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DETERMINATION OF CADMIUM AND ZINC AND ITS RELATIONSHIP TO METALLOTHIONEIN LEVELS IN SWINE KIDNEY

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ABSTRACT

Cadmium is considered a hazardous environment pollutant. Animals exposed to this metal show cadmium accumulation mainly in their kidneys. Over 60 % of cadmium binds to metallothioneins, i.e., proteins that act as detoxification agents. The purpose of this study was to isolate metallothioneins in swine kidneys and to determine cadmium, zinc, and copper link with this protein. Twenty kidneys were selected and total concentration of cadmium, zinc, and copper were analyzed by atomic absorption spectroscopy and total metallothioneins using the silver saturation method. Metallothioneins were isolated by gel filtration and ion exchange chromatography. AAS-graphite furnace was used to monitor cadmium, zinc, and copper in eluted fractions. Total concentrations of cadmium, zinc, copper, and metallothioneins were 1.20-2.40 μg/g, 20.37-39.82 μg/g, 3.99-14.80 μg/g, and 4.26-101.68 μg/g, respectively. Results show a positive correlation between metallothioneins levels and each of the metals; a Zn-thionein and a Cd-thionein were also identified.

Palabras clave: metalotioneína, cadmio, zinc, indicador ambiental

RESUMEN

El cadmio es considerado un contaminante ambiental tóxico. Los animales expuestos a este metal lo acumulan principalmente en el riñón. En este órgano aproximadamente el 60 % del cadmio se encuentra ligado a metalotioneínas, estas proteínas actúan como agentes desintoxicantes. El objetivo de este estudio fue aislar las metalotioneínas (MT) en riñón porcino y determinar el cadmio, zinc y cobre ligado a esta proteína . Se seleccionaron 20 riñones, a los cuales se les detectó el contenido de cadmio, zinc y cobre por medio de la espectrofotometría de absorción atómica y de metalotioneínas totales por el método de saturación con plata. Las metalotioneínas se aislaron por cromatografía de filtración en gel y por intercambio iónico y se monitoreó cada uno de los metales en las fracciones de elusión por medio del horno de grafito. Las concentraciones totales de cadmio, zinc, cobre y de las MT fueron 1.20-2.40 μg/g, 20.37-39.82 μg/g, 3.99-14.80 μg/g y 4.26-101.68 μg/g, respectivamente. Los resultados de este estudio mostraron correlaciones positivas entre las MT y los metales, además se identificó una Zn-tioneína y una Cd-tioneína.

INTRODUCTION

Cadmium (Cd) is considered a hazardous environment pollutant widely distributed in nature that has a broad spectrum of toxic effects in mammalians, including nephrotoxicity, hypertension and osteomalacia (Torra *et al.* 1994, Kaji *et al.* 1996).

After ingestion and absorption a significative fraction of Cd is accumulated in organs showing a long biological average life. Kidneys are the main target organs for both

accumulation and toxicity, leading to nephrotoxicity. A possible indicator of such metal exposure is the presence of metallothioneins (MT), a cysteine-rich low-molecular-weight bioligand that has been postulated as the most important mechanism for protection against Cd toxicity (Torra *et al.* 1994, Bordin *et al.* 1996, García-Rico *et al.* 1999).

MT are cytoplasmic proteins of extremely high metal and sulfur content. They appear to play several roles in the homeostatic control of metals, e.g., in the intracellular fixation of essential trace elements like zinc (Zn) and copper (Cu). MT regulates the concentration of free ions of these metals, and regulates their flow to appropriate cellular destinations. Also, MT neutralizes the harmful influences of exposure to toxic concentrations of Cd and mercury (Hg) (Scheuhammer and Cherian 1986, Cherian 1989, Bordin *et al.* 1996).

A great amount of research has been devoted to the understanding of the detoxifying mechanism of MT against Cd, mainly focusing in the study of the induction of MT synthesis by high levels of Cd in animal models (Scheuhammer and Cherian 1986, Carpené and Vašák 1989, Chan and Cherian 1993).

