Prediction of Cadmium (Cd) Toxicity in Cattle

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Hepato- and nephrotoxicity can be induced by the exposure to cadmium (Cd). This toxicity can be detected by the elevation of blood biomarkers such as ALT, AST, ALP, BUN and creatinine. These elevations are found in small animals, e.g. mice and rats. However, the alteration of biomarkers did not investigate in large animals, e.g. cattle.

Hepato- and nephrotoxicity induced by cadmium can also be examined by the alterations of metallothionein (MT) and metal transcription factor-1 (MTF-1). To present study the expressions of these markers, the cattle were classified into five groups according to the levels of cadmium in the kidneys. ALT, AST and ALP were analyzed to determine liver damage whereas BUN and creatinine were examined for kidney damage. The results showed that blood biomarkers were not sensitive enough to be correlated markers to cadmium induced hepato- and nephrotoxicity in cattle.

The expressions of MT and MTF-1 protein were investigated by immunofluorescence method. The expressions of MT and MTF-1 proteins were firstly found in the cattle group which had low cadmium concentration in tissues (< 0.5 mg/kg). Thus, these proteins could be used as the sensitive markers to determine the cadmium exposure. The MT and MTF-1 gene expressions were also studied. However, there was no correlation between the level of RNA and the protein expressions due to the concentration of protein levels bearing unclear relationship with the mRNA level. The investigation of these protein expressions is very useful because the result can be used as a protective method to prevent consumption of cadmium-contaminated beef.

Keywords: *Cadmium, biomarkers, metallothionein, metal transcription factor-1, hepato- and nephro-toxicity*

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Cadmium (Cd) is a highly toxic environmental pollutant found in soil and water. The primary route of cadmium exposure for humans and animals is via the ingestion of cadmium-contaminated food. Exposure of Cd can damage various organs such as bone, lungs, liver and kidneys. Severity of Cd intoxication depends on dose, route and duration of exposure[5,12,14]. Since cattle are one of the major sources of food for Thai people, the consuming of beef in long term can cause the health problems.

Even though Cd induced hepato- and nephrotoxicity can be detected by blood chemical profiles such as alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) and blood urea nitrogen (BUN)[2,6], the elevations of theses markers have been examined only in small animals. The question about the effects of Cd to theses markers in large animal are required.

In addition to the blood biomarkers, Cd induced hepato- and renal dysfunction can be investigated by the alteration of gene expressions. These genes are metallothionein (MT) and metal-responsive transcription factor-1 (MTF-1).

MT is cysteine-rich, low molecular weight protein that binds many metal ions, including zinc, cadmium and copper. This protein functions in metal homeostasis, protection from oxidative stress, and metal detoxification. MT synthesis is controlled at the level of transcription and can be induced by multiple stimuli including exposure to zinc, cadmium and cytokine[1,9-11,14].

MTF-1 plays an important role in the cellular responses to heavy metal stress. MTF-1 contains six zinc fingers of the C_2H_2 type. Via the zinc fingers, it binds to DNA sequence motifs with the consensus binding site TGCRCNC, known as metal response elements (MREs). Binding of MTF-1 to MRE sequence is dependent on heavy metal cations such as zinc, cadmium and copper. In quiescent cells, MTF-1 preferentially resides in the cytoplasm but translocates

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to the nucleus upon several stress conditions, notably heavy metal load[3,4,7,8,10,15,16]. Therefore, MTF-1 is the key regulator of MT expression. Since MT and their transcription factor MTF-1 plays and important role in Cd exposure, then, the possibility of using MT and MTF-1 protein expressions in tissues as the biomarkers of Cd exposure is very interesting for the researchers to investigate.

Material and Method

Blood chemical analysis

Forty-five cattle were divided into 5 groups according to the levels of Cd in kidneys analyzed by Inductively Coupled Plasma Mass Spectrometry (ICP-MS) technique as shown in Table 1.

Blood was collected from the cattle before they were sacrificed. Serum were collected by centrifugation at 3,000 g for 5 minutes and kept at -20°C until the analysis occurred. BUN and creatinine were analyzed for the investigation of the renal functions where as ALT, AST and ALP were analyzed to examine the liver functions. All of blood biochemical tests were analyzed by Reflovet machine.

