Jurnal Sains Kesihatan Malaysia Isu Khas 2019: 65-71
DOI : http://dx.doi.org./10.17576/JSKM-2019-09

Histological Differences in the Livers and Kidneys of Two Populations of Rice Frog (*Fejervarya limnocharis*) Naturally Exposed to Different Environmental Cadmium Levels

Perbezaan Histologi dalam Hati dan Ginjal Dua Populasi Katak Sawah (*Fejervarya limnocharis)* yang Terdedah pada Aras Kadmium Semulajadi yang Berbeza

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ABSTRACT

Histological observation has always been important in the study of sentinels. In this research, histological differences in the liver and kidney of two wild populations of Fejervarya limnocharis exposed to different environmental cadmium levels were observed. Liver and kidney samples from 30 rice frogs caught from reference site (Mae Pa) and contaminated site (Mae Tao) were sectioned and stained with Haemotoxylin and Eosin and then observed under light microscope. It was found that liver sections from frogs caught from Mae Tao had higher hepatic macro-melanophage count (MMC) (0.949 ± 0.267 cells/1000 µm²) as compared to those from Mae Pa (0.672 ± 0.299 cells/1000 µm²). Other observable histological features found in frog's liver from Mae Tao include possible necrotic areas, cellular swellings and chromatin margination. The kidney sections from frogs caught from Mae Tao showed the presence of tumor-like cell aggregation and hemorrhage. However, the proportion of tumor-like cell aggregation and hemorrhage were not significantly different than were expected from random occurrences. The result of the histological study revealed that F. limnocharis caught from Mae Tao had higher hepatic MMC and higher prevalence of renal tumor-like aggregation and renal hemorrhage than the rice frogs caught from Mae Pa. Cellular swelling, possible necrotic area and possible apoptotic cell could also be observed in the liver of contaminated frogs.

Keywords: Histology, Liver, Kidney, Fejervarya limocharis, Cadmium

ABSTRAK

Pemerhatian histologi amat penting dalam kajian spesies sentinel. Dalam kajian ini, perbezaan histologi hati dan ginjal dua populasi liar Fejervarya limnocharis yang terdedah kepada aras kadmium persekitaran yang berbeza telah dikaji. Sampel hati dan ginjal dari 30 ekor katak sawah yang ditangkap dari kawasan rujukan (Mae Pa) dan kawasan tercemar (Mae Tao) telah dihiris dan diwarnakan dengan Haemotoxylin dan Eosin dan dilihat di bawah mikroskop cahaya. Kajian mendapati seksyen hati dari katak yang ditangkap dari Mae Tao mempunyai bilangan melanofaj hepar makro (0.949 ± 0.267 sel/1000 µm2) yang tinggi berbanding katak dari Mae Pa (0.672 ± 0.299 cells/1000 µm2). Pemerhatian histologi lain yang dilihat dalam hati katak dari Mae Tao termasuklah kawasan nekrotik, pembengkakan sel dan marginasi kromatin. Seksyen ginjal katak yang ditangkap dari Mae Tao menunjukkan kehadiran agregasi sel mirip tumor dan pendarahan. Namun, bilangan agregasi sel bak-tumor dan pendarahan ini tidak berbeza secara signifikan berbanding kehadiran secara rawak. Hasil kajian histologi menunjukkan bahawa F. limnocharis yang ditangkap dari Mae Tao mempunyai MMC hepatik yang tinggi, dan prevalens yang tinggi bagi kehadiran agregasi sel bak-tumor dan pendarahan renal berbanding katak sawah yang ditangkap dari Mae Pa. Pembengkakan sel, kemungkinan kawasan nekrotik dan sel apoptotik juga boleh dilihat dalam hati katak tercemar.

