Preliminary Report

Investigation of Sensitive Biomarkers to Determine Cadmium Inducing Hepato- and Nephro-toxicity in Cattle by Immunofluorescence Method

Shutipen Buranasinsup BSc, PhD*,
Arunee Jangsangthong BS*, Piyanat Toniti DVM, MSc*

** Department of Pre-clinic and Applied Animal Science, Faculty of Veterinary Science, Mahidol University, Nakornpathom, Thailand*

Cadmium inducing hepato- and nephro-toxicity can cause the alteration of protein expression such as metallothionein (MT), i.e. cadmium binding protein, and the metal transcription factor-1 (MTF-1), which plays a significant role in cellular responses to the heavy metal stress. To study the expression of these proteins, the cattle were classified into five groups resulting from the levels of cadmium in the kidneys. Next, the blood biochemical profiles were analyzed to estimate the liver and kidney functions. The expressions of MT and MTF-1 proteins both in the liver and kidneys were investigated by immunofluorescence method. This study found that the blood biochemical profiles were not correlated with the level of cadmium in these tissues; however, the expressions of MT and MTF-1 proteins were earlier detected in the bovine which had a low level of cadmium contamination (0.5 mg/kg). Thus, these proteins could be used as the sensitive markers to determine the cadmium in tissue.

Keywords: *Biological markers, Cadmium, Cattle, DNA-Binding proteins, Fluorescent antibody technique, Kidney, Liver, Metallothionein, Transcription factors*

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Cadmium (Cd) is a highly toxic environmental pollutant found in soil and water. The primary route of Cd exposed to humans and animals is via the ingestion of Cd-contaminated food. Cd is preferentially accumulated not only in the liver and kidneys, but also in other tissues under certain circumstances[1-3]. This metal has a variety of adverse effects to humans and animals due to the dose, route and duration of the exposure. It can damage several organs especially lungs, testis, bone, liver and kidneys. Additionally, the Cd exposure causes negative effects both directly and indirectly on the bone mass: affecting directly on the osteoblast and osteoclast functions, and interfering indirectly on the metabolism of bone resulting in osteopenia and osteoporosis[4-7]. Cadmium inducing hepato- and nephro-toxicity can be detected by blood chemical profiles such as alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), blood urea nitrogen (BUN) and creatinine. However, the elevations of blood chemical profiles are detected only when the hepato and renal functions are severely damaged.

Recently, the hepato and renal dysfunctions can be detected by the alteration of protein expression. The alterations of protein expression for cadmium inducing the hepato and renal injuries are varied, for instant, oxidative stress species, metallothionein, metal-responsive transcription factor-1 (MTF-1) and apoptotic enzymes.

Metallothionein (MT) is the Cd binding protein with low molecular weight. It is rich in cysteine and abundant in the liver and kidneys. The precise physiological role of MT has not been fully elucidated. Nevertheless, the proposed roles include a) the participation in maintaining the homeostasis of essential transition metals, b) the detoxification of

Correspondence to: Buranasinsup S, Department of Pre-clinic and Applied Animal Science, Faculty of Veterinary Science, Mahidol University, Nakornpathom 73710, Thailand. Phone: 0-2441-5242 ext. 1530, E-mail: vssbr@mahidol.ac.th

non-essential metals and c) the protection against intracellular oxidative stress[8-10]. The cysteine residues work on binding and storing the metal ions. One molecule of MT is capable of binding 7 atoms of Cd[11]. Under the Cd load, the MT expression is strongly induced at the level of transcription. The promoter regions of the MT genes contain the acceptably called metal-responsive elements (MRE) which are responsible for the induction of heavy metals. The factor binding to the MRE promoter sequences was described as the metal-responsive element binding the transcription factor-1, in other words, the metal transcription factor-1 (MTF-1). MTF-1 is a ubiquitously expressed zinc finger protein that is essential for the heavy metal inducing the expression of MT. In quiescent cells, MTF-1 preferentially resides in the cytoplasm but translocates to the nucleus upon several stress conditions, notably the heavy metal load. Therefore, MTF-1 is the key regulator of the MT expression[12-17]. As MT and the transcription factor (MTF-1) play the important roles in the Cd exposure, the possibility of using MT and MTF-1 protein expressions in tissues as the biomarkers of Cd exposure is interesting to be investigated.