MT and metal MTF-1 detection by immunofluorescence method

Kidneys and liver from the cattle were collected and preserved in formalin buffer solution. The tissues were processed by paraffin embeds. The staining method was performed as described below.

The tissue sections were deparafinnized, rehydrated and antigen retrieving. Nonspecific reaction was blocked by using 10% normal horse serum. The mixture of 1:400 rabbit anti-bovine metallothionein (Santa Cruz Biotechnology, Inc.) and 1:400 goat anti-bovine MTF-1 (Santa Cruz Biotechnology, Inc.) in 1:1 ratio were added on the tissues and incubated for 1 hour. The excess primary antibody was washed by using phosphate buffer saline (PBS) containing 0.1% Tween-20 for 3 times, 10 minutes in each. After that, the mixture of 1:600 goat anti-rabbit IgG conjugated with fluorescence isothiocyanate (FITC, Santa Cruz Biotechnology, Inc.) and 1:600 donkey anti-goat IgG conjugated with texas red (TR, Santa Cruz Biotechnology, Inc.) in 1:1 ratio were added on the tissues again and incubated for 30 minutes. The excess secondary antibody was washed in similar method with primary antibody washing. The negative control was performed by using PBS instead of primary antibody. The fluorescence signal was detected under fluorescence microscopy.

Table 1. Classification of cattle into 5 groups according to the levels of Cd in kidney tissues

Levels of Cd in kidneys	Group
< 0.5 mg/kg	1
0.5-1 mg/kg	2
1.1-3 mg/kg	3
3-5 mg/kg	4
> 5 mg/kg	5

MT and MTF-1 gene expression by RT-PCR method

The expressions of MT and metal MTF-1 were determined by using RT-PCR method. Total RNA was extracted from kidney and liver tissues by using TRIzol reagent (Invitrogen, USA). One microgram of RNA was reversely transcribed by using RevertAid M-MuLV reverse transcriptase (Promega). cDNA was used as a template for PCR amplification. GAPDH gene expression was used as an internal control of the RNA extraction method and RT-PCR conditions. The primer sequences for MT were F: 5'-ATCGGGTGTCTCTTCTTTGC-3', R: 5'-GGAATGTCGTATCGCATCAA-3'; for MTF-1 F: 5'-CGGAGAACACTTGCCTTT TC-3', R: 5'-TGTTGAA CGCCTTC TCACAG -3' and GAPDH F: 5'-GGGTC ATCATCTCTGCACCT-3', R: 5'-CCC TGTTGCTGTA GCCAAAT-3'.

Statistical analysis

The data was statistically analyzed by using one way ANOVA test. The level of significance considered was $p < 0.05$.

Results

Blood chemical analysis

ALT, AST, ALP, BUN and creatinine were analyzed to determine hepato- and renal function. The result was shown in Fig. 1 that the alterations of these blood biochemicals were not increased along with the levels of Cd in tissues ($p > 0.05$). The alterations of these blood biochemical elements should have been theoretically increased in Group 5 at the highest level, but the results did not concur with the assumptions.

The alterations of all blood biochemical results were not significantly increased at $p > 0.05$.

MT and MTF-1 detection by immunofluorescence method

To determine the MT and MTF-1 expressions, the liver and kidneys of the cattle were stained with the

mixture of rabbit anti-bovine metallothionein and goat anti-bovine MTF-1 following with the mixture of goat anti-rabbit IgG conjugated with fluorescence isothiocyanate (FITC) and donkey anti-goat IgG conjugated with texas red (TR). The expressions of MT and MTF-1 protein both in kidneys and liver were firstly detected in Group 1 (Cd < 0.5 mg/kg) as shown in Fig. 2 and 3.

MT and MTF-1 gene expression by RT-PCR method

The MT and MTF-1 gene expression can be summarized in form of percentage in each cattle group as shown in Table 2.

Discussion

Cd intoxication can cause negative effects in various tissues especially liver and kidneys due to Cd preferentially accumulated in these tissues. The toxicity of Cd to liver can be detected by increasing of hepatic enzyme such as ALT, AST and ALP as described in the experiment of rats by Karmakar et al[6] and in the experiment of mice by Brzoska et al[2] whereas the toxicity of Cd to kidneys can be examined by the increase of BUN investigated in the research of Brzoska et al[2]. In this experiment; however, the blood biochemical profiles, *e.g.* ALT, AST, ALP, BUN and creatinine did not elevate ($p > 0.05$). Thus, the blood biochemical profiles were not sensitive and could not be the correlated markers to Cd induced hepato- and nephrotoxicity in cattle.