Kata kunci: Histologi, Hati, Ginjal, Fejervarya limnocharis, Kadmium

INTRODUCTION

Histological analysis is one of the important parameters often included in the study of sentinels. Hutchingson and Pickford (2002) stated that histology has been included as priority endpoints in the new OECD test guidelines for environmental monitoring. Often the initial effects of heavy metal pollution are evident only at the cellular or tissue level, thus causing histological changes before significant changes can be identified in a higher level (Pereira et al. 2016: van Dyk et al. 2007). Some of the histological effects of cadmium exposure to a tissue include fibrosis, apoptosis and necrosis (Habeebu et al. 1998; Thijssen et al. 2007; Rosa et al. 2008), hyalinization, vacuolation and cellular swelling (van Dyk et al. 2007) and cadmium deposition (Itokawa et al. 1978).

One of the most notorious effects of cadmium toxicity to a tissue is the formation of tumor cells. According to Alvarez et al. (2004) cadmium has been classified by the International Agency for Research on Cancer as category I (human) carcinogen. This means that cadmium has the ability to induce abnormal and uncoordinated cell proliferation. A study on prostate epithelial cells by Martin et al. (2002) revealed that low level of cadmium is enough to mimic the effect of androgen and stimulate cell proliferation, thus propagating cancer cells.

Another common effect of cadmium in the tissue is the production of macro-melanophage centers. Macro-melanophage centers are produced in response to the presence of parasites and foreign materials in the tissue. Cadmium is reported to cause a depression in the immune system. (Loumbourdis & Vogiatzis 2002). As a result of this depression, the tissue is prone to massive invasion of parasites which will then lead to the production of macro-melanophage centers. Apart from that, cadmium is reported to cause the rupture of small blood vessels in the tissue. This leads to the release of red blood cells in the tissue hence the activation of the aggregation of Kupfer cells to form macro-melanophage centers. Macro melanophage centers function to scavenge the parasites, red blood cells and other foreign materials in the tissue. In addition, since macro-melanophage centers are from Kupfer cells, its function also involves cadmium accumulation and scavenging (Loumbourdis & Vogiatzis 2002). Ibraheem et al. (2016) also reported that the appearance of many phagocytic cells may be attributed to exposure to cadmium. The presence of macro-melanophage centers has been reported in the liver, kidney and testis (El-Refaiy & Eissa 2013; Loumbourdis & Vogiatzis 2002)

The effect of cadmium toxicity would result in different histopathological changes depending on what tissue it exerts its effect on. In the liver, cadmium was found to induced pathological changes including diffused hepatocyte and eosinophilic cytoplasm (Dudley & Klaasen 1984); and hyalinization of hepatocytes, increased vacuolation associated with lipid accumulation, congestion of blood vessels and cellular swellings (van Dyk et al. 2007). Cadmium is also reported to cause liver damage and induce oxidative stress in the liver (Wu et al. 2017). In the kidney, cadmium was known to activate glomerular or interstitial infiltrated inflammatory cells, which would eventually lead to the over production of extracellular matrix component along with an increase in vacuolization and an increased amount of lysosomes (Thijssen et al. 2007). Interstitial fibrosis was also found in tissues suffering the effect of cadmium toxicity (Rosa et al. 2008).

In this study, *F. limnocharis* has been chosen to be a sentinel species for cadmium contamination based on a few criteria, including 1) not threatened by extinction 2) ubiquitous 3) of suitable size 4) has large distribution area and 5) has a stable population (IUCN, Conservation International and NatureServe 2014). This study aimed to document histological differences of the liver and kidney of two populations of rice frogs exposed to different environmental cadmium levels.

MATERIALS AND METHOD

Frogs were caught live at night time during visual encounter survey (Crump & Scott 1994) and were collected on a monthly basis during November 2007 and October 2008 from several rice fields in Mae Tao and Mae Pa in Mae Sot District, Tak Province. The contaminated site, Mae Tao, was located at 16°45'13"N; 98°35'25"E. This area is irrigated by the Mae Tao Creek. The reference site, Mae Pa, was located 8.4 km north of the contaminated site at 16°40'43"N; 98°35'36"E. The area is irrigated by Huay Luek Creek and not on the path of the cadmium contamination plume.