Material and method

Blood chemical analysis

The kidneys and livers from forty-five cattle living in the Cd contaminated area were analyzed for the Cd levels by Inductively Coupled Plasma Mass Spectrometry (ICP-MS) technique. According to the Cd levels in kidneys, the cattle were divided into 5 groups: Cd level < 0.5 mg/kg as group 1, Cd level 0.5-1 mg/kg as group 2, Cd level 1.1-3 mg/kg as group 3, Cd level 3-5 mg/kg as group 4 and Cd level > 5 mg/kg as group 5 (Table 1).

Before collecting the tissues, the serum from forty-five cattle were collected by centrifugation and kept at -20°C until the analysis took place. The blood chemical analysis for testing the liver and renal functions was conducted: blood urea nitrogen (BUN, Reflotron®), creatinine (Reflotron®), alanine aminotransferase (ALT, Reflotron®), aspartate aminotransferase (AST, Reflotron) and alkaline phosphatase (ALP, Reflotron®) by Reflovet machine.

Metallothionein and metal-responsive transcription factor (MTF-1) detection were done by immunofluorescence method.

Both forty-five livers and kidneys of the cattle were preserved in the formalin buffer solution and performed for paraffin embeds. The sections were deparaffinized and rehydrated, followed by the antigen retrieving method using the sodium citrate buffer. Non-specific binding antibodies were blocked by using 10% normal horse serum. The deparaffinized tissue sections were incubated by the mixture of 1:400 rabbit anti-bovine metallothionein (Santa Cruz Biotechnology, Inc.) and of 1:400 goat anti-bovine MTF-1 (Santa Cruz Biotechnology, Inc.) in 1:1 ratio for 1 hour. After that, the tissue sections were washed in the phosphate buffer saline (PBS) containing 0.1% Tween-20 for 3 times, 10 minutes for each. Then, the tissue sections were incubated by the mixture of 1:600 goat anti-rabbit IgG conjugated by the fluorescence isothiocyanate (FITC, Santa Cruz Biotechnology, Inc.) and of 1:600 donkey anti-goat IgG conjugated by Texas red (TR, Santa Cruz Biotechnology, Inc.) for 30 minutes followed by washing in PBS containing 0.1% Tween-20 for 3 times, 10 minutes for each. The fluorescence signal was detected under the fluorescence microscopy. The livers and kidneys from two mice were used as the negative control.

Table 1. Five study groups divided by the cadmium concentration

Cadmium concentration (mg/kg)				
Group 1	Group 2	Group 3	Group 4	Group 5
<0.5	0.5-1.0	1.1-3.0	3.0-5.0	>5.0

Results

Blood chemical analysis

To determine the liver function, the serum biochemistry was analyzed: alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP). The results shown in Fig. 1 indicated that the alterations of these enzymes were not correlated to the levels of Cd in tissues. The alterations of the enzymes in Group 5 were raised to the highest level of the other groups, but the results did not concur with the assumptions. To evaluate the renal function, the levels of blood urea nitrogen (BUN) and creatinine were examined. The levels of BUN were normal in all groups, including the kidney samples with the highest amount of Cd. The levels of blood creatinine were also not correlated with the levels of cadmium in kidneys.

The bar chart represented the percentage of blood biochemistry in each bovine group. It showed

Table 2. The color responses in the study groups

Liver		Kidney	
Metallothionein	MTF1	Metallothionein	MTF1
Group 1-5 Green-color stain in cytoplasm	Group 1-5 Red-color stain in cytoplasm	Group 1-5 Green-color stain in cytoplasm	Group 1-5 Red-color stain in cytoplasm