The alternative biomarkers to determine toxicity of Cd are recently metal associated protein including MT and MTF-1. Cd can alter the expression of MT mRNA but not in the protein levels in rats[13]. The similar results described by Hispard et al in 2008[5] were that MT was the sensitive biomarkers to examine the Cd exposure in rats even in the low concentration. The expressions of MT mRNA in cattle exposed to Cd were found in the present study. However, the MT mRNA expressions did not correlate with the levels of Cd in tissues. Moreover, the expression of MTF-1 mRNA also showed in similar way. Surprisingly, the MT and MTF-1 proteins were detected by immunofluoresece staining method independent with mRNA expression and Cd level in tissues. It can be described that the concentration of MT protein levels

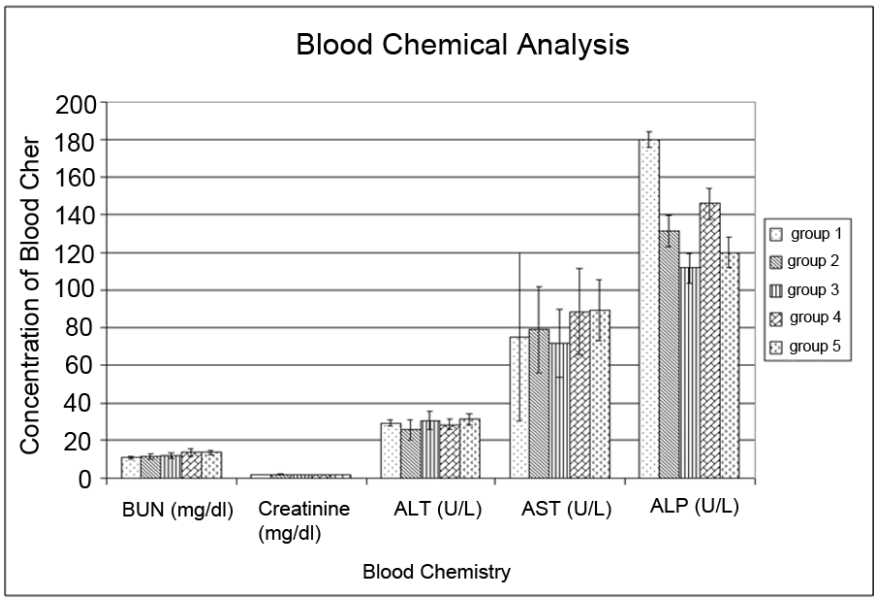

Fig. 1 The blood biochemistry analysis
The blood biochemical analysis was done by the dry chemical assay. These bar charts represented the concentration of each blood biochemical result
Bar chart represented the blood biochemical result of the cattle in Group 1
Bar chart represented the blood biochemical result of the cattle in Group 2
Bar chart represented the blood biochemical result of the cattle in Group 3
Bar chart represented the blood biochemical result of the cattle in Group 4
Bar chart represented the blood biochemical result of the cattle in Group 5