The frogs were transported live to the lab where they individually subjected to cold anaesthesia procedure before sacrificed by double-pith at the brain and spinal cord (Tharp & Woodman 2015). Each frog was then biopsied. Liver (n = 30) and kidney (n = 30) tissues were fixed in 10% neutral buffered formalin before they were processed. Tissues were dehydrated and processed through graduated changes of 70% ethanol, 90% ethanol, 95% ethanol, n-butanol and xylene before they were embedded in paraffin wax. Each paraffin blocks were trimmed and then sections were cut from the block. The thickness of each section was 5 μm. The sections were mounted onto slides and then dried. The sections were then stained by Haemotoxylin and Eosin staining procedure involving graduated immersion into xylene, n-butanol, 95% ethanol, 90% ethanol, 70% ethanol, water, haematoxylin solution, differentiator and eosin solution. The slides were then finally cleaned with xylene before the sections were mounted with DPX mounting medium (distyrene and tricresyl phosphate in xylene, BDH Laboratory Supplies). The slides were dried and then observed under light microscope.

The difference in the average value of macro-melanophage center count was statistically analyzed by t-test. Prevalence of tumor-like aggregation and hemorrhage were statistically analyzed by Fisher Exact Test for proportions. All statistical analyses were performed on the SigmaStat 2.0 program.

RESULTS AND DISCUSSION

We have previously reported cadmium concentrations in water and soil samples from Mae Tao, the contaminated site and Mae Pa, the reference site (Othman et al. 2009). Preliminary analysis showed that the cadmium concentrations from the contaminated site ranged from 0.0019 to 0.0021 mg/L in water samples, and 2.9260 to 3.2888 mg/kg in sediment samples. The concentration ranges at the reference site were 0.0018 to 0.0020 mg/L (water) and 0.1013 to 0.2206 mg/kg (sediment). We also found that the result also showed that frogs from contaminated site had significantly higher hepatic, renal and gonadal cadmium levels than frogs from reference site. In addition, we also documented that frogs from the contaminated sites had lower hepatosomatic index and renosomatic index as compared to those from the reference site (Othman et al. 2016)

Figure 1 showed the liver sections of *Fejervarya limnocharis* caught from Mae Sot, Tak. The sections showed that there was an observable difference in the number of macro-melanophage centers (MMC) or Kupfer cells in liver sections of frog caught from both sites. This observation is further confirmed in Figure 2 that shows average macro-melanophage center count in liver section from *Fejervarya limnocharis* caught from Mae Sot, Thailand. It was found that frogs caught from Mae Pa had an average hepatic MMC count of 0.672 ± 0.299 cells/1000 μm^2 (n = 15). For Mae Tao, the average hepatic MMC count was 0.949 ± 0.267 cells/1000 μm^2 (n = 15). The mean differences between station is statistically significant a P = 0.049. Hyalinization and vacuolation were not observed in the liver sections. However, the hepatocytes of frogs from the contaminated site showed polygonal hepatocyte shape and an increase in the cytoplasmic area. Figure 1(d) also showed some possible necrotic cells/necrotic area similar to those found by Dudley and Klaasen (1984). The liver section also revealed chromatin margination which is a morphologic sign of apoptosis.