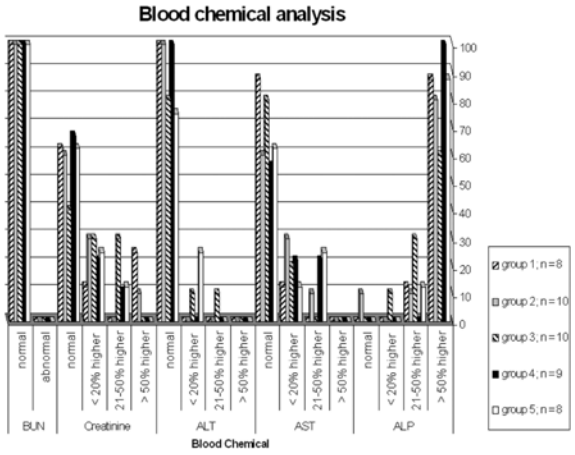

Fig. 1 The blood biochemical analysis by dry chemical assay

the levels which were normal, higher than normal within 20 percent, higher than normal within 20-50 percent and higher than normal over 50 percent. The blocks in blue, yellow, pink, green and red represented the blood biochemistry of the bovine in Group 1, 2, 3, 4 and 5 respectively.

Metallothionein and metal-responsive transcription factor (MTF-1) were detected by the immunofluorescence method

To determine the metallothionein and MTF-1 expressions, the bovine liver and kidney of the study groups and the control group were stained by the mixture of rabbit anti-bovine metallothionein and of goat anti-bovine MTF-1, then followed by the mixture of goat anti-rabbit IgG conjugated by the fluorescence isothiocyanate (FITC) and of donkey anti-goat IgG conjugated by Texus red (TR). The results of these protein expressions in the study groups were shown in green and red colors in cytoplasm compared with the control group which were not stained (Table 2).

Discussion

The ingestion of cadmium (Cd) contaminated food can cause negative effects in various organs especially the liver and kidneys as Cd preferentially accumulates in these tissues. Hepato- and nephrotoxicity can be evaluated by blood biochemical profiles such as alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), blood urea nitrogen (BUN) and creatinine. In fact, these are not the sensitive and correlated markers whereas metals associated with the proteins, *e.g.* metallothionein (MT) and metal transcription factor-1 (MTF-1) are the earlier markers and can be detected even in bovine having the level of cadmium < 0.5 mg/kg. From the experiment, this study suggests the protein expressions as the sensitive markers to determine cadmium contaminated tissue.

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References

1. Akesson A, Bjellerup P, Lundh T, Lidfeldt J, Nerbrand C, Samsioe G, et al. Cadmium-induced effects on bone in a population-based study of women. Environ Health Perspect 2006; 114: 830-4.
2. Trinchella F, Riggio M, Filosa S, Volpe MG, Parisi E, Scudiero R. Cadmium distribution and metallothionein expression in lizard tissues following acute and chronic cadmium intoxication. Comp Biochem Physiol C Toxicol Pharmacol 2006; 144: 272-8.
3. Liu J, Liu Y, Michalska AE, Choo KH, Klaassen CD. Metallothionein plays less of a protective role in cadmium-metallothionein-induced nephrotoxicity