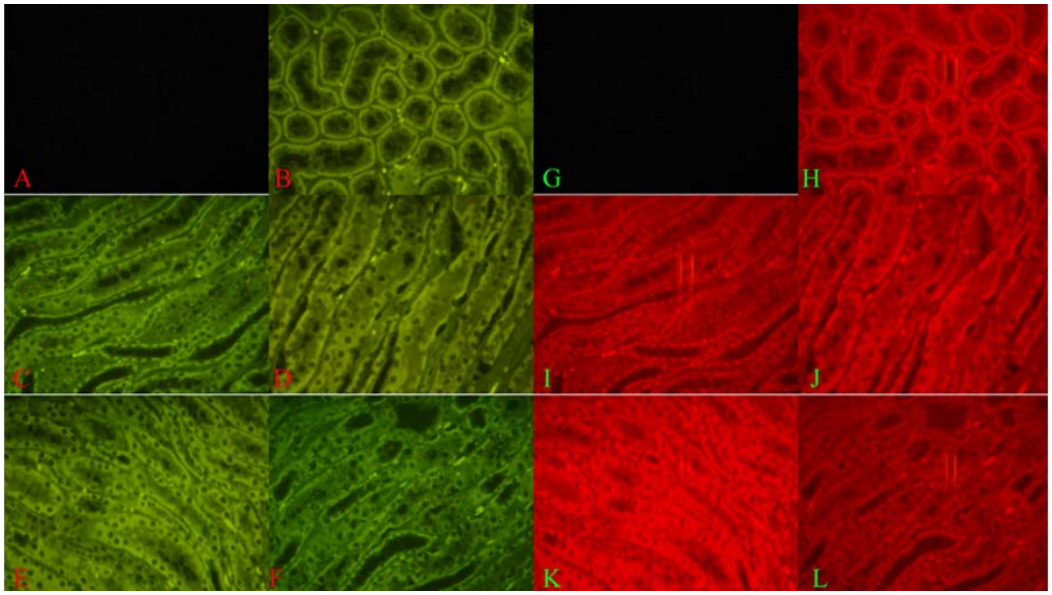

Fig. 2 The MT and MTF-1 protein expressions in kidneys determined by immunofluorescence method
Kidneys were examined for the MT and MTF-1 expressions. Kidneys named A to F were examined for the MT expression whereas G to L kidneys were examined for the MTF-1 expression. A and G kidneys were negative control. B and H were the kidneys in Group 1, C and I in Group 2, D and J in Group 3, E and K in Group 4 and finally F and L in Group 5. The positive results for the MT expression were shown in green color while the positive results of the MTF-1 expression were shown in red color

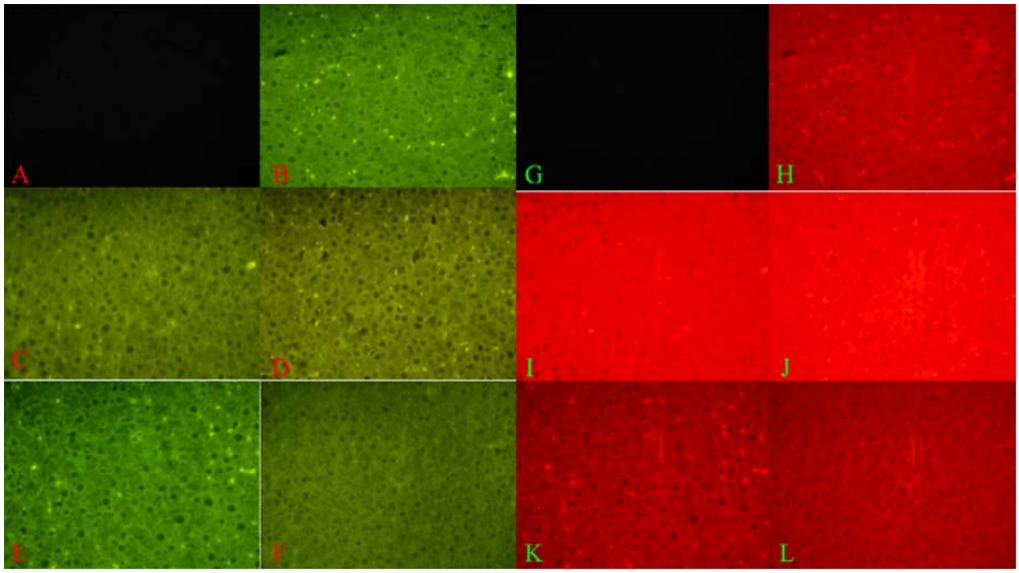

Fig. 3 The MT and MTF-1 protein expressions in liver determined by immunofluorescence method
The livers were examined for the MT and MTF-1 expressions. A to F were livers which were examined for the MT expression whereas G to L livers were examined for the MTF-1 expression. A and G livers were negative control. B and H livers were in Group 1, C and I in Group 2, D and J in Group 3, E and K in Group 4, and finally F and L in Group 5. The positive results for MT expression were shown in green color while the positive results of MTF-1 expression were shown in red color

Table 2. MT and MTF-1 gene expressions

	MT expression (%)		MTF-1 expression (%)	
	kidney	liver	kidney	liver
Group 1	37.5	62.5	100	100
Group 2	20	20	100	100
Group 3	20	40	100	100
Group 4	11.11	44.44	33.33	55.56
Group 5	37.5	12.5	75	87.5

bears no clear relationship with mRNA level[13].