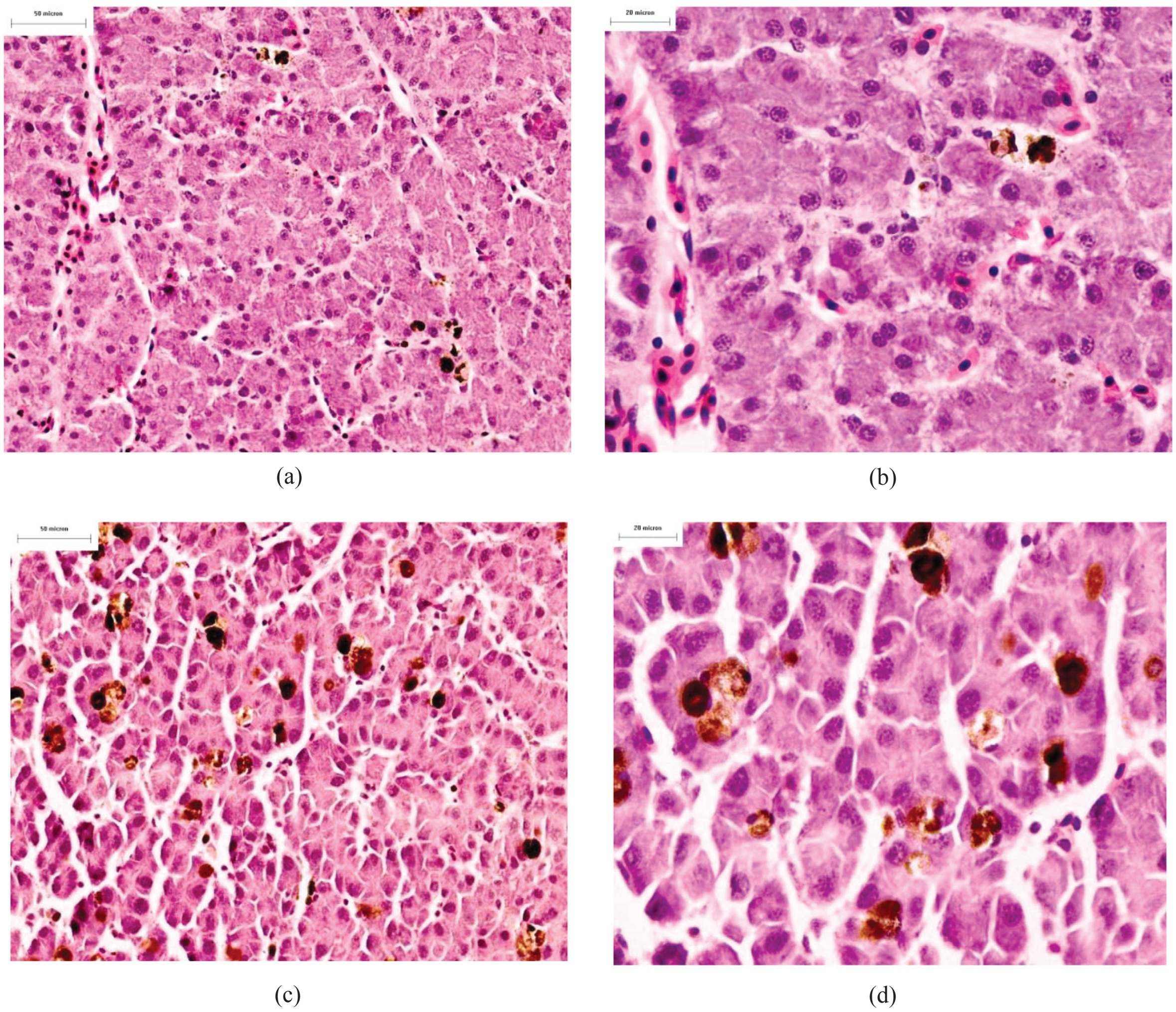

FIGURE 1. Liver sections of Fejervarya limnocharis caught from Mae Pa (a & b) and Mae Tao (c & d) at 20x (left; bar = 50 µm) and 40x (right; bar = 20 µm) magnifications (H&E staining). The sections showed macro-melanophage centers (M), necrotic cell area (N) and chromatin margination (C)