MT has also been used as biomarker of environment contamination by metals (Chung *et al.* 1986, Imber *et al.* 1987, Bebiano *et al.* 1994). However, most of these studies are based on laboratory assays in which exposure conditions differ greatly from those in natural environments. Furthermore, the metal concentrations used to induce MT synthesis in these experiments often are several orders of magnitude higher than those found even in the most polluted environments (Chou *et al.* 1993, Ferrarello *et al.* 2000). A major concern has been to determine the possible toxic effects of Cd at low concentrations or at natural background exposure in farm animals. The type of MT expressed at low metal concentration is scarce.

The state of Sonora is located in the northwest of Mexico and its area covers a surface of 184 934 km^2. Sonora has a great diversity of industrial activities, including mining, agriculture and livestock. It ranks among the top breeders of swine in the country. As a consequence of such intense economic activity the Sonoran environments are also characterized by traces of metals like Cu, Pb, As, and Cd. The purpose of this study was to isolate and identify the Cd- thionein and Zn- thionein in swine exposed to natural background Cd concentration.

MATERIALS AND METHODS

Reagents

The following chemicals were used: concentrated HNO_3 (70 % v/v, Mallinckrodt, Phillipsburg, NJ), hydrogen peroxide (30 % v/v, Productos Químicos Monterrey, Mexico) and standard stock solutions of Ag, Cd (Sigma Chemical Co, St. Louis, MO), and Zn (Mallinckrodt). A Sephadex G-75 and DEAE-Cellulose chromatography matrix, and the low molecular gel filtration kit came from Sigma Chemical Co. The other reagents came from Sigma Chemical Co, analytical grade.

Equipment

The atomic absorption spectrophotometer was a Varian model SpectrAA-20 equipped with head burner, hollow-cathode lamp, and GTA-96 graphite furnace (Varian, Victoria, Australia). An air-acetylene flame was used and argon employed as carrier gas.

The microwave digestion system was a MDS-81D model (CEM Corp., Matthews, NC) equipped with a 600 W magnetron adjustable in 1 % increments, a rotating turntable, and a variable-speed exhaust fan to dispose any corrosive fumes to a hood. Time, pressure, and power were controlled by a programmable microprocessor. Digestions were performed in 100 mL lined digestion vessels (CEM Corp.) equipped with safety relief valves.

The low pressure chromatography system came from Pharmacia (Uppsala, Sweden) equipped with a monitor uv-1, peristaltic pump-1, fraction collector Frac-100 and recorder Rec-100.

All glassware was washed with Pierce solution 20 % v/v (Pierce, Rickford, IL), rinsed with cold tap water followed by 20 % v/v nitric acid, and then rinsed with double-distilled water.

Preparation of metal standards

Mixed flame working standards of Cd, Cu, and Zn were prepared by diluting 1000, 1025, and 1000, µg/mL, respectively, stock reference solutions with 0.5 mL (50 % v/v) HNO_3 and 25 mL double-distilled water to produce concentrations of 0.5, 1.0, 2.0, 3.0, and 5.0 µg/mL. For GTAAS (Graphite Tube Atomizer Atomic Spectro-photometer) the stock reference solutions above mentioned were used and diluted with a 30 mM TRIS-HCl buffer to produce 4, 8 and 12 ng/mL concentrations.

Metals quality control

Standard Reference Material, NBS 1566a, reagent blanks and duplicate samples were run with each digestion series. Relative standard deviation (RSD) for flame AAS standards was below 0.5 % and below 1 % for GTAAS methods. Precision was estimated from the coefficient variation (CV) calculated from replicate analyses of five samples of Standard Reference Material in different days. All CV values were below 10 %. The methods accuracy was confirmed though intercomparison studies. The detection limits (DL) for each element (AAS) were estimated following Varian (Analytical Methods. Flame Atomic Absorption Spectrometry, 1989) and multiplied by volume over weigh

(μg/g fresh wt): Cd 0.06, Cu 0.12, Zn 0.03. For GTAAS, DL were calculated using the reagent blank method (ng/mL): Cd 0.0093, Cu 0.0086 , Zn 0.0114.