In conclusion, the expressions of MT and MTF-1 proteins based on this experiment are strongly believed that they are the alternative and sensitive biomarkers to determine Cd induced hepato- and nephrotoxicity since they can be detected even though in cattle which has the level of Cd < 0.5 mg/kg.

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Potential conflicts of interest

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ตัวชี้วัดที่มีความไวต่อการทำนายความเป็นพิษของแคดเมียมในโคขุน

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แคดเมียมสามารถเหนี่ยวนำให้เกิดความเป็นพิษในตับและไต ซึ่งความเป็นพิษนี้สามารถตรวจวิเคราะห์ได้จากค่าเคมีในเลือด ได้แก่ ค่าเอนไซม์อลานีนอะมิโนทรานส์เฟอเรส แอสปาเทตอะมิโนทรานส์เฟอเรส แอลคาไลน์ฟอสฟาเทส ยูเรียในเลือด และครีเอตินีน การเพิ่มขึ้นของค่าเคมีต่างๆ ในเลือดนี้ มีการศึกษามาแล้วในสัตว์ที่มีขนาดเล็ก เช่น หนูแรทและหนูไมท์ อย่างไรก็ตาม ยังไม่มีการศึกษาถึงการเปลี่ยนแปลงของค่าเคมีในเลือดของสัตว์ที่มีขนาดใหญ่ เช่น โคขุน

ความเป็นพิษที่เกิดจากแคดเมียมในตับและไต สามารถตรวจสอบได้จากการแสดงออกของโปรตีนเมทัลโลไธโอนีน และเมทัลทรานส์คริปชั่นแฟคเตอร์ 1 เพื่อศึกษาการแสดงออกของโปรตีนทั้งสอง ผู้วิจัยได้ทำการแบ่งโคขุนออกเป็น 5 กลุ่มตามระดับของค่าแคดเมียมในไต เอนไซม์อลานีนอะมิโนทรานส์เฟอเรส แอสปาเทตอะมิโนทรานส์เฟอเรส และแอลคาไลน์ฟอสฟาเทส และมีการวิเคราะห์เพื่อประเมินความเสียหายของตับ ในขณะที่ค่ายูเรียในเลือดและครีเอตินีน ถูกวิเคราะห์เพื่อประเมินความเสียหายของไต จากผลการทดสอบค่าเคมีในเลือดพบว่าค่าเคมีในเลือดไม่มีความไวและไม่สัมพันธ์กับความเป็นพิษที่เกิดจากแคดเมียมในตับและไตของโคขุน

การแสดงออกของโปรตีนเมทัลโลไธโอนีน และเมทัลทรานส์คริปชั่นแฟคเตอร์ 1 ถูกศึกษาโดยวิธีอิมมูโนฟลูออร์เรสเซนต์ ผู้นิพนธ์พบว่าการแสดงออกของโปรตีนทั้ง 2 ถูกพบตั้งแต่โคขุนกลุ่มแรกๆ ซึ่งมีระดับแคดเมียมในไตน้อยกว่า 0.5 มิลลิกรัมต่อกิโลกรัม ดังนั้น โปรตีนทั้ง 2 น่าจะเป็นตัวชี้วัดที่มีความไวในการตรวจหาการสัมผัสกับแคดเมียม นอกจากนี้ ผู้นิพนธ์ยังได้ทำการศึกษาการแสดงออกของ เมทัลโลไธโอนีน และเมทัลทรานส์คริปชั่นแฟคเตอร์ 1 ในระดับยีน พบว่าการแสดงออกของอาร์เอนเอ และโปรตีนของเมทัลโลไธโอนีนและเมทัลทรานส์คริปชั่นแฟคเตอร์ 1 ไม่มีความสัมพันธ์กัน อาจเนื่องมาจากการแสดงออกของโปรตีน และเอ็มอาร์เอนเอของเมทัลโลไธโอนีน และเมทัลทรานส์คริปชั่นแฟคเตอร์ 1 ไม่มีความเกี่ยวข้องกัน ประโยชน์ที่ได้รับจากงานวิจัยชิ้นนี้ คือ การตรวจสอบการแสดงออกของโปรตีนดังกล่าวอาจถูกใช้เป็นการป้องกัน การได้รับแคดเมียมจากเนื้อสัตว์โดยการใช้วิธีการตรวจที่ไวยิ่งขึ้น