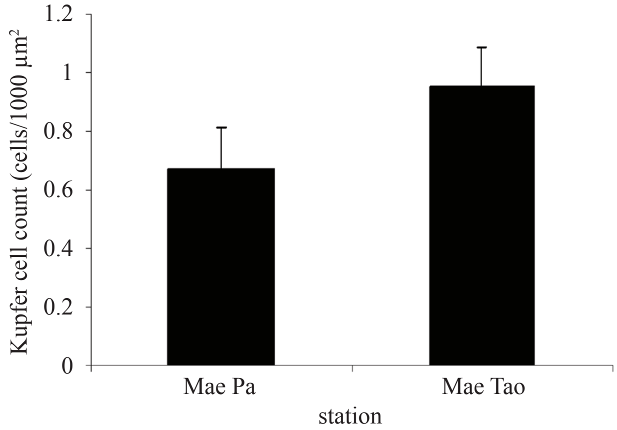

FIGURE 2. Mean macro-melanophage center count in liver section from Fejervarya limnocharis caught from Mae Pa (n = 15) and Mae Tao (n = 15). Mean differences between station is statistically significant (P = 0.049)

For kidney, the section is presented in Figure 3. The sections showed the presence of tumor-like cell aggregation in the kidney of *Fejervarya limnocharis* caught from Mae Tao. The cells within the aggregation had a denser nucleus with much reduced cytoplasm. The prevalence of this tumor-like aggregation in the kidneys of *Fejervarya limnocharis* caught from both Mae Pa and Mae Tao is presented in Figure 4. The result of this study revealed that the prevalence of tumor cell in the kidney of Mae Tao's frog was 15% (n = 20), while the prevalence in Mae Pa's population was 0% (n = 20). Apart from that, the result also showed that the prevalence of hemorrhage in the kidney of *Fejervarya limnocharis* caught from the contaminated site was 5% (n = 20), while none were observed in the kidney sections of the frogs caught from Mae Pa (n = 20). For both observations, Fisher Exact Test revealed that the proportion of tumor-like cell aggregation and hemorrhage were not significantly different than were expected from random occurrences (P = 0.112 and P = 1.00, respectively).