than in cadmium chloride-induced hepatotoxicity. J Pharmacol Exp Ther 1996; 276: 1216-23.
4. Prozialeck WC, Edwards JR, Woods JM. The vascular endothelium as a target of cadmium toxicity. Life Sci 2006; 79: 1493-506.
5. Brzoska MM, Moniuszko-Jakoniuk J. Disorders in bone metabolism of female rats chronically exposed to cadmium. Toxicol Appl Pharmacol 2005; 202: 68-83.
6. Zhu G, Wang H, Shi Y, Weng S, Jin T, Kong Q, et al. Environmental cadmium exposure and forearm bone density. Biometals 2004; 17: 499-503.
7. Liu J, Habeebu SS, Liu Y, Klaassen CD. Acute CdMT injection is not a good model to study chronic Cd nephropathy: comparison of chronic CdCl2 and CdMT exposure with acute CdMT injection in rats. Toxicol Appl Pharmacol 1998; 153: 48-58.
8. Liu Y, Liu J, Habeebu SS, Klaassen CD. Metallothionein protects against the nephrotoxicity produced by chronic CdMT exposure. Toxicol Sci 1999; 50: 221-7.
9. Klaassen CD, Liu J, Choudhuri S. Metallothionein: an intracellular protein to protect against cadmium toxicity. Annu Rev Pharmacol Toxicol 1999; 39: 267-94.
10. Adams TK, Saydam N, Steiner F, Schaffner W, Freedman JH. Activation of gene expression by metal-responsive signal transduction pathways. Environ Health Perspect 2002; 110 (Suppl 5): 813-7.
11. Liu J, Kadiiska MB, Corton JC, Qu W, Waalkes MP, Mason RP, et al. Acute cadmium exposure induces stress-related gene expression in wild-type and metallothionein-I/II-null mice. Free Radic Biol Med 2002; 32: 525-35.
12. Lichtlen P, Schaffner W. The "metal transcription factor" MTF-1: biological facts and medical implications. Swiss Med Wkly 2001; 131: 647-52.
13. Lichtlen P, Wang Y, Belser T, Georgiev O, Certa U, Sack R, et al. Target gene search for the metal-responsive transcription factor MTF-1. Nucleic Acids Res 2001; 29: 1514-23.
14. Zhang B, Georgiev O, Hagmann M, Gunes C, Cramer M, Faller P, et al. Activity of metal-responsive transcription factor 1 by toxic heavy metals and H_2O_2 in vitro is modulated by metallothionein. Mol Cell Biol 2003; 23: 8471-85.
15. Chu WA, Moehlenkamp JD, Bittel D, Andrews GK, Johnson JA. Cadmium-mediated activation of the metal response element in human neuroblastoma cells lacking functional metal response element-binding transcription factor-1. J Biol Chem 1999; 274: 5279-84.
16. Gunes C, Heuchel R, Georgiev O, Muller KH, Lichtlen P, Bluthmann H, et al. Embryonic lethality and liver degeneration in mice lacking the metal-responsive transcriptional activator MTF-1. EMBO J 1998; 17: 2846-54.
17. Wang Y, Wimmer U, Lichtlen P, Inderbitzin D, Stieger B, Meier PJ, et al. Metal-responsive transcription factor-1 (MTF-1) is essential for embryonic liver development and heavy metal detoxification in the adult liver. FASEB J 2004; 18: 1071-9.

การศึกษาดรรชนีที่มีความไวเพื่อวัดการสะสมพิษของแคดเมียมในตับและไตของโคขุนด้วยวิธี immunofluorescence

ชุติเพ็ญ บูรณะสินทรัพย์, อรุณี แจ้งแสงทอง, ปิยะนาถ โตนิติ

ความเป็นพิษในตับและไตที่มีสาเหตุจากแคดเมียมทำให้เกิดการเปลี่ยนแปลงการแสดงออกของโปรตีนบางชนิด เช่น metallothionein โปรตีนซึ่งทำหน้าที่ในการจับกับแคดเมียม และ metal transcription factor-1 โปรตีนควบคุมการเริ่มต้นของขบวนการถอดรหัส และมีบทบาทสำคัญในการตอบสนองของเซลล์ต่อโลหะหนัก เพื่อศึกษาการแสดงออกของโปรตีนทั้งสอง ผู้นิพนธ์ได้แบ่งโคขุนเป็น 5 กลุ่มตามระดับแคดเมียมที่ตรวจพบในไต จากนั้นทำการวิเคราะห์หาค่าชีวเคมีในเลือดเพื่อประเมินสภาวะการทำงานของตับและไต และได้ศึกษาการแสดงออกของโปรตีน metallothionein กับ metal transcription factor-1 ในชิ้นเนื้อตับและไตด้วยวิธี immunofluorescence พบว่าค่าชีวเคมีในเลือดไม่สัมพันธ์กับระดับแคดเมียมที่ตรวจพบในไต อย่างไรก็ตาม การแสดงออกของโปรตีน metallothionein กับ metal transcription factor-1 สามารถพบได้ตั้งแต่ในชิ้นเนื้อของโคขุนที่มีระดับแคดเมียมปนเปื้อนน้อย (0.5 mg/kg) ดังนั้นโปรตีนทั้งสองอาจใช้เป็นดรรชนีชี้วัดที่มีความไวในการตรวจการปนเปื้อนของแคดเมียมในชิ้นเนื้อ