Sampling

Sampling was conducted according to the National Program of Sampling for Toxic Residues and Meat Products. 144 swine kidneys were obtained from slaughterhouses located in Sonora according to annual random sampling. All swine used in this study were raised under an intensive breeding system, therefore metal contribution by soil was not expected to be significant, but it was expected to come from water, food and via coprophagy practices.

Sample preparation and selection

Fat and connective tissue were thoroughly removed from the organ. Each sample was then ground in a food processor for 30 s, placed in a plastic container, and frozen at –20 ºC prior to the analysis. Swine kidneys were analyzed for total Cd and those that presented concentrations above the FAO/WHO tolerance limit (1 μg/g) were selected for the study (20 kidneys total).

Total cadmium, zinc, and copper

Digestion was performed according to García-Rico and Jara-Marini (1996). Briefly, 1.250 ± 0.003 g of kidney tissue was weighted in digestion vessels and 5 mL 50 % v/v HNO_3 were added. First, they were digested for 3 min at 95 % power, 5 min at 90 % power and 15 min at 85 % power. Then 3 mL 30% H_2O_2 was slowly added to the vessels, which were kept in a hood until all bubbling ceased. The vessels were returned to the microwave oven, and a second digestion process of 3 min at 100 % power and 20 min at 90 % power took place. Pressure was set at 90 psi during both procedures. Samples were cooled, vented, and diluted with double-distilled water after microwave digestion. Samples were diluted to 50 mL and then analyzed by AAS flame.

Total metallothionein

The silver saturation method of Scheuhammer and Cherian (1991) was used. Kidneys were briefly homogenized (1:4) in 0.25 M sucrose solution for 2 min and then centrifuged at 20 000 x g for 20 min. 400 μL of glycine 0.02 M were added to 400 μL of the supernatant and incubated with 500 μL of 20 μg/g Ag solution for 5 min. 100 μL of human red blood cell hemolysate were added, boiling heated for 1.5 min and centrifuged at 12 000 x *g* for 5 min. The last step was repeated twice more. The supernatant was centrifuged at 15 000 x *g* for 6 min, transferred to a volumetric flask and Ag was analyzed by AAS. The amount of MT in the sample was calculated using the following equation:

$$\mu g\ MT/g\ tissue = (C_{AG} - CB_{KG}) \times 3.55 \times Vt \times SDF / S_V$$

where C_{AG} is the concentration of silver in the final supernatant; CB_{KG} is the background reading in the supernatant of the blank; Vt is the total volume in the assay sample; SDF is the sample dilution factor, and S_V is the sample volume used in the assay.

Metallothionein purification

The crude extract (CE) was prepared as follows: kidneys were homogenized 1:2 in 30 mM (pH 8.0) TRIS-HCl buffer (buffer A) and centrifuged at 31 000 x g for 20 min at 0 ºC. Supernatant was heated at 100 ºC for 6 min and centrifuged again at 47 400 x g for 23 min at 4 ºC, then concentrated to 3 mL by ultrafiltration using a 3 kDa cutt-off membrane (Amicon 8400, Beverly, MA).

The first step of purification was a gel-permeation chromatography. The crude extract (68.55 mg/mL) was applied to the Sephadex G-75 column (1.5 x 95 cm) previously equilibrated with buffer A at 0.6 mL/min. Low molecular weight standards used included Blue Dextran, Alcohol Dehydrogenase, Carbonic Anhydrase and Aprotinin. Eluted proteins were collected in 0.85 mL fractions and monitored at 250 nm. Cd, Zn, and Cu were analyzed in each fraction.