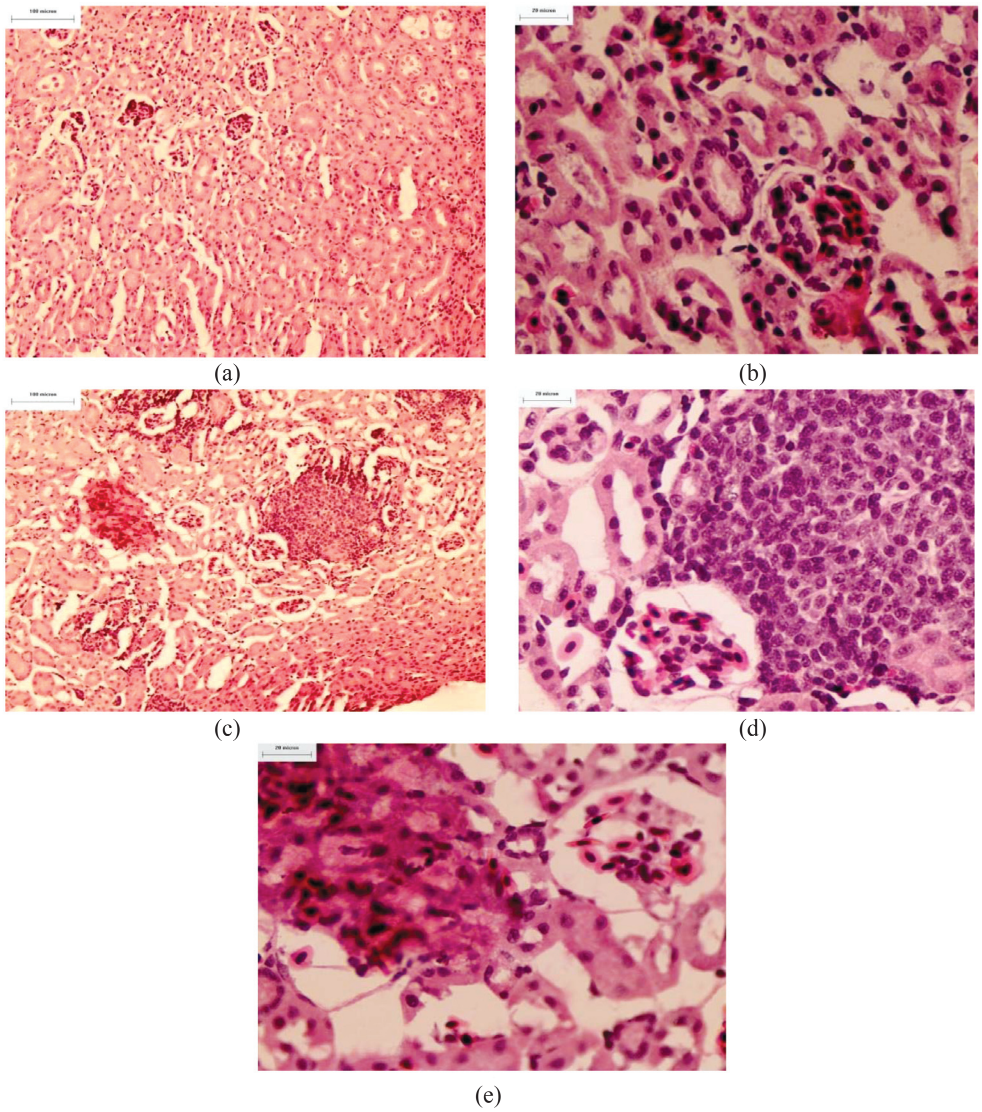

FIGURE 3: Kidney sections of Fejervarya limnocharis caught from Mae Pa (a & b) and Mae Tao (c, d and e) at 10x (bar = 100µm) and 40x (bar = 20µm) magnifications (H&E staining). The sections showed glomerulus (G), tumor-like cell aggregation (N) and hemorrhage (H).

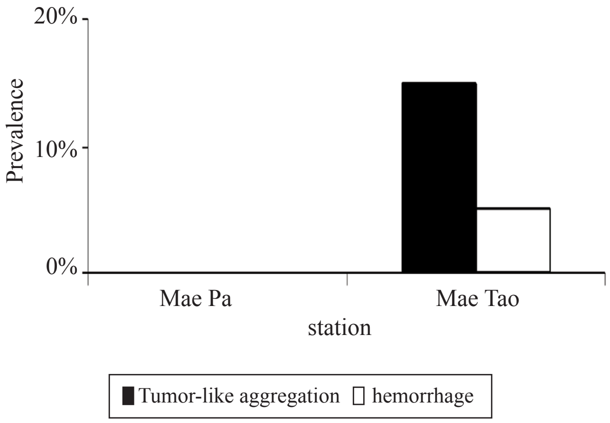

FIGURE 4: Prevalence of tumor-like cell aggregation and hemorrhage in the kidney sections from Fejervarya limnocharis caught from Mae Pa (n =15) and Mae Tao (n =15)

The presence of macro-melanophage centers in the livers of the frogs in both reference and contaminated site was expected. This is because frogs living in the wild are often exposed to parasitic infections. However, this study found that the macro-melanophage center count of *Fejervarya limnocharis* caught from the contaminated site was statistically significantly higher than the macro-melanophage count of those caught from reference site. To explain, there is an association between cadmium accumulations with the production of macro-melanophage centers. Cadmium was known to cause a depression in the immune system (Loumbourdis & Vogiatzis 2002). When this occurs, the body lacks the defense against parasitic infection, which would cause a massive invasion of parasites in the liver. As a response to this parasitic invasion, the liver would produce Kupfer cells to scour the parasites. The presence of Kupfer cells that has engulfed the parasites and foreign bodies is also often termed the macro-melanophage centers. Apart from that, Loumbourdis and Vogiatzis (2002) also reported that cadmium may cause the rupture of small vessels in the tissue. This would cause red blood cells to be discharged into the surrounding tissue. The presence of red blood cells in the tissue would also trigger the aggregation of Kupfer cells that would act as scavenger of the foreign bodies, forming the macro-melanophage centers.

