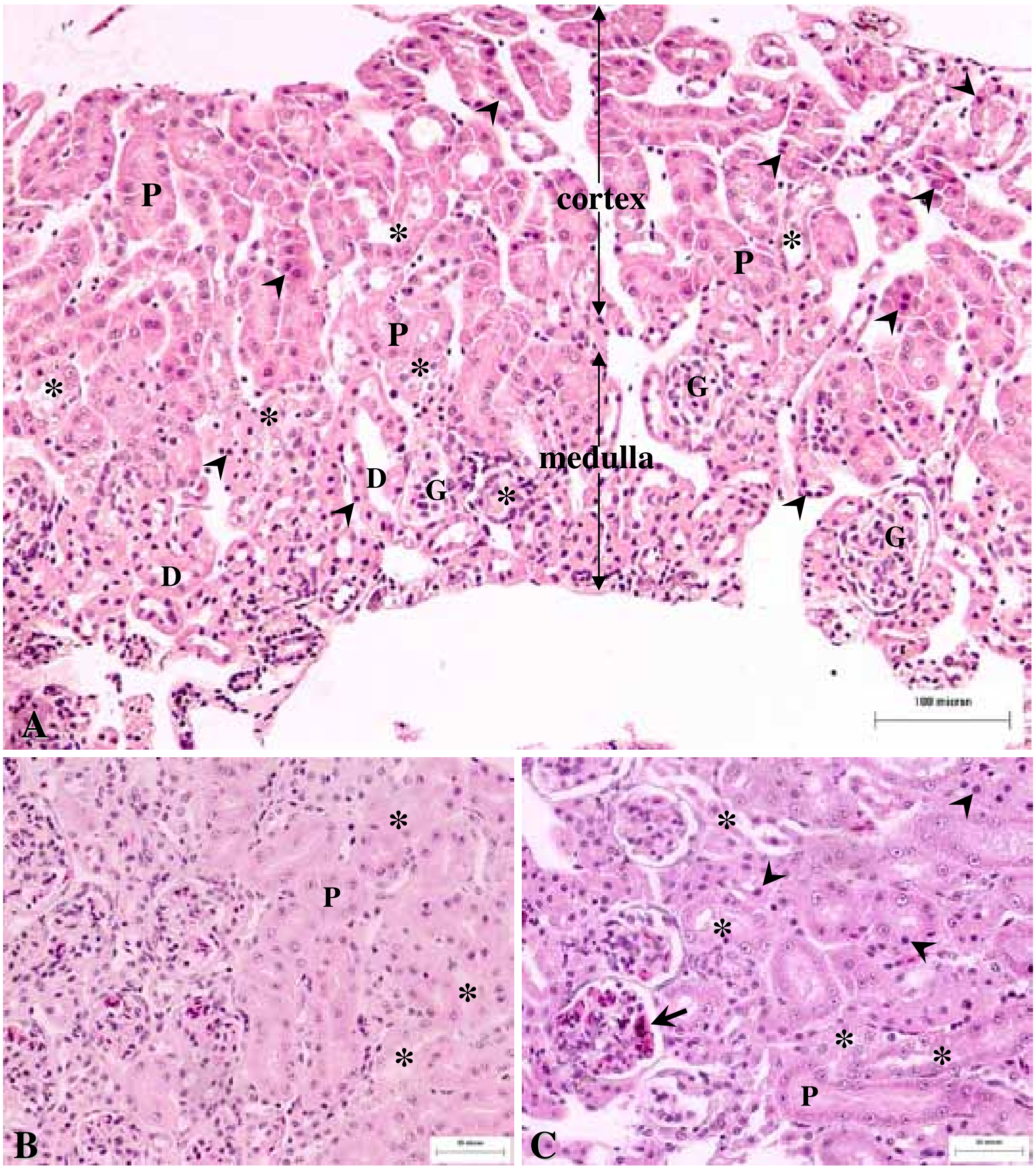

Figure 3. Micrograph of the kidney tissues of *Microhyla fissipes*. (A) Overall structure of kidney of the frog from low-Cd habitat separating into cortex and medulla regions. Pyknosis of tubular cells (*arrow heads*) were found in both proximal (*P*) and distal (*D*) tubules. Degeneration of the renal tubules and glomerulus (*asterisks*) was evidenced. *Bar = 100 µm*. (B) Edema in proximal tubules (*asterisks*) found in the renal cortex of the frog from low-Cd habitat. *Bar = 50 µm*. (C) Pyknosis of proximal tubular cells (*arrow heads*) and blood congestion in glomerular capillary (*arrow*) in the kidney of the frog from high-Cd habitat. Collapses of some proximal tubules were also evidenced (*asterisks*). *Bar = 50 µm. H&E*

may not involve in lowering their metabolism due to hibernation. Endothelium of blood vessels has been known as a target tissue of cadmium toxicity (Nolan and Shaikh, 1986). But from the results, no sign of hepatic hemorrhage was observed in the frog liver, so formation of MMCs may not involve in phagocytosing leaked red blood cells. From the evidences of liver injuries in term of necrosis, fatty degeneration and fibrosis, the formation of MMCs possibly involve with scavenging cell debris and cytotoxic substances due to chronic exposure to environmental cadmium. MMCs were also reported to accumulate some metals, they possibly accumulate cadmium (Loumbourdis and Vogiatzis, 2002). Moreover, high degree of MMC formation found in this study may imply that the immune function of these frogs was altered leading to infections of microorganisms and parasites.

Kidney histological analyses revealed some major histopathological alterations in both frog populations from low and high environmental cadmium habitats. In the frog from low-Cd habitat, edema of proximal tubules was observed (Fig. 3B). Pyknosis and necrosis of renal tubular cells leading to collapses of renal tubular structures were observed in the kidney tissues of the frog from both habitats (Figs. 3A and 3C). Blood congestion in glomerular capillary was also found in the kidney of the frog from high-Cd habitat (Fig. 3C). After chronic exposure to cadmium, cadmium can accumulate in many internal organs especially kidney and elicit nephrotoxicity (Degraeve, 1981). Renal tubular cell necrosis and edema of kidney tissues were frequently reported as major lesions of cadmium toxic effects in mammals (Aughey *et al*., 1984; Brzoska *et al*., 2003; Jihen *et al*., 2008). Non-inflammatory edema of renal tubules may be a result of retention of salt and water due to kidney malfunction (Robbins *et al*., 1995) and can be a warning sign of kidney injury in the frog living in low-Cd habitat. Severe alterations such as pyknosis and necrosis of renal tubular cells indicate that the renal injury entered the point of no return. As it was reported that cadmium-induced renal injury occurred after hepatic damage (Dudley *et al*., 1985), the renal injury found in this study therefore indicates chronic exposure to environmental cadmium. From overall histological results, it indicates that environmental cadmium contamination in Mae Sot, Thailand, even at low level, may cause hepatotoxic and nephrotoxic effects on the frog living in this area. So, the frog populations living in both low and high environmental cadmium habitats are in vulnerable status.

The results from gravimetric analyses indicate that only gravimetrical biomarkers are not sufficient to interpret the effects of environmental contamination of a certain toxic compound. Using of histological biomarkers clarified the toxic responses of animal organ system to the toxicant. Overall, the results indicate that exposure to environmentally relevant level of cadmium may interfere with the frog health. This suggests an important implication for overall health of animals/ human in the areas of Mae Sot District, Tak Province, Thailand.

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