An ion exchange chromatography was applied. Fractions containing metallothionein collected from G-75 gel filtration column were applied to a 1.5 x 30 cm DEAE-Cellulose column previously balanced with buffer A, at 0.6 mL/min. MT was eluted using a liner gradient of 0-1M NaCl in buffer A. Elution was monitored at 250 nm. Cd, Zn, and Cu were analyzed in each fraction.

AAS measurements

Cd, Zn, and Cu in digested samples, and Ag in total MT were determined by AAS flame at 228.8, 213.9, 324.8, and 328.1 nm, respectively.

Cd, Zn, and Cu in the chromatographic fractions were determined by graphite furnace AAS. Standard curves were prepared diluting the stock standard solutions.

Statistics analysis

A multiple and simple regression and a Pearson's correlation analysis were performed using the NCSS 6.0 computational system.

RESULTS AND DISCUSSION

Total Cd, Zn, Cu, and MT

Metal concentrations in kidneys are summarized in **table I**. Zn was the most abundant element (26.7 μg/g), followed by Cu and Cd. Cd concentrations range from 1.20 to 2.40 μg/g. These variations may indicate the origin, age and sex of the animals (Goyer 1991). In this study group most animals were about six months old, females, and raised in the central (71 %) and southern (29 %)

regions of Sonora. Swine raised in these two areas has been reported to have the highest Cd concentrations in kidneys, and about 68 % of the samples exceed the action level of 1 µg/g (García-Rico *et al.* 1999); the obtained values were slightly higher than those found in previous reports. Tissues of swine raised in these regions with no detectable Cd were used as a control group. The Zn and Cu concentrations in the control group were similar to those found in the study group.

TABLE I. TOTAL CONCENTRATIONS OF CADMIUM, ZINC, AND COPPER (µg/g, FRESH WT) IN KIDNEY SAMPLES

Element	Mean ± SD[a]	Range
Cadmium	1.6 ± 0.33	1.20 - 2.40
Zinc	26.0 ± 5.02	20.37 - 39.82
Copper	8.9 ± 3.00	3.99 - 14.80

[a] Standard Deviation

The ratio of Cd/Zn was low (0.06) and may be associated with elevated urinary excretion of metals due to changes in the renal function. Cd can alter the functioning of the kidneys tubules and also have toxic effects on the reproductive system (Santiago *et al.* 1998). Higher accumulations of Cd in kidneys compared to livers in deer, boar, domestic slaughter animals by a factor of 4 to 11 have also been reported (Santiago *et al.* 1998). In the present study almost renal-Cd/hepatic-Cd fractions were closely to 1 and hepatic-Cd/renal-Cd were lower than 1. This may indicate that animals had been constantly exposed to Cd, which could be explained by the feeding habits. Cd is extensively used for industrial purposes; it is also a contaminant in fertilizers and urban sludge used to fertilize pastures or crops. This widespread distribution has resulted in feed stuffs as a major source of Cd for domestic animals (Spiric and Saicic 1998, Santiago *et al.* 1998). It is important to note that Sonora shows a great diversity of natural mineral deposits like silver, lead, zinc, gold, and copper. Studies in Sonora found Cd levels of 0.012 µg/g in water wells (Wyatt *et al.* 1998), 0.4 to 1.43 µg/g in oysters from Sonoran coasts (García-Rico *et al.* 2001), and 1.40 µg/g in swine kidneys. These concentrations in water and oysters can reflect nearby industrial activity as well as the composition of local rocks and soil. Moreover, the accumulations of Cd in the trophic chain might be explained by the atmospheric route.

Mexican regulatory agencies have established 5 µg/g as maximum concentrations for Cd in swine kidneys (PROY-MOD-NOM-004-ZOO-1994); so the results of this study are within the norm. However, based on the international tolerance standard of 1µg/g (Santiago *et al.* 1998) our results are likely to contain Cd levels that entail potential hazards to public health.

Total MT concentrations in kidneys ranged from 4.26 to 101.68 µg of MT/g tissue. These values agree with the basal levels of total MT reported in experimental animal studies (Scheuhammer and Cherian 1991, Chan and Cherian 1993), the concentration of MT in control animals was 3.31 µg MT/g tissue. When animals are exposed or treated with sublethal doses of Cd or other metals, synthesis of MT takes place in the liver, kidneys, spleen, and other tissues. Cherian and Goyer (1978) indicated that trace amounts of Cd can induce the synthesis of MT in the liver, kidneys and spleen. On the contrary, high concentration of Zn (above 30 µg/g tissue) induces the synthesis in liver. Unlike Cd, Zn does not have any influence on the synthesis of renal MT. However, MT has been recently reported in both liver and kidneys (Chung *et al.* 1986, Carpené and Vašák 1989, Torra *et al.* 1994).

A significant positive linear relation was observed between MT levels and Cd or Zn levels in kidney cortex samples ($p < 0.05$); MT-Zn presented the highest correlation ($r = 0.944$), followed by MT-Cd ($r = 0.929$). Significant correlation values may indicate that the MT synthesis is produced even at Cd and Zn low levels (Carpené and Vašák 1989, Hao *et al.* 1993). These results could suggest that MT might serve as a major intracellular storage protein for Cd in kidneys and probably Zn in both liver and kidneys. Furthermore, a specific role of MT has been found as a Zn regulator in the liver and as a Cd detoxifier (Whanger 1991).

As indicated by Whanger (1991), MT was first isolated and characterized from the kidneys of a big animal; however most of the work done on metals and MT metabolism has been performed with small animals, mainly rats. Therefore, metabolic links between metals and MT in animals like swine require further study. Swine raised in natural environments may be useful as a human model to understand the relation between metals and MT.

The elution profile of MT from gel permeation column is shown in **figure 1**. Two mayor peaks (I and II) were found. Peak I corresponds to a high molecular weight protein (200 to 24 kDa) and to a protein concentration of 49.19 mg (72 % of the crude extract). Peak II corresponds to a low molecular weight protein (< 24 kDa), MT (6.5 kDa) as previously reported (Cherian 1989, Hao *et al.* 1993). The protein content of peak II was 0.74 mg, accounting for 1.1 % of the protein contained in the crude extract.

Monitoring of metals in gel-permeation fractions is shown in **figure 1**. Zn levels ranged from 106-1339 ng/mL in peak I and from 96-526 ng/mL in peak II. In contrast, Cd was detected at trace levels (10-100 ng/mL) in peak I and from 86 to 603 ng/mL in peak II. The high concentration of Zn in peak I indicates the role of this metal as a cofactor in proteins of high molecular weight. Both Cd and Zn in peak II suggest affinity with low mo-

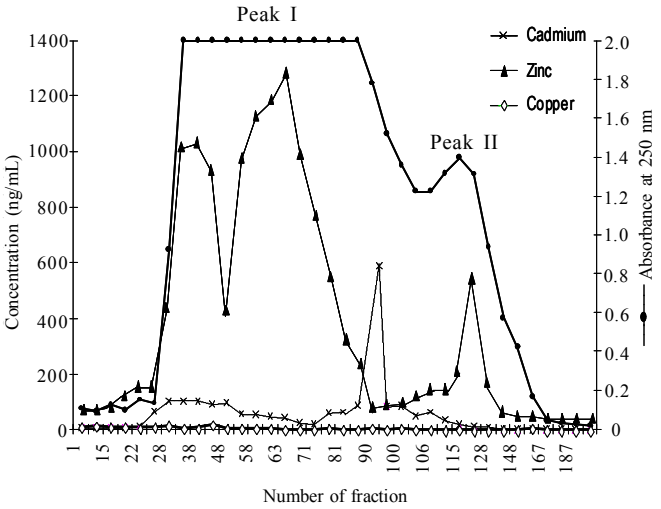

Fig. 1. Gel-permeation chromatography of crude extract (peak I: first fraction of elution, peak II: second fraction of elution) and monitoring of metals in both fractions

lecular weight proteins. Despite MT selectivity, higher for Zn than Cd (Cherian 1989), Cd and Zn were found at similar levels in peak II. This behavior may indicate the role of MT.

The highest concentration of zinc did not coincide with the highest concentration of protein in peak II (**Fig. 1**). This is due to the presence of different proteins with similar molecular weight. For this reason, this peak was re-chromatographied for exchange chromatography.

Fractions of peak II were pooled and concentrated by ultrafiltration and applied to a DEAE-Cellulose ion exchange column (**Fig. 2**). The flow trough contained three minor no metalothionein peaks. Two MT (peak I′ and II′) eluted using a NaCl gradient, the first at the beginning of the gradient (0.01 to 0.05 M), and the second at 0.1 M NaCl. Similar results were reported by Carpené *et al.* (1992) in goldfish kidney studies. They identified I′ and II′ as isoforms MT-I and MT-II, respectively.

The metal monitoring of ion exchange chromatography is shown in **figure 2**. The Zn range was 100-980 ng/mL in peak I′ and in peak II′ was detected at low levels (13.75-90.3 ng/mL). In contrast, Cd was in three defined fractions ranging from 120-1374 ng/mL in peak II′ and was detected at trace levels (72-87 ng/mL) in peak I′. This behavior may indicate the elution of MT as a Zn, Cd-thionein in peak I′ and a Cd, Zn-thionein in peak II′. In both chromatographs the concentration of Cu was below 3 ng/mL.

In animals, the amount and type of metals associated with MT depends on the source of the protein and can be quite varied. In most mammalian tissues, Zn or Cu is the most common metal component of MT. However, even when isolated from a single source, MT can be found to contain more than one metal, e.g., Zn and Cu in fetal bovine liver or Cd and Zn in equine kidney cortex (Falchuk and Czupryn 1991). Reports of purification and characterization of MT in different tissues where the synthesis of MT was not induced have predominantly shown a Zn-thionein (Huffman 1992, Hao *et al.* 1993, Scudiero *et al.* 1995). In contrast, induced MT studies proved similar to the present report, yielding a Cd-thionein and a Zn-thionein (Cherian and Goyer 1978, Carpené and Vašák 1989, Carpené *et al.* 1992, Chan and Cherian 1993).

In summary, low levels of Cd, Zn, and Cu in swine kidneys showed a positive correlation with MT. Moreover, MT were identified as Cd-thionein and Zn-thionein.

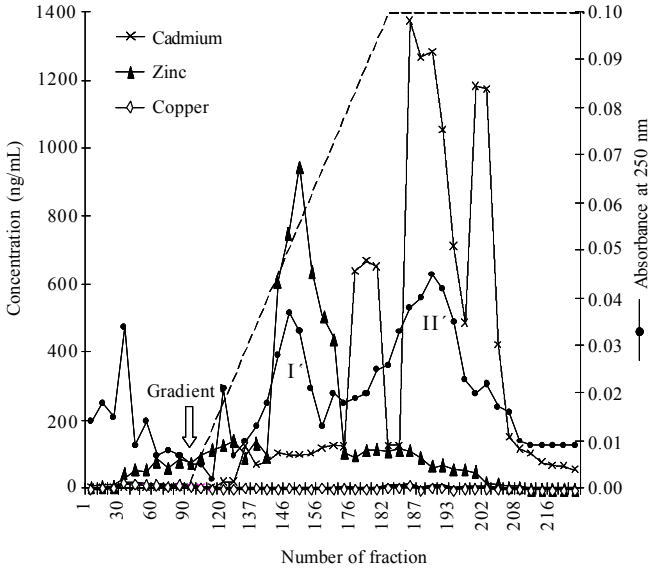

Fig. 2. Ion exchange chromatography of peak II (I′: first peak of elution, II′: second peak of elution) and monitoring of metals in both fractions

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