It was reported that some of the common manifestations of cadmium toxicity to the microstructure of the liver were hyalinization, vacuolation and cellular swelling. (El-Refaiy & Eissa 2013: van Dyk et al. 2007). In this study, however, hyalinization and vacuolation were not observed in the liver sections. Yet, moderate cellular swelling can be observed in the section from frog from Mae Tao, signified by polygonal-shaped hepatocytes and an increase in the cytoplasmic area and swelling (Witeska et al. 2011: Dudley & Klaasen 1984). This is because, van Dyk et al. (2007) stated that cadmium contamination could cause water influx into the cell, thus causing hydropic degeneration or cellular swelling. Possible necrotic cell/necrotic area similar to those found by El-Refaiy & Eissa (2013) and Dudley & Klaasen (1984) were also found in the sections in this study. This might be due to the formation of highly reactive radicals and subsequent lipid peroxidation induced by cadmium. The liver section also revealed chromatin margination which is a morphologic sign of apoptosis (Habebu et al. 1998).

In the kidney, the result showed that tumor-like cell aggregation and inflammation were found only in the sections from frogs caught from the contaminated site. A study by Martin et al. (2002) showed that cadmium was shown to stimulate the proliferation of epithelial cells of the prostate. Rana et al. (2018) also reported that cadmium may initiate renal damage through inflammatory cell infiltration and inducing oxidative stress. The presence of cellular inflammation and hemorrhage is also reported by Ibraheem et al. (2016). In another study, Stoica et al. (2000) found that cadmium has the potential to increase the risk of breast cancer via the activation of estrogen receptors while Alvarez et al. (2004) reported that cadmium has been classified by the International Agency for Research on Cancer as carcinogen. Therefore, cadmium may be able to activate the proliferation of the kidney cells, hence explaining the presence of tumor-like cell aggregation in the kidney of contaminated frogs. However, great care should be practiced before making the relationship between cadmium and cancer of the kidney. Since the diagnosis of cancer cells requires more extensive tests, therefore this study would only label the finding as tumor-like cell aggregation, distinguishing them from the actual tumor cells or neoplasm. Also, the presence of renal hemorrhage can also be attributed, albeit indirectly, to cadmium. As stated before, cadmium has the ability to cause the rupture of blood vessels, hence causing hemorrhage in the tissue.

CONCLUSION

This research found that frogs from the contaminated sites had higher macro-melanophage centers in the liver and higher prevalence of tumor-like cell aggregation and inflammation in the kidney. However, only hepatic macro-melanophage count showed statistically significant difference between the two sites. Cellular swelling, possible necrotic area and possible apoptotic cell could be observed in the liver sections. Despite these evidences, any conclusion should be approached with great caution because any abnormalities that occur in the tissue most definitely cannot be traced back to cadmium contamination alone.

ACKNOWLEDGEMENT

This research is part of a research project titled “Using the Rice Frog (*Fejervarya limnocharis*) as Sentinel Species for Cadmium Contamination in Tak Province, Thailand”.

Financial support of this work was obtained from the National Center of Excellence for Environmental and Hazardous Waste Management (NCE-EHWM), the 90th Anniversary of Chulalongkorn University Fund, The Ratchadaphiseksomphot Endowment Fund and the MUA-TRF research grant (MRG4980120) to NK. An educational grant from the Malaysian Ministry of Higher Education to MSO is fully acknowledged. Additional support was also obtained from NIH Fogarty ITREOH (D43TW007